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Immunogenicity of the B Monomer of *Escherichia coli* Heat-Labile Toxin Expressed on the Surface of *Streptococcus gordonii*

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The B monomer of the *Escherichia coli* heat-labile toxin (LTB) was expressed on the surface of the human oral commensal bacterium *Streptococcus gordonii*. Recombinant bacteria expressing LTB were used to immunize BALB/c mice subcutaneously and intragastrically. The LTB monomer expressed on the streptococcal surface proved to be highly immunogenic, as LTB-specific immunoglobulin G (IgG) serum titers of 140,000 were induced after systemic immunization. Most significantly, these antibodies were capable of neutralizing the enterotoxin in a cell neutralization assay. Following mucosal delivery, antigen-specific IgA antibodies were found in feces and antigen-specific IgG antibodies were found in sera. Analysis of serum IgG subclasses showed a clear predominance of IgG1 when recombinant bacteria were inoculated subcutaneously, while a prevalence of IgG2a was observed upon intragastric delivery, suggesting, in this case, the recruitment of a Th1 type of immune response.

Enterotoxigenic strains of *Escherichia coli* cause acute watery diarrhea, known as traveler's diarrhea, by colonizing the small intestine and producing enterotoxins such as the heat-labile toxin (LT) (19). The crystal structure of LT revealed that it is composed of one A subunit (LTA) (27 kDa) and five noncovalently associated B subunits (LTB) (11.6 kDa each) forming a ring-like pentamer (46). LTA has ADP-ribosylating activity, whereas LTB is able to bind to the ganglioside GM1 and to other related receptors present on enterocytes (3, 18).

LT and cholera toxin are structurally, functionally, and immunologically related, although not identical (8, 47). Both toxins, as well as their B subunits (LTB and CTB), are strong immunogens, able to induce serum and mucosal antibodies when orally delivered (14, 49, 57). While it is generally accepted that the holotoxins are mucosal adjuvants, the actual adjuvanticity of the B subunits is questioned (11, 13). However, there is evidence indicating that CTB and LTB can function as carriers for mucosal delivery of vaccine antigens (7, 10, 25, 30, 43, 45, 52) and possess some adjuvant activity (9, 15, 16, 53, 56).

An effective vaccine against cholera or traveler's diarrhea should be able to induce an immune response at the level of the intestinal mucosa capable of conferring protection by inhibiting toxin activity and preventing bacterial colonization (19). CTB and LTB are currently used for the formulation of oral vaccines, since the antitoxin immune response is essentially directed against the B subunit (17, 19, 44, 48).

In this work, we constructed a recombinant gram-positive bacterium expressing the LTB monomer on the cell surface, to test the possibility of delivering to the immune system the B subunit attached to a live microorganism rather than as a soluble protein antigen. While a similar approach has been tried using attenuated strains of *Salmonella* (5, 15, 32, 50), here we used *Streptococcus gordonii*, a human commensal bacterium which we developed as a vaccine delivery vector (41). The genetic system for heterologous gene expression in *S. gordonii* allows surface expression of recombinant proteins, since it is based on the use of the streptococcal surface protein M6 as a fusion partner (35, 37, 39, 41). Recombinant strains of *S. gordonii* expressing heterologous antigens were shown to induce systemic and local immune responses by colonizing the host mucosal surfaces (12, 26, 27, 33, 41). Here, we show that the LTB monomer expressed on the surface of *S. gordonii* can induce LTB-specific local and systemic antibodies after immunization with recombinant bacteria.

MATERIALS AND METHODS

***Streptococcus gordonii* strains.** *S. gordonii* strain GP1221.1 was used as a recipient for transformation (35). *S. gordonii* GP246, expressing the E7 protein of human papillomavirus type 16 (HPV-16) (37), and GP1246, expressing LTB (this work), were used for immunization experiments.

LTB-encoding DNA. The *eltB* gene (encoding the LTB monomer), from the *E. coli* strain H74-114 of human origin, was PCR-amplified from plasmid pAM23 (23). The primers (forward, 5′-GGT ACC GCT CCT CAG TCT ATT ACA-3′; reverse, 5′-A AGC TTT TGA GTT TTC CAT ACT GAT-3′), containing the restriction sites *Kpn*I and *Hin*dIII, respectively, were designed to amplify the *eltB* gene, excluding the leader peptide coding sequence. PCR conditions were as follows: annealing at 52°C for 10 s, extension at 72°C for 10 s, and denaturation at 92°C for 10 s for a total of 25 cycles.

Construction of recombinant *S. gordonii*. The LTB-expressing *S. gordonii* was constructed using the host-vector system previously described (35). The 309-bp *eltB* gene, obtained by PCR and cut with *Kpn*I and *Hin*dIII, was inserted in frame into the *emm6* gene of plasmid pSMB55, digested with *Kpn*I and *Hin*dIII. In the new recombinant plasmid, designed pSMB120, the *emm6-eltB* gene fusion included the M6 signal sequence, the sequences coding for the first 122 N-terminal amino acids and for the last 140 C-terminal amino acids of M6, while the central region of *emm6* was deleted and replaced with the *eltB* gene. Plasmid pSMB120 was then used to transform competent cells of *S. gordonii* GP1221.1 to obtain the

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recombinant strain GP1246. Procedures for cloning gene fusions in *E. coli*, transformation of *S. gordonii*, and scoring and genetic analysis of transformants were as previously described (37–39).

Immunofluorescence and Western blotting of cellular fractions. Immunofluorescence staining of whole streptococcal cells was performed as previously described (40). The methods for cell fractionation of *S. gordonii* and for Western blotting have been already described (34, 40). For cell fractionation, four different fractions are obtained: supernatant; cell wall, containing fragments of the cell wall after digestion with lysozyme and protoplast formation; envelope, which represents the protoplast surface containing the cell membrane together with cell wall fragments associated to the protoplast; and cytoplasm. Briefly, 10-ml cultures of *S. gordonii* GP1246 and GP1221.1 grown to late stationary phase in Todd-Hewitt broth (Difco) were harvested by centrifugation. The supernatant was precipitated with trichloroacetic acid and resuspended in 0.1 ml of 0.25 mM Tris (pH 6.8) to obtain the supernatant fraction. Bacterial cells were washed in phosphate-buffered saline (PBS), resuspended in 0.1 ml of protoplasting buffer (100 mM Tris [pH 7.2], 30% raffinose, 5 mM EDTA, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, lysozyme [1 mg/ml], and mutanolysin [0.1 mg/ml]), and kept for 1 h on ice. The protoplasts were centrifuged for 3 min at $16,000 \times g$, and the supernatant obtained represented the cell wall fraction. The complete lysis of the bacterial cells was obtained by repeating the following procedure five times: (i) resuspend the pellet in 1.5 ml of H_2O, (ii) centrifuge the suspension for 3 min at $16,000 \times g$, and (iii) save the supernatant. The five supernatants were pooled and ultracentrifuged for 1 h at $100,000 \times g$. The pellet (envelope) was resuspended in 0.1 ml of 0.25 mM Tris (pH 6.8), whereas the supernatant (cytoplasm) was precipitated with trichloroacetic acid and then resuspended in 0.1 ml of 0.25 mM Tris (pH 6.8). Each 0.1-ml fraction represented about 10^{10} cell equivalents. The protein content of cellular fractions was separated on sodium dodecyl sulfate (SDS)–10% polyacrylamide gel and transferred onto nitrocellulose membranes. The presence of the M6-LTB fusion protein was visualized using either a rabbit anti-LTB serum or an M6-specific rabbit serum (prepared by Cesare Berneri, Istituto Zooprofilattico Sperimentale of Lombardia and Emilia, Brescia, Italy).

GM1-binding assay. A GM1 enzyme-linked immunosorbent assay (ELISA) was used to analyze the capacity of whole cells of recombinant streptococci to bind to the GM1 receptor. Ten milliliters of each bacterial culture (GP1246, GP1221.1, or GP246) was grown in tryptic soy broth (TSB) without glucose (Difco) and harvested 1 h after the beginning of the stationary phase. Cells were washed once with fresh medium and resuspended in 1 ml of PBS, and 100 μl of each bacterial sample (containing approximately 10^9 CFU) was added to microtiter plates (medium binding capacity; Greiner) previously coated with 100 μl of GM1 (15 μg/ml; Sigma) per well and titrated in twofold dilutions. A pure preparation of LTB, purified from the marine *Vibrio* sp. 60 essentially as described previously (1, 24), was used as a positive control and to calculate the minimal antigen concentration required to obtain a positive optical density reading in the ELISA (3 ng/ml). Following 2 h of incubation at 37°C, plates were washed and LTB- or M6-specific antibodies were added at a 1:1,000 dilution. After 2 h of incubation at 37°C, plates were washed, and alkaline phosphatase-conjugated goat-anti rabbit immunoglobulin G (IgG) (Sigma) was added at a 1:10,000 dilution. Plates were incubated at 37°C for 2 h and washed, and 200 μl of *p*-nitrophenyl phosphate substrate (Sigma) freshly dissolved in diethanolamine buffer (pH 9.8) at a final concentration of 1 mg/ml was added to each well; absorbance at 405 nm was recorded using a 340 ATC ELISA reader (SLT Labinstruments, Salzburg, Austria).

Animals. Six-week-old female BALB/c mice were obtained from Charles River (Lecco, Italy) and maintained in our animal facilities for the duration of the experiment. All animal procedures were in accordance with institutional guidelines.

Subcutaneous immunization. Two groups of eight mice each were inoculated either with the vaccine strain GP1246 or with the control GP246. Both strains, grown in TSB without glucose (Difco), were harvested at the beginning of the stationary phase, washed once with PBS, and resuspended in fresh growth medium (1:1,000 of the original culture volume). Mice were inoculated subcutaneously (s.c.) three times (weeks 0, 3, and 6) with 10^9 CFU of bacteria in 100 μl of PBS.

Intragastric immunization. Two groups of eight mice were immunized intragastrically (i.g.) with live *S. gordonii*. Each group was inoculated either with GP1246 (vaccine) or with GP246 (control). Both strains, grown in TSB (Difco), were harvested at the beginning of the stationary phase, washed with PBS, and resuspended in fresh medium containing 3% skim milk (1:1,000 of the original culture volume). Mice were immunized twice (4 weeks apart), and each immunization consisted of two doses (24 h apart). Animals were inoculated by i.g. intubation with a ball-ended feeding needle (Popper and Sons Inc., Hyde Park, N.Y.) on days 0, 1, 28, and 29. One dose consisted of 100 μl of the bacterial suspension containing 10^{10} CFU. Food and water were removed from animals 2 h and 30 min before inoculation, respectively, and returned 30 min afterwards.

Sample collection. Blood samples were collected as previously described (26, 27) at weeks 0, 5, and 8 (s.c.), and at weeks 0, 4, and 7 (i.g.). Fecal samples were collected at weeks 0, 2, 4, 5, 6, 7, and 8. Feces evacuated during a 15-min period from each animal were weighed and carefully resuspended (at 100 mg/ml) in ice-cold PBS containing 1% bovine serum albumin. Samples were centrifuged at $12,000 \times g$ at 4°C for 10 min, and protease inhibitors and sodium azide (27) were added to supernatants before storage at −35°C.

Bacterial counts. To check for the presence of recombinant *S. gordonii* in feces, dilutions of fecal samples were plated on blood agar plates containing streptomycin (500 μg/ml). Bacterial colonies were counted, tested for resistance to erythromycin (1 μg/ml) and for expression of LTB in immunoblot, as previously described (12, 26, 27, 39).

ELISA. Determination of serum titers of LTB-specific total IgG and IgG subclasses was performed using microtiter plates (high binding capacity; Greiner) coated with 100 μl of purified recombinant LTB pentamer (1 μg/ml) (1, 24). Serum samples from individual mice and pooled sera were used to measure toxin-specific total IgG and IgG subclasses, respectively. Serum samples were initially diluted 1:16 and titrated in twofold dilutions. After a 3-h incubation at 37°C, plates were washed and alkaline phosphatase-conjugated goat anti-mouse γ-chain-specific IgG (1:3,000; Sigma), or IgG1, IgG2a, IgG2b, or IgG3 (1:1,000; Southern Biotechnology Associates), was added. Absorbance at 405 nm was recorded using a 340 ATC ELISA reader (SLT Labinstruments). Concentrations of total antibodies were determined using microtiter plates coated with 1 μg of goat anti-mouse IgG, IgG1, or IgG2a (Southern Biotechnology Associates) per ml. Pooled serum samples were diluted 1:20,000 (IgG and IgG1) or 1:2,000 (IgG2a) and titrated in twofold dilutions. To calculate the concentrations of total and specific antibodies, a standard curve for mouse myeloma IgG, IgG2a (Southern Biotechnology), and IgG1 (Cappel) was used. The concentrations of LTB-specific antibodies were normalized to those of total antibodies, and results were expressed in micrograms of LTB-specific IgG, IgG1, or IgG2a per milligram of total IgG, IgG1, or IgG2a, respectively. To reduce sample variability, the amounts of LTB-specific IgA or IgG were normalized to the concentrations of total IgA or IgG calculated for each sample (12, 26, 27). The total IgA or IgG content was determined using microtiter plates (medium binding capacity; Greiner) coated with 100 μl of goat anti-mouse IgA or IgG (1 μg/ml; Southern Biotechnology Associates). To dose total IgA and IgG, fecal samples were diluted 1:2,500 and 1:5, respectively, and titrated in a series of threefold dilutions. To determine the concentration of LTB-specific IgA or IgG, pools of fecal samples respectively containing 10 μg of total IgA or 70 ng of total IgG from each sample per ml were prepared. Samples were then diluted 1:2, added in triplicate to LTB-coated plates, and titrated in twofold dilutions. Concentrations of total and specific IgA or IgG were calculated against a standard curve of mouse myeloma IgA or IgG (Cappel) determined simultaneously in the same plate. Results were expressed in micrograms of specific IgA or IgG per milligram of total IgA or IgG.

Neutralization assay. Sera of mice immunized s.c. and i.g. were tested for the ability to inhibit the toxicity of LT on Y1 adrenal cells, as previously described (36). A total of 80 pg of LT toxin was added to each well of microtiter plates containing sera initially diluted 1:40 and then titrated in a series of twofold dilutions. Toxin and sera were incubated at 37°C for 3 h, and then 5×10^4 cells were added to wells. After 24 to 48 h, cells were examined for the typical cell rounding. Titer was defined as the reciprocal of the highest serum dilution showing complete neutralization of the toxin.

Data analysis. Samples from two separate experiments were analyzed individually. Results were expressed as the mean ± the standard deviation (SD). Statistical significance was determined by the Student *t* test, and the significance level was set at $P < 0.05$.

RESULTS

Expression of the M6-LTB fusion protein on the surface of *S. gordonii* GP1246 was demonstrated on whole cells by immunofluorescence (data not shown) and on cellular fractions by Western blotting (Fig. 1), using LTB- and M6-specific antibodies. Using whole bacterial cells in the assay, no evidence of GM1 binding was found for the LTB-expressing strain GP1246. The immunogenicity of the LTB monomer expressed on the surface of *S. gordonii* GP1246 was evaluated first by immunizing mice s.c. without adjuvant and then by i.g. delivery. As a control, mice were inoculated with *S. gordonii* strain GP246 expressing the E7 protein of HPV-16, already used for immunizing mice by oral and vaginal colonization (27, 33).

Systemic antibody response. Mice inoculated parenterally with strain GP1246 showed high serum titers of LTB-specific IgG (Fig. 2A). Animals immunized with the vaccine strain showed a significant increase of LTB-specific IgG antibodies (mean ± SD = 139,264 ± 81,625) compared to mice inoculated with the control strain GP246 (mean ± SD = 42 ± 19, $P = 0.002$), 2 weeks after the second inoculation (Fig. 2A). When GP1246 was delivered i.g., LTB-specific serum IgG peaked 3 weeks after the second inoculation (Fig. 2B). A

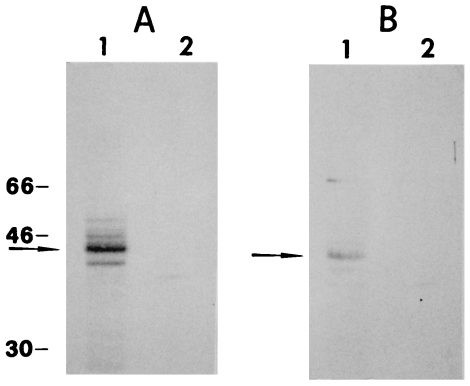

FIG. 1. Immunoblot analysis of the surface proteins of recombinant *S. gordonii*. The envelope fractions of recombinant strain GP1246, expressing the M6-LTB fusion protein (lanes 1), and of control strain GP1221.1 (lanes 2) were analyzed by Western blotting. The arrows indicate a major protein band of 44 kDa (the expected molecular mass of the recombinant protein was 41 kDa) that was found to be reactive with both anti-M6 (panel A) and anti-LTB (panel B) polyclonal antibodies. The positions of the molecular weight standards are indicated on the left (in kDa).

significant difference in the IgG titer between vaccinated animals (mean ± SD = 1,056 ± 676) and controls (mean ± SD = 58 ± 34; $P = 0.004$) was found. Analysis of the LTB-specific IgG subclasses showed that IgG1 were predominant after the parenteral (s.c.) immunization, with an IgG1-to-IgG2a ratio of 64 (Table 1). Relatively high titers of IgG2a and IgG1 were induced by mucosal (i.g.) delivery, with an IgG1-to-IgG2a ratio of 0.5 (Table 1). Further analysis of the IgG response, performed by normalizing the LTB-specific IgG1 and IgG2a concentrations to the total IgG1 and IgG2a, respectively, confirmed that the IgG1 subclass was predominant after parenteral immunization (11.9 μg per mg of total IgG1, at week 5), while IgG2a was the dominant subclass produced following mucosal delivery (5.4 μg per mg of total IgG2a, at week 7) (Fig. 3). The IgG1-to-IgG2a ratio was 12.4 for the parenteral immunization (week 5) and 0.13 for the mucosal delivery (week 7).

TABLE 1. Subclasses of LTB-specific IgG after immunization with recombinant streptococci

Type of immunization (weeks after first inoculum)	ELISA titer				IgG1/IgG2a ratio
	IgG1	IgG2a	IgG2b	IgG3	
Parenteral (5)	131,072	2,048	2,048	1,024	64
Parenteral (8)	131,072	4,096	4,096	2,048	32
Mucosal (7)	512	1,024	1,024	256	0.5

Enterotoxin neutralization. Serum samples from both the s.c. (week 8) and i.g. (week 7) immunization experiments were pooled and tested for the ability to neutralize the biological activity of the LT toxin using Y1 adrenal cells. Antibodies from

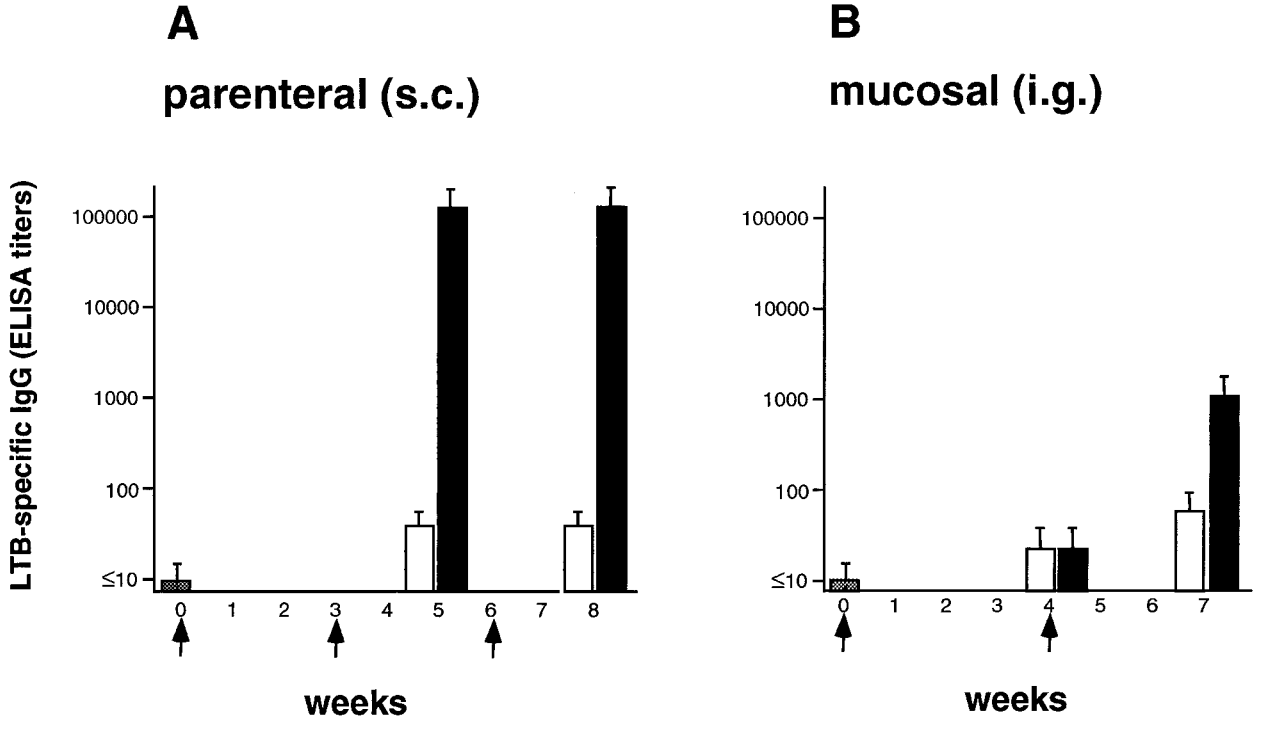

FIG. 2. ELISA titers of serum LTB-specific total IgG following s.c. (A) and i.g. (B) immunizations with recombinant *S. gordonii*. Two groups of eight BALB/c mice each were inoculated s.c. at weeks 0, 3, and 6 with 10^9 CFU of *S. gordonii* vaccine strain GP1246 expressing LTB (solid bars) or with the control strain *S. gordonii* GP246 expressing an unrelated antigen (E7 of HPV-16) (open bars). For the i.g. inoculum, two groups of eight BALB/c mice each were inoculated with live recombinant *S. gordonii* GP1246 (solid bars) or with control strain GP246 (open bars). Mice were immunized twice (weeks 0 and 4), and each immunization consisted of two doses (24 h apart) of 10^{10} CFU of live bacteria. Serum samples were collected from animals immunized s.c. and i.g. at weeks 0, 5, and 8 and 0, 4, and 7, respectively. Samples from each animal were titrated in twofold dilutions and analyzed by ELISA for the presence of LTB-specific IgG. Antibody titers are expressed as the reciprocal of the highest serum dilution with an absorbance per hour value of >0.2 after subtraction of the background value. The titer of preimmune sera is also reported (gray bars). Arrows indicate the times of inoculations. Results are expressed as the mean ± SD. Significant differences between vaccine and control animals inoculated s.c. and i.g. were calculated ($P = 0.002$ and $P = 0.004$, respectively).

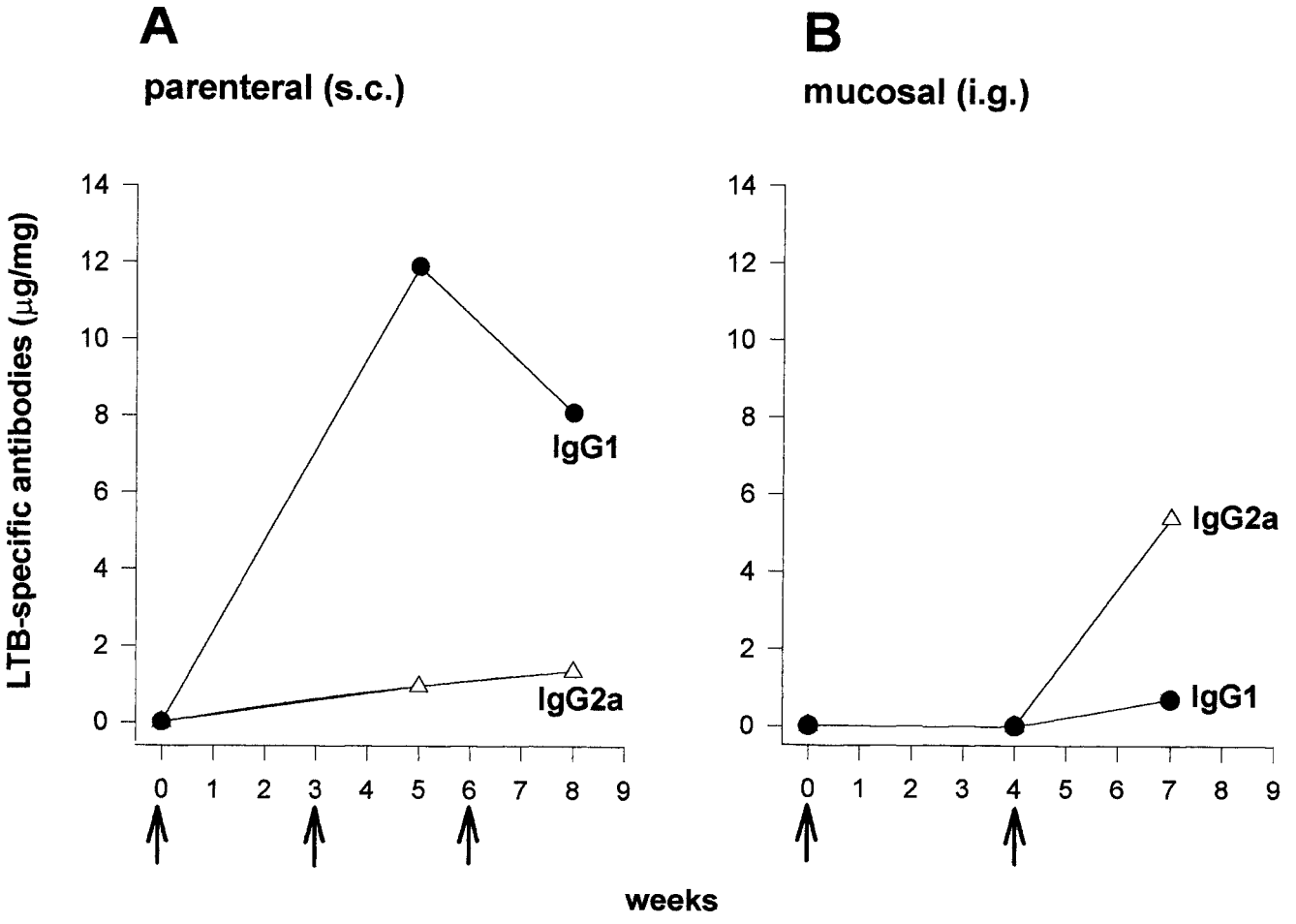

FIG. 3. LTB-specific serum antibodies after s.c. (A) and i.g. (B) immunizations with recombinant *S. gordonii*. The concentrations of total and LTB-specific IgG1 and IgG2a antibodies were determined by ELISA using standard curves of mouse myeloma IgG1 and IgG2a. The amounts of LTB-specific IgG1 and IgG2a were normalized to the concentrations of total IgG1 and IgG2a, and results were expressed in micrograms of LTB-specific IgG1 or IgG2a per milligram of total IgG1 or IgG2a, respectively. Serum samples were collected at weeks 0, 5, and 8 (s.c.) and 0, 4, and 7 (i.g.). On the *x* axes, arrows indicate the times of inoculations.

animals immunized parenterally with the LTB-expressing strain GP1246 were capable of neutralizing the enterotoxin, with the antitoxin IgG titer being 1,280, while the IgG titer from the control mice was 160. There was no difference in the neutralizing capacity of serum antibodies from animals immunized i.g. with the vaccine *S. gordonii* strain GP1246 or the control strain GP246 (titer for both groups was 160).

Local antibody response. LTB-specific IgA were found in fecal samples from mice immunized intragastrically with GP1246, with a peak at week 5 (0.65 μg per mg of total IgA). Positive samples were found from week 5 to the end of the experiment (week 8) (Fig. 4). It should be noted that LTB-specific IgA antibodies were not present in serum samples and that LTB-specific IgG antibodies were not found in feces (data not shown).

Control immune response to the E7 protein of HPV-16. Since we had successfully used *S. gordonii* GP246 expressing the E7 protein of HPV-16 as a vaccine strain in previous oral and vaginal colonization experiments (12, 27, 33), we investigated the E7-specific antibody response as a control in mice inoculated with GP246. While local and systemic LTB-specific antibodies were induced in mice by i.g. delivery (see above), no E7-specific antibodies were detected in serum and fecal samples of animals inoculated with GP246 (titer, 10). When GP246 was inoculated subcutaneously, the E7-specific IgG serum titers peaked 2 weeks after the second inoculum (titer, 80). These antibody titers are over 1,000-fold lower than those obtained with s.c. inoculation of the LTB-expressing strain GP1246 (see above).

Bacterial persistence in mouse intestine. As previously observed in oral colonization experiments (D. Medaglini, unpublished results), recombinant strains of *S. gordonii* were found capable of persisting in the mouse intestine after i.g. delivery. Three weeks after the first inoculation, 13 out of 16 animals (81.2%) were positive for the isolation of *S. gordonii* from fecal samples, and 12 of these 13 mice were still positive 3 weeks after the second inoculation. Positive samples contained 87.8 ± 9.9 CFU per mg of feces (mean ± SD).

DISCUSSION

Diarrheal diseases represent one of the leading health problems. Among these enteropathies, cholera is the most severe, while infections with enterotoxogenic strains of *E. coli* cause the largest number of cases (19). A variety of vaccines against cholera and traveler's diarrhea have been produced, and the B subunit is an essential component of many of these (19, 54). In fact, synthetic vaccines based on selected epitopes of the B

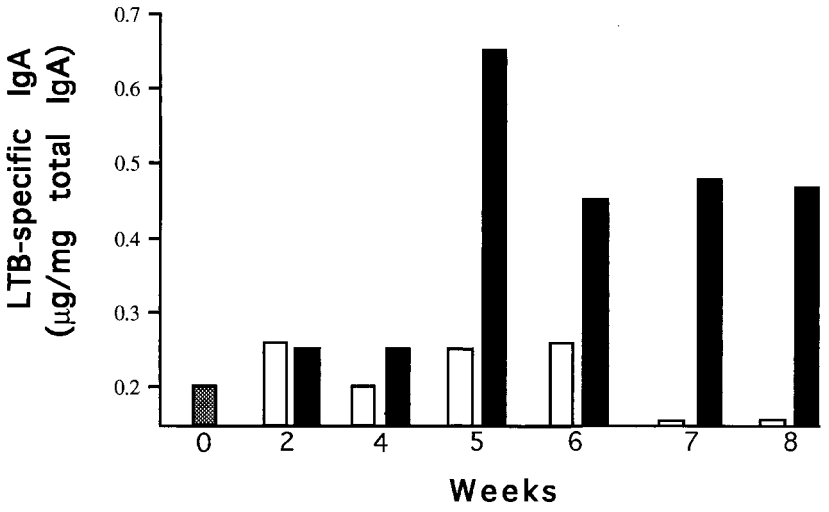

FIG. 4. LTB-specific IgA in fecal samples of mice inoculated i.g. Two groups of eight BALB/c mice each were immunized with either the vaccine strain GP1246 (solid bars) or the control strain GP246 (open bars) as described for Fig. 2. Inoculations were performed at weeks 0 and 4. Feces were collected at weeks 0, 2, 4, 5, 6, 7, and 8. To calculate the concentrations of total and LTB-specific IgA, a standard curve of mouse myeloma IgA was used. The concentration of LTB-specific IgA was determined in pools of fecal samples containing 10 μg of total IgA from each sample per ml. The amount of LTB-specific IgA was normalized to the concentration of total IgA and expressed in micrograms of specific IgA per milligram of total IgA. Antigen-specific IgA in pooled preimmune fecal samples is also reported (gray bar).

subunit have also been developed (20–22, 50). Since the parenteral route of administration was demonstrated to be a limiting factor for conferring a protective immune response against cholera and traveler's diarrhea (19, 54), the optimal approach may be based on oral delivery in order to induce IgA in the gut, where it plays a crucial role in mucosal immunity (2, 19, 55). LTB and CTB have been shown to be powerful mucosal immunogens when administered via the oral route as soluble proteins or when delivered by attenuated *Salmonella* strains (5, 15, 17, 29, 32, 48–50).

The purpose of this work was to investigate the immunogenicity of the LTB monomer expressed on the surface of the human oral commensal *S. gordonii*, for the development of a mucosal vaccine against traveler's diarrhea. The data obtained indicated the following: (i) the LTB monomer expressed on the *S. gordonii* surface is highly immunogenic in mice by parenteral immunization, inducing serum IgG titers over 1,000 times higher than those obtained using recombinant *S. gordonii* expressing a different antigen; (ii) the antibodies elicited after the parenteral immunization were capable of neutralizing the LT toxin in vitro; (iii) the LTB-expressing bacteria induce local and systemic antibody responses after mucosal (i.g.) delivery; and (iv) the analysis of serum IgG subclasses showed a predominance of IgG1 (indicative of a Th2 type of immune response) when recombinant bacteria were delivered parenterally, while a predominance of IgG2a was observed upon mucosal delivery, suggesting the involvement of the Th1 arm of the immune response.

When *S. gordonii* expressing antigens such as the E7 protein of HPV-16, the V3 domain of HIV-1 gp120, or proteins of the measles virus, are inoculated subcutaneously into mice, serum titers of IgG specific for the recombinant antigen typically reach values around 160 (41). However, subcutaneous injection of recombinant *S. gordonii* expressing the LTB monomer induces extremely high titers of LTB-specific serum IgG, even in the absence of an adjuvant. These data clearly indicate that the LTB monomer is an extremely good immunogen when expressed on the surface of gram-positive bacteria.

The LTB-specific serum IgG antibodies induced after the parenteral immunization were also capable of neutralizing the biological activity of the LT toxin, with an antitoxin titer of 1,280. Therefore, approximately 1% of the total IgG evoked after the second inoculation (mean ± SD = 139,264 ± 81,625) (Fig. 2A) resulted in protective antibodies. On the contrary, the fact that the serum IgG titer after the intragastric immunization was much lower (mean ± SD = 1,056 ± 676) (Fig. 2B), might explain why we failed to detect neutralizing antibodies.

Colonization of the murine oral and vaginal mucosa with recombinant *S. gordonii* had been previously shown to induce local and systemic antibody responses against heterologous vaccine antigens, such as the E7 protein of HPV-16 (27, 33). In the present case, the protocol of i.g. immunization was not aimed at obtaining bacterial colonization of the murine gut, but rather at using recombinant streptococci as microparticles delivering the antigen to the mucosal immune system, as in the case of the nonreplicating bacterial vaccine vehicle *Lactococcus lactis* (4). For this reason, repeated inoculations of high doses of bacteria (10^{10} CFU) were administered. In fact, recombinant bacteria were isolated from fecal samples, indicating that they can persist in the mouse intestine, although their exact localization in the gut (small or large intestine) remains to be determined. Delivery carried out i.g. with *S. gordonii* expressing LTB was also able to evoke antigen-specific IgA in the gut. The production of LTB-specific IgA is a key point for inducing a protective antitoxin immune response in the perspective of a vaccine against traveler's diarrhea, and in fact, it has been shown for experimental animals that antitoxin intestinal IgA are capable of conferring protection against cholera (19). The potent mucosal immunogenicity of LTB appears to depend upon GM1 binding that mediates the efficient uptake into the enterocytes (31). In our case, the LTB monomer expressed in *S. gordonii*, despite being unable to bind GM1, was clearly able to induce a mucosal immune response following i.g. delivery. Hence, the requirement for GM1 binding appears to be bypassed by expression of the LTB monomer on the bacterial cell surface.

The serum antibody response after i.g. immunization with

the recombinant streptococci was characterized by the production of an IgG2a response. In fact, the LTB-specific IgG2a concentration was 5.4 μg/mg, with an IgG1/IgG2a ratio of 0.13, suggesting a Th1 type of response (Fig. 3B). On the other hand, a Th2 type of response appeared to occur following parenteral immunization, as indicated by an IgG1/IgG2a ratio of 12.4 (Fig. 3A).

It is commonly accepted that Th1 cells are preferentially involved in cell-mediated immunity, while Th2 cells are more effective in the regulation and support of B-cell responses (6, 28). LTB is known to induce a mixed Th1-Th2 immune response when administered orally in a soluble form (29, 51). Here, we have shown that, after parenteral immunization with LTB-expressing streptococci, there is a clear predominance of IgG1, while IgG2a antibodies are produced upon mucosal delivery, indicating the importance of the route of immunization for the type of the immune response induced. Moreover, the ability of a nonintracellular commensal bacterium to elicit the production of IgG2a (indicative of a Th1 type of response) has important implications for using *S. gordonii* as a vector for vaccines aimed at stimulating a cell-mediated immune responses. Furthermore, this capacity of inducing Th1 responses is in accordance with data we recently obtained by studying the interaction between immature mouse dendritic cells and recombinant *S. gordonii*. In fact, we found that *S. gordonii* is internalized by conventional phagocytosis and stimulates maturation and activation of dendritic cells, and we demonstrated that the recombinant antigen expressed on the bacterial surface is presented by the dendritic cells in association not only with major histocompatibility complex class II, but also—and very efficiently—with class I molecules (42).

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