INFECTION AND IMMUNITY, May 1993, p. 1859–1866
0019-9567/93/051859-08$02.00/0

Vol. 61, No. 5

Immune Responses to *Streptococcus sobrinus* Surface Protein Antigen A Expressed by Recombinant *Salmonella typhimurium*

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In this study, we used a vaccine strain of *Salmonella typhimurium* to express antigenic determinants of the SpaA antigen of *Streptococcus sobrinus*, which is involved in the caries-forming process. We cloned either a single repeat (pYA2901) or three tandem repeats (pYA2905) of the 0.48-kb fragment of the *spaA* gene, which codes for an important component of the SpaA protein, plus a 1.2-kb minor antigenic determinant and measured the resulting immune responses to SpaA in orally immunized BALB/c mice. The single or triple repeat of the *spaA* gene fragment was inserted into the Asd$^+$ vector pYA292 and was transformed into the *S. typhimurium* Δ*cya* Δ*crp* vaccine strain χ4072 containing Δ*asd* in the chromosome. Female BALB/c mice were then orally immunized with two doses of the *S. typhimurium* containing either of the two SpaA constructs, and the immune responses to the expressed SpaA protein were assessed. Significant serum immunoglobulin G (IgG) anti-SpaA titers were detected in mice immunized with χ4072(pYA2905) but not χ4072(pYA2901). Salivary anti-SpaA IgA titers were minimal and were only detected in mice immunized with *S. typhimurium* expressing the SpaA encoded by pYA2905. Intestinal anti-SpaA IgA titers, however, were detected in both groups of mice, particularly in mice immunized with χ4072(pYA2905). An oral booster 26 weeks after the initial series of immunizations resulted in increased serum IgG titers in both χ4072(pYA2901)- and χ4072(pYA2905)-immunized animals, particularly in the χ4072(pYA2905)-immunized animals. No anamnestic IgA response was detected in the saliva following the booster immunization.

Dental caries is a major infectious disease that afflicts more than 95% of the human population worldwide. *Streptococcus mutans*, the principal etiologic agent of dental caries, represents a group of microorganisms that includes *S. cricetus*, *S. mutans*, *S. sobrinus*, *S. rattus*, and *S. downei*. Each of these species expresses major cell wall-associated proteins, of which antigens I/II and III are displayed predominantly at the cell surface (33). Of these two antigens, only one, antigen I/II, has been implicated in the induction of immunity to dental caries. Antigen I/II, also known as antigen B (34), SpaA (17), or antigen PI (12), may function as an adhesin (26) and/or a virulence factor (6) in the caries-forming process.

Since a large number of infectious agents either colonize or penetrate mucosal surfaces, it would be desirable to generate protective responses at these surfaces, thus preventing widespread colonization during the early stages of infection. This is particularly applicable to the prevention of dental caries. For instance, rats (29), monkeys (3), and humans (27) upon oral immunization with *S. mutans* elicit a secretory immunoglobulin A (SIgA) response, which is characterized by the appearance of antistreptococcal antibodies in the saliva. In rats, an increase in specific SIgA antibodies has been correlated with the reduced ability of *S. mutans* to colonize the oral cavity (28).

A variety of attenuated *Salmonella* strains have been generated in laboratories worldwide (1, 5, 7, 9, 10, 13, 16, 18, 20, 24). *Salmonella typhimurium* Δ*cya* Δ*crp* mutants, in particular, are both avirulent and immunogenic in orally immunized mice, while retaining their ability to colonize and persist in the gut-associated lymphoid tissue (7). These and other *Salmonella* strains can potentially serve as carriers by delivering heterologous antigens to the immune system upon introduction of cloned gene products (2, 10, 21, 30, 31, 36, 45). In this study, we report on the immune response of orally immunized mice to the immunodominant determinants of the antigen I component of *S. sobrinus* 6715 SpaA using such a bivalent antigen delivery system. Two constructs were generated with either a single (1×) or a triple (3×) repeat of the *spaA* fragment containing a major antigenic determinant plus a 1.2-kb minor antigenic determinant, pYA2901 and pYA2905, respectively, and were introduced into the *S. typhimurium* vaccine strain χ4072 (Δ*cya* Δ*crp*), which harbors a Δ*asd* mutation in the chromosome (30). The ability of these genetically engineered *Salmonella* strains to induce immune responses against the expressed SpaA protein was assessed.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* χ6097 and *S. typhimurium* χ4072 (30) were used. Bacterial cultures were maintained and stored as previously described (7). Plasmids constructed for this study are described below.

Construction of plasmids. Below we describe the generation of two plasmids containing either a single repeat or three tandem repeats of the 0.48-kb *spaA* fragment, encoding the major antigenic determinant. These *spaA* fragments were fused to a 1.2-kb *spaA* fragment which encodes a minor antigenic determinant and is required to generate the immunodominant determinants of SpaA (17).

A single copy of the 0.48-kb fragment of the *spaA* gene fragment, which codes for an important component of the major antigenic determinant of the SpaA protein, was in-

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serted into the *Sst*I site of pYA170 that contained the 1.2-kb *spaA* sequence, coding for a minor antigenic determinant, yielding a 1.68-kb *spaA* fragment, pYA177 (17). pYA2901 was constructed by ligating the Klenow-filled *Nco*I-to-*Hin*dIII 1.68-kb *spaA* fragment from pYA177 into the Asd$^+$ vector pYA292 (14) cut with *Eco*RI and blunt ended with Klenow. pYA2905 was constructed by purifying from agarose gels the *Eco*RI-to-*Bam*HI fragment of pYA262 (30), containing three tandem repeats of the 0.48-kb *spaA* fragment fused to a single copy of the 1.2-kb *spaA* fragment. This 2.6-kb *spaA* fragment was then ligated into pYA292 which had been cut with *Eco*RI and *Bam*HI. The Asd$^+$ *E. coli* transformants were screened for the loss of *lacZ*α complementation and then for *spaA* gene expression by colony immunoblot (35).

DNA analysis, restriction mapping, transformation, and LPS analysis. Plasmid DNA was extracted from both *E. coli* and *S. typhimurium* as described by Sambrook et al. (35). DNA restriction fragments were isolated from agarose gels with Prep-A-Gene (Bio-Rad Laboratories, Richmond, Calif.). Digestion with restriction endonucleases and fill-in reactions with the Klenow fragment of DNA polymerase I were performed as recommended by the supplier (Promega, Madison, Wis.). *E. coli* cells were transformed by the $CaCl_2$ protocol of Dagert and Erlich (8). *Salmonella* strains were transformed by electroporation, as recommended by the manufacturer (Bio-Rad), and transformants were examined for completely smooth lipopolysaccharide (LPS) by polyacrylamide gel electrophoresis (PAGE) and silver staining (19).

SDS-PAGE and Western blot (immunoblot) analysis. *Salmonella* cells were grown with aeration for 12 h in L broth and lysed in 0.1% sodium dodecyl sulfate (SDS) for 5 min at 65°C (35). The protein content of each sample was estimated by a bicinchoninic acid protein assay (Pierce, Rockford, Ill.) and adjusted to 500 μg of protein per ml with water followed by dilution in 2× loading buffer, and the sample was placed in boiling water for 3 min (35). Protein samples (40 μg per lane) were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes with a TE series Electrophoresis Transfer Unit (Hoefer, San Francisco, Calif.). Blots were first reacted with rabbit antiserum raised against recombinant SpaA (this laboratory), preabsorbed with heat-killed *S. typhimurium* and then with an alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma Chemical Co., St. Louis, Mo.), and developed as previously described (35).

Isolation and purification of antigens. LPS from the host *S. typhimurium* χ4072 (Δ*cya* Δ*crp* Δ*asd*) was prepared by hot phenol extraction (15). The extract was dialyzed against several changes of distilled water for 24 h and then lyophilized to a dry powder. SpaA was purified from the culture supernatant fluid of *S. sobrinus* 6715 (UAB 66) cultures grown anaerobically in a defined medium (44) at 37°C. Ammonium sulfate (300 mg/liter) was added to the culture supernatant fluids and mixed for 2 h at 45°C, and the resulting precipitate was collected by centrifugation at 27,000 × *g* for 50 min. The precipitate was then dissolved in and dialyzed against 25 mM sodium acetate (pH 5.5). SpaA was purified on a MonoQ anion-exchange column (Pharmacia-LKB, Piscataway, N.J.), using a low-high salt gradient (0 to 500 mM sodium chloride in 25 mM histidine buffer [pH 5.5]), at a flow rate of 0.5 ml/min. The fractions collected were tested for SpaA content by immunoblot with rabbit anti-SpaA and goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma). Fractions displaying SpaA activity, determined by spot-blot with rabbit anti-SpaA, were pooled, lyophilized, and stored at −20°C until reconstituted.

Growth of bacteria and immunization of animals. Female BALB/c mice aged 8 and 20 weeks were purchased from SASCO Inc. (Omaha, Nebr.). Immunized mice were isolated and maintained in autoclavable filter-top cages. Overnight cultures of *S. typhimurium* χ4072 containing either pYA2901 (1×), pYA2905 (3×), or pYA292 (vector alone) were diluted 1:30 in fresh L broth and grown at 37°C with aeration to an optical density at 600 nm of 0.6 (3×10^9 CFU/ml). *S. sobrinus* cultures were grown anaerobically in FMC medium (43) to an optical density at 600 nm of 0.5 (approximately 5×10^8 cells per ml). Five milliliters of each of the bacterial cultures was washed twice with buffered saline with gelatin (BSG) for 10 min at 3,000 × *g* and finally resuspended in BSG to the original volume. Mice, deprived of food and water for 4 h prior to immunization, were anesthetized with ether and intubated intragastrically with a 21-gauge feeding needle (Popper & Sons Inc., Hyde Park, N.Y.) with 100 μl of *S. typhimurium* χ4072 containing pYA292, pYA2901 (1×), or pYA2905 (3×); BSG alone (control); or *S. sobrinus*. Food and water were returned 30 min later. A second immunization was repeated 10 days later. An oral booster immunization with recombinant *Salmonella* strains was administered 26 weeks after the initial immunizations under the same protocol.

Sample collection. Blood samples were collected from the retro-orbital sinus of anesthetized mice prior to immunization and then collected at 2, 3, 4, 6, 8, and 10 weeks after the second immunizing dose. Similarly, blood samples were collected at the same times following the booster immunization. Salivation was induced by intraperitoneal injection of pilocarpine (0.1 ml [7.5 mg/kg of body weight]) dissolved in 0.85% NaCl. Saliva was collected prior to immunization, 10 days after the second immunization, and then at weekly intervals and at similar time points after the booster immunization. Gut secretions were collected from euthanized mice 10 and 24 days postimmunization, together with serum samples. The small intestine, from cecum to duodenum, was excised and washed three times in ice-cold 0.85% NaCl. Intestinal contents were removed, and the mucus was scraped from the luminal surface, collected in a centrifuge tube containing 5 μl each of 0.1 M phenylmethylsulfonyl fluoride in ethanol, 1% bovine serum albumin (BSA), and 1% sodium azide and then centrifuged at 12,000 × *g* for 10 min at 4°C. Supernatant fluids were collected to which were added an additional 5 μl of 0.1 M phenylmethylsulfonyl fluoride and 1% sodium azide before storage at −20°C until use.

Colonization of lymphoid tissues by *S. typhimurium*. Mice were immunized as above with *S. typhimurium* containing the construct pYA292 (cloning vector), pYA2901, or pYA2905. Mice were subsequently euthanized at 2, 5, 10, and 20 days after the second oral immunization, and the Peyer's patches, mesenteric lymph nodes, and spleens were processed as previously described (7) to estimate the extent of colonization by *S. typhimurium*. Bacteria recovered from immunized animals were enumerated with Difco MacConkey base agar with 1% maltose and 40 μg of nalidixic acid per ml.

Measurement of antibody response. Anti-SpaA and anti-LPS antibodies in intestinal secretions, saliva, and sera were quantitated by an enzyme-linked immunosorbent assay (ELISA). Specifically, 96-well Immunlon-1 plates (Dynatech, Chantilly, Va.) were coated with either 10 μg of methylated LPS per ml (39) or 5×10^8 formalin-killed *S. sobrinus* 6715 cells per ml (29) in 0.2 M bicarbonate-carbon-

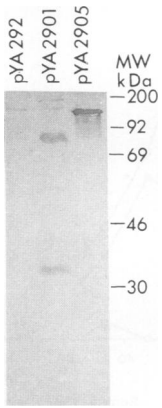

FIG. 1. Western blot of an SDS-10% PAGE gel of whole-cell lysates of *S. typhimurium* containing the expression vector pYA292 or the SpaA construct pYA2901 (1×) or pYA2905 (3×). Each well was loaded with 40 μg of protein.

ate buffer (pH 9.6). The total IgA and IgM content of saliva and intestinal secretion samples was determined with plates coated with goat anti-mouse IgA or IgM (Southern Biotechnology, Birmingham, Ala.) at a dilution of 1:1,000 in the same buffer and mouse myeloma IgA and IgM standards (Cappel Research, Durham, N.C.). Nonspecific binding sites were blocked with 1% BSA (Sigma) in phosphate-buffered saline (PBS) plus 0.1% Tween 20 (pH 7.4) (blocking buffer). Initially, saliva samples were diluted 1:1 and intestinal mucus samples were diluted 1:5 with blocking buffer. A volume of 100 μl was added in duplicate to the plates and then diluted in a series of twofold dilutions, incubated at 37°C for 2 h, and washed with PBS plus 0.05% Tween 20. The presence of IgA or IgM in the samples was detected with biotin-avidin-labelled goat anti-mouse IgA and IgM (Southern Biotechnology) and alkaline phosphatase-labelled Extravidin (Sigma) used at a dilution of 1:1,000 and 1:4,000, respectively, in blocking buffer. Serum samples were initially diluted 1:100 in blocking buffer and then placed in a series of twofold dilutions and processed as above with biotin-labelled goat anti-mouse IgA and IgG. The optical density of the resulting substrate reaction, *p*-nitrophenylphosphate (Sigma) (1 mg/ml) in 0.1 M diethanolamine buffer (pH 9.8), was read at 405 nm with an automated reader (BioTek, Burlington, Vt.).

RESULTS

Expression of SpaA. The SpaA protein expressed by both *E. coli* and *S. typhimurium* that contained either pYA2901 or pYA2905 reacted strongly with the antiserum raised against the whole protein extracted from culture supernatant. Figure 1 shows a Western blot of whole-cell lysates of *S. typhimurium* χ4072 containing the expression vector pYA292 or the SpaA construct pYA2901 (1×) or pYA2905 (3×). The estimated molecular masses of the proteins encoded by pYA2901 and pYA2905, 86 and 145 kDa, respectively, are significantly larger (approximately 33%) than the predicted sizes as deduced from the lengths of the inserts. This particular phenomenon has been observed with other *S. sobrinus spaA* constructs as well as other proteins (22). It was also observed that a smaller protein, approximately 45 kDa, was produced when a lysate of χ4072(pYA2901) was prepared, and this protein is believed to be a cleavage product of the 86-kDa protein.

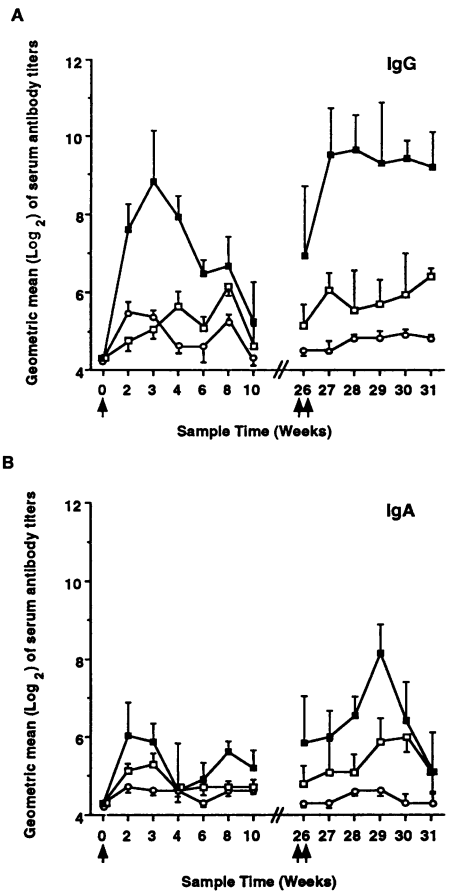

FIG. 2. Serum anti-SpaA response in 8-week-old mice following two oral immunizations, 10 days apart (↑), with *S. typhimurium* χ4072 containing pYA2901 (□) or pYA2905 (■) or with BSG alone as a control (○). A booster immunization was administered 26 weeks later using the same constructs (↑↑). A preimmunization serum sample was taken on day 0, and time points are weeks after the second immunization. Serum IgG (A) and IgA (B) levels are expressed as the geometric mean ($\log_2$) of the titers, where $n = 10$.

Colonization of lymphoid tissue by *S. typhimurium*. Mice were orally immunized with two doses, 10 days apart, of *S. typhimurium* χ4072 containing one of three plasmids, pYA292, pYA2901, or pYA2905, and the colonization of the Peyer's patches, mesenteric lymph nodes, and spleen of these mice by the *Salmonella* cells was monitored over a 20-day period. *Salmonella* cells could be recovered from all groups of mice during the 20-day time course, and the extent of colonization was found to be almost equivalent (data not shown). Peak *Salmonella* levels were found within the first 10 days postimmunization in all immunized groups, and at the 20-day sample point, *Salmonella* cells were isolated at moderately lower levels. *Salmonella* cells could also be isolated from mice 30 days postimmunization but in significantly lower numbers.

Serum antibody responses. Mice immunized with *S. typhimurium* expressing the SpaA protein encoded by pYA2905 (3×) raised significant humoral IgG titers to the expressed protein with no significant difference between the titers detected by immunizing 8-week-old mice (Fig. 2A) and

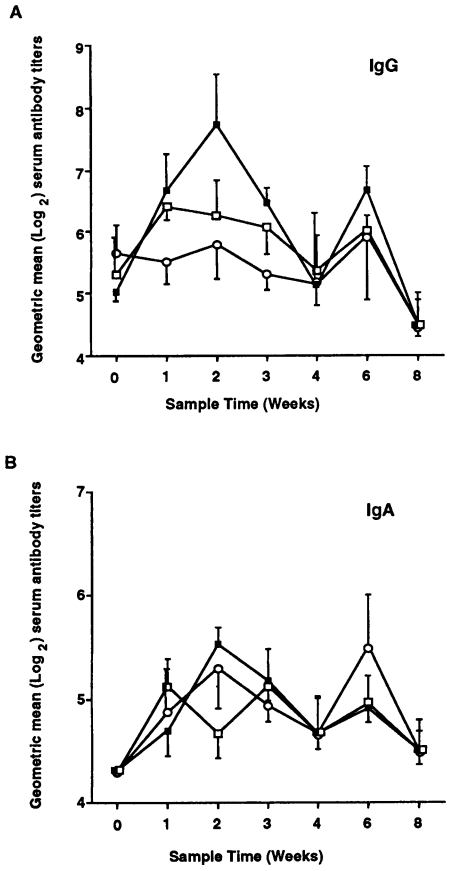

FIG. 3. Serum anti-SpaA responses of 20-week-old BALB/c mice following two oral immunizations 10 days apart with *S. typhimurium* χ4072 containing either pYA2901 (□) or pYA2905 (■). Serum IgG (A) and IgA (B) are expressed as the geometric mean (log_2) of the titers, where $n = 8$. ○, BSG control. A preimmunization sample was taken at day 0, and time points are weeks after the second immunization.

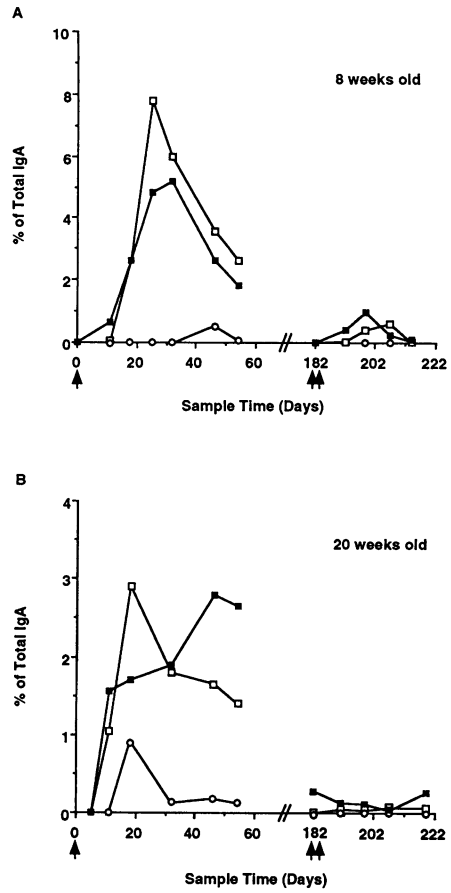

FIG. 4. Anti-LPS IgA levels in the saliva of 8-week-old (A) and 20-week-old (B) BALB/c mice after two oral immunizations 10 days apart (↟) with *S. typhimurium* χ4072 containing either pYA2901 (□) or pYA2905 (■) or with BSG alone as a control (○), followed by a booster immunization 26 weeks later (↟↟). Results are expressed as the percentage of the total IgA present in the saliva that is specifically directed against *Salmonella* LPS. Saliva samples were pooled and run in duplicate, where for 8-week-old mice $n = 10$ and for 20-week-old mice $n = 8$. Day 0 indicates the preimmunization sample, and time points thereafter are following the second initial immunization.

20-week-old mice (Fig. 3A). Serum IgA titers, however, remained low in both groups of mice (Fig. 2B and 3B) and were only significantly raised above background in the 8-week-old mice. Those mice immunized with *S. typhimurium* containing pYA2901 (1×) raised only minimal IgG titers to the expressed SpaA and very low IgA titers. Following a booster immunization, 26 weeks after the initial series of immunizations, an anamnestic response was detected in the sera of mice originally immunized at 8 weeks of age, particularly in those mice immunized with *Salmonella* strains containing pYA2905 (3×) (Fig. 2A). This IgG response remained high and was accompanied by an IgA response which peaked 3 weeks after the booster immunization. Those mice immunized with *S. typhimurium* containing pYA2901 (1×) had a considerably lower anamnestic IgG response which was accompanied by a moderate IgA response. Mice originally immunized at 20 weeks of age also received a booster immunization; however, samples taken 3 and 4 weeks later indicated only minor rises above the baseline of control animals and are not shown. The control mice (BSG fed) were found to have low levels of naturally occurring serum antibodies to SpaA, particularly the IgA isotype, and these values were used as a baseline to indicate positive responses to the recombinant SpaA. Serum IgG responses to *Salmonella* LPS were monitored following the initial immunizations, and these were found to be considerably greater than those raised in response to the expressed antigen, SpaA (data not shown).

Salivary antibody responses. The salivary IgA titers to both SpaA and *Salmonella* LPS were evaluated in both age groups of mice and expressed as the percentage of the total IgA present that was specifically directed against either of the two antigens. In both 8- and 20-week-old mice, significant anti-LPS IgA levels were detected in the saliva of immunized animals, with maximum levels usually detected 17 to 25 days after the second immunization (Fig. 4A and B). Salivary antibodies to the expressed protein were, however, difficult to detect in the 8-week-old mice, with a maximum level of 0.17% being detected in mice immunized with *S. typhimurium* containing the construct pYA2905 (3×) (Fig. 5A). In the saliva of 20-week-old mice immunized with *S. typhimurium* containing the same construct, a higher titer was detected, a maximum of 0.51% (Fig. 5B). Booster immunization did not result in elevated levels of either

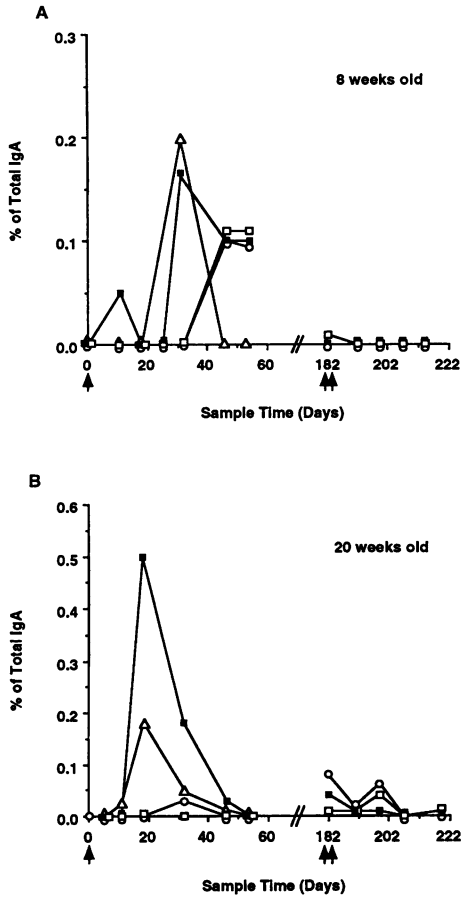

FIG. 5. Anti-SpaA IgA levels in the saliva of 8-week-old (A) and 20-week-old (B) BALB/c mice following two oral immunizations 10 days apart (↟) with *S. typhimurium* χ4072 containing either pYA2901 (□) or pYA2905 (■) and a booster immunization 26 weeks later (↟↟) or two oral immunizations with *S. sobrinus* (△). Results are expressed as the percentage of the total IgA present in the saliva specifically directed against SpaA. Samples were pooled and run in duplicate. For 8-week-old mice $n = 10$ and for 20-week-old mice $n = 8$. ○, BSG control. Day 0 indicates a preimmunization sample, and time points thereafter are samples taken after the second initial immunization.

anti-SpaA or anti-LPS salivary IgA antibodies in any of the groups of mice. Mice immunized with two oral doses of *S. sobrinus* were also found to have salivary anti-SpaA IgA titers. In 8-week-old mice, the levels detected were comparable to those in the pYA2905 (3×) group (Fig. 5A) but were lower than the peak response detected in the saliva of 20-week-old mice (Fig. 5B) also immunized with *S. typhimurium* containing pYA2905 (3×). No salivary IgM antibodies to either LPS or SpaA were detected in immunized mice, and the presence of IgG antibodies in saliva was not evaluated.

Intestinal antibody responses. Intestinal secretions from control mice and those orally immunized with *S. typhimurium* containing either pYA2901 (1×) or pYA2905 (3×) were collected (and pooled) at 10 and 24 days postimmunization. An ELISA for specific anti-SpaA antibodies demonstrated that IgA antibodies to SpaA were present in the intestinal secretions of mice immunized with *S. typhimurium* containing either of the SpaA constructs (Table 1). Those mice

TABLE 1. Serum and intestinal IgA anti-SpaA levels in BALB/c mice immunized with SpaA-expressing *S. typhimurium*

Immunization group	Intestinal secretions (% of total IgA)[a]		Serum IgA titer[b]	
	Day 10	Day 24	Day 10	Day 24
Control	0	0.71	0	0
pYA2901	0	0.92	100	0
pYA2905	2.68	3.75	250	200

[a] Anti-SpaA IgA levels in intestinal secretions are expressed as the percentage of the total IgA present specifically directed against SpaA. Samples were pooled ($n = 3$).

[b] Serum titers are expressed as the last dilution that gave an optical density at 405 nm of 0.1 ($n = 3$).

orally immunized with *S. typhimurium* expressing the SpaA protein encoded by pYA2905 (3×) gave particularly high levels of specific antibodies at both time points. High titers of anti-LPS IgA antibodies were also detected in the intestinal secretions of those mice immunized with *S. typhimurium* but not in the control group. Intestinal secretion samples contained low levels of IgM, and IgG was detected in some samples, but no antigen-specific antibodies of either isotype were detected.

DISCUSSION

The Δ*cya* Δ*crp* *Salmonella* vaccine strains have been shown to be both avirulent and immunogenic in mice (7), and the introduction of the Asd^+ plasmid into the Δ*asd* *Salmonella* mutants completely restores virulence (30). These mutants express high levels of cloned gene products and are very stable, both in vivo and in vitro, without adverse effects on the growth of the bacteria (30). The SpaA hybrid proteins expressed by *S. typhimurium* χ4072 and *E. coli* χ6097 containing either of the *spaA* constructs pYA2901 (1×) or pYA2905 (3×) reacted strongly with the antisera raised against the recombinant SpaA protein and did not affect the ability of *S. typhimurium* to colonize the Peyer's patches and reach the spleen. That these proteins exhibited higher molecular weights than was predicted is not an unusual feature as other workers have reported similar observations with other gene products (22).

Initial studies in which mice were orally immunized with only one dose of *S. typhimurium* with either of the two SpaA constructs resulted in only minor or no antibody responses (unpublished data). In this study, two oral doses at 10-day intervals resulted in significant serum IgG antibody responses, particularly with *S. typhimurium* containing the plasmid with the triple repeat of the 0.48-kb *spaA* fragment, pYA2905. However, Chatfield et al. (4) have been successful in inducing protective serum IgG antibodies to fragment C of tetanus toxin after a single oral dose with a recombinant *aroA aroD* *S. typhimurium*. The need for multiple doses of recombinant *S. typhimurium* for the induction of immune responses to the heterologous antigen is contrasted by the observation that one dose of avirulent *S. typhimurium* protects against subsequent challenge with isogenic wild-type strains (7), with induction of high systemic antibody titers against *Salmonella* antigens (8a).

More recently, Takahashi et al. (42) demonstrated that immune responses to the cell surface protein antigen (PAc) of *S. mutans* and a peptide of the protein antigen, PAc(301–319), were affected by the *H-2* haplotype of the mouse strain used. The serum antibody (IgG) response to both the peptide

and recombinant protein following subcutaneous immunization of a number of mouse strains indicated that they could be divided into high (BALB/c *H-2*^{d}), intermediate (C57BL/6 *H-2*^{b}), and low (DBA/1 *H-2*^{s}) responders. This was also observed in our earlier studies with recombinant *S. typhimurium* expressing the analogous *S. sobrinus* SpaA. In these studies, when comparing the response to SpaA in BALB/c and C57BL/6 mice immunized with recombinant *S. typhimurium*, we observed that C57BL/6 mice did not respond to the SpaA expressed by *S. typhimurium* with pYA2901 and only weakly to pYA2905 (unpublished data). The involvement of the *H-2* complex in the response to antigens expressed by recombinant *S. typhimurium* has been demonstrated by Sjöstedt et al. (37). Using *S. typhimurium* χ4072 to express the 17-kDa lipoprotein (TUL4) of *Francisella tularensis*, they were able to show that mice of different haplotypes recognized different synthetic peptides of TUL4, *H-2*^{s} mice not recognizing any of the peptides.

Reports of secretory immune responses to the antigens expressed by recombinant *Salmonella* strains have been varied. Intestinal anti-SpaA IgA titers were detected in mice orally immunized with *S. typhimurium* containing either of the constructs, particularly those animals immunized with *S. typhimurium* expressing the SpaA protein encoded by pYA2905 (3×); however, the desired site for a protective antibody response against a colonization antigen of oral streptococci would be the oral mucosa. Salivary anti-SpaA IgA responses were detected in this study, particularly in older animals, but only in those animals immunized with *S. typhimurium* expressing the SpaA protein encoded by pYA2905 (3×), containing the triple repeat of the 0.48-kb SpaA antigenic determinant. Unlike the anti-SpaA response, salivary anti-LPS IgA titers were considerably greater and were present in all groups immunized with recombinant *S. typhimurium*. In similar experiments in which germfree rats were immunized with χ4072(pYA2905), salivary anti-SpaA IgA titers were detected and preceded the serum anti-SpaA response (42). A booster immunization 21 days later was found to potentiate the mucosal response to the SpaA expressed by the recombinant *S. typhimurium*. Whether the salivary anti-SpaA antibodies are capable of reducing or preventing the cariogenic process is unknown and will be evaluated with the germfree rat model, an established caries model system.

Apart from the induction of specific mucosal antibody responses, significant serum anti-SpaA IgG titers were detected particularly in mice immunized with *S. typhimurium* containing pYA2905 (3×) and were observed in both 8- and 20-week-old animals. The induction of specific systemic antibody responses to a variety of antigens expressed by orally delivered recombinant *S. typhimurium* has been reported previously (2, 4, 21, 36, 37, 40, 41, 44). In some instances, these serum responses were accompanied by protection against a subsequent challenge with the pathogen (4, 31, 37, 44), while serum from mice orally infected with *S. typhimurium aroA* mutants expressing the *E. coli* heat-labile toxin B subunit (LT-B) was capable of neutralizing the holotoxin in tissue culture assays (25). These serum antibodies may also be of importance in protection at mucosal surfaces. IgG detected in the gingival crevices of mice parenterally immunized with *S. mutans* resulted in reduced colonization of the oral mucosa by cariogenic streptococci accompanied by high serum IgG titers (3, 23, 38). These serum immunoglobulins are thought to be sequestered into the oral cavity via the gingival crevicular fluid (38), contributing to the overall levels of IgG present in saliva.

Of particular interest is that booster immunization with *S. typhimurium* containing either pYA2901 (1×) or pYA2905 (3×) resulted in elevated serum IgG and IgA anti-SpaA titers, particularly with the latter construct. While a serum anamnestic response was observed following an oral booster, specific salivary IgA antibodies to either *Salmonella* LPS or SpaA were not. Booster oral immunization with *S. typhimurium aroA* mutants expressing LT-B also resulted in slightly elevated IgG anti-LT-B titers together with an increase in anti-LT-B IgA levels in the intestine (9). It does appear that it is possible to generate a memory response to heterologous antigens expressed by *S. typhimurium*, but this may depend on the period between the initial immunizations and the subsequent booster immunization. To a certain degree, it also appears that this memory antibody response is short-lived and is generally not greater than that observed following the first immunization, and the peak response is delayed in onset compared with the initial IgA response.

Using the recombinant *Salmonella* system, we were able to demonstrate the induction of both systemic and mucosal immune responses to the expressed antigen SpaA. The difference in the salivary response between the 8- and 20-week-old mice is, perhaps, not unexpected as Ebersole and Steffen (11) have shown in rats that the age at immunization is an important parameter when measuring SIgA immune responses. When monitoring SIgA to T-dependent antigens, they observed that the antibody responses were greater in adult rats (90 to 120 days old) than in weanling rats (21 to 35 days old), and such differences could be analogous to the responses that were detected in this study. Oral boosting with *S. typhimurium* expressing the same antigen results in a serum IgG anamnestic response but not a salivary mucosal response. Substantially higher serum anti-SpaA antibodies were detected in mice immunized with *S. typhimurium* containing the plasmid pYA2905 (3×), which encoded three repeats of the major antigenic determinant of SpaA (17). We are now evaluating the cellular immune response to the SpaA protein encoded by the two constructs. We are also investigating the possibility of enhancing the salivary mucosal response by generating fusion proteins in which SpaA (pYA2905) will be fused to LT-B. It is hoped that the LT-B will act as an adjuvant eliciting greater mucosal responses to SpaA, particularly in the oral cavity.

ACKNOWLEDGMENTS

We thank Grace Harris, Lisa Burns-Keliher, Mary Wilmes-Riesenburg, and Steve Tinge for their critical reviewing of the manuscript and S.-Y. Wanda for help with the purification of SpaA.

This work was supported by Public Health Service grants DE-06669, DE-06673, and AI-26186 (to Josephine Clark-Curtiss) from the National Institutes of Health and by an unrestricted grant from Bristol Meyers-Squibb.

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