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Oral Immunization with Recombinant *Salmonella typhimurium* Expressing Surface Protein Antigen A of *Streptococcus sobrinus*: Persistence and Induction of Humoral Responses in Rats

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Recombinant *Salmonella typhimurium* has been used as an oral vaccine for various microbial pathogens. Here we report immune responses in Fischer rats orally immunized with a recombinant *S. typhimurium* strain encoding surface protein antigen A (SpaA) of *Streptococcus sobrinus*. The attenuated *S. typhimurium* χ4072 Δ*cya* Δ*crp* Δ*asd* mutant used in this study contains the Asd$^+$ plasmid pYA2905 expressing a fragment of the SpaA protein. *Salmonella* cells were cleared from spleens by 7 days and from Peyer's patches by 14 days in rats receiving a single oral immunization of 10^9 CFU of χ4072. In animals receiving multiple (i.e., days 0 and 7 or days 0, 7, and 21) immunizations, *Salmonella* cells were cleared from the Peyer's patches by 25 days following the initial immunization. Antigen-specific systemic and mucosal antibody responses were greater in rats receiving multiple immunizations than in those receiving a single immunization. Serum anti-*Salmonella* activity was potentiated following boosting on day 21. Mucosal immunoglobulin A antibody responses were also greater in rats receiving multiple immunizations than in rats receiving a single immunization. Anti-*Salmonella* and anti-*Streptococcus* immunoglobulin A activity persisted longer in rats boosted on day 21 than in rats immunized on days 0 and 7. These data indicate that oral immunization of rats with the recombinant *S. typhimurium* χ4072(pYA2905) vaccine induces systemic as well as mucosal antibody responses specific to the *Salmonella* cells and to the cloned SpaA protein. This is the first report of the use of an attenuated mutant of the murine pathogen *S. typhimurium* as an oral vaccine in rats.

The major way that microbial pathogens cause disease is by colonization of, or invasion through, mucosal surfaces. Therefore the induction of immune responses to a pathogen at these surfaces should serve as a first line of defense against infection. Immunoglobulin A (IgA) is the predominant isotype of antibody in mucosal secretions (34). The oral administration of antigen has been shown to result in IgA antibody responses in a variety of secretions (e.g., saliva) via stimulation of the common mucosal immune system (32, 33). When antigen is given orally, it is taken up by the specialized M cells interspersed throughout the luminal epithelium of Peyer's patches in the gut-associated lymphoid tissue (GALT) and delivered to the underlying lymphoid cells. The antigen-stimulated T and B cells leave Peyer's patches, migrate to numerous mucosal tissues, and mediate an antigen-specific IgA immune response in the external secretions (27).

Since most orally administered nonviable antigens are inefficient in stimulating the GALT, several approaches are currently being investigated to develop oral vaccines which are more effective in inducing mucosal IgA responses. Inert particulate carriers such as microspheres and liposomes, in addition to live delivery systems such as bacterial and viral carriers, are being studied for use as oral vaccines (32). Recombinant *Salmonella* strains are particularly useful as oral vaccines since *Salmonella* organisms colonize Peyer's patches in the GALT (4). Several types of attenuated *Salmonella* strains have been developed for use as oral vaccine delivery systems, including those with mutations in aromatic biosynthetic metabolism (*aroA* and *aroC* mutants), UDP-galactose synthesis (*galE* mutants), phosphate metabolism (*phoP* mutants), and regulation of outer membrane proteins (*ompR* mutants) (reviewed by Curtiss [5] and Doggett [10]). Ideally, *Salmonella* vaccine strains should have two attenuating mutations to prevent reversion to the virulent phenotype. For example, *Salmonella typhimurium* Δ*cya* Δ*crp* mutants, which lack adenylate cyclase and the cyclic AMP receptor protein, have been shown to be stable and safe for in vivo use (6–8). Oral immunization with these mutants has been shown to induce humoral and cellular responses to *Salmonella* strains (44) and to cloned gene products expressed by this live vector (3).

Streptococcus mutans (serotype *c*) and *Streptococcus sobrinus* (serotype *g*) are considered major etiologic agents of human dental caries (31). The pathogenesis of this disease involves at least two initial stages: a sucrose-independent stage, in which the bacteria adhere to glycoproteins in the pellicle coating the tooth surface, followed by a sucrose-dependent stage involving adherence and colonization of mutans streptococci (15, 20). Finally, acid production by the colonizing *S. mutans* and *S. sobrinus* results in demineralization of the tooth surface. A virulence factor believed to be involved in adherence of *S. mutans* is Ag I/II (28, 39). This protein functions as a receptor for salivary agglutinin or as an adhesin which interacts with the saliva-coated tooth surface (1, 19). *S. sobrinus* produces a protein called SpaA, which is very similar to Ag I/II. Within the middle region of the two proteins, SpaA and Ag I/II share 66% amino acid identity (29, 46).

Previous studies have tested the effectiveness of Ag I/II in inducing protective antibody responses against *S. mutans* infection (39). Subcutaneous immunization of Rhesus monkeys with Ag I/II in incomplete Freund's adjuvant resulted in IgG antibodies to Ag I/II in serum as well as in gingival crevicular fluid, and the presence of these antibodies correlated with

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protection from caries formation (30). Oral administration of Ag I/II does not result in notable immune responses. However, salivary IgA responses were induced when Ag I/II was given in liposomes (35) or when Ag I/II was coupled to the B subunit of cholera toxin and given along with a small amount of cholera toxin as adjuvant (9, 35, 41). The induction of salivary IgA and anti-Ag I/II responses in rats correlated with protection against *S. mutans*-induced dental caries (35). In other studies it has been shown that intranasal immunization of mice (47) or rats (24) with Ag I/II also resulted in the induction of salivary IgA anti-Ag I/II antibodies and that their presence in the latter model correlated with protection.

The goal of this study was to determine the effectiveness of a recombinant *S. typhimurium* vaccine in inducing immune responses to the cloned SpaA protein in rats. The recombinant SpaA is expressed by *S. typhimurium* χ4072(pYA2905), a live, avirulent Δ*cya* Δ*crp* Δ*asd* mutant of *S. typhimurium* (8). Plasmid pYA2905 contains three tandem 0.5-kb repeats of a fragment of the SpaA gene encoding a major antigenic determinant followed by a 1.2-kb fragment encoding a minor antigenic determinant of SpaA (17, 18). In this study we measured the persistence of the attenuated *S. typhimurium* strain in Peyer's patches and spleens of rats after different oral immunization regimens. The serum and mucosal antibody responses to both the recombinant SpaA and the *Salmonella* carrier were also measured. The relationship between persistence and the immune response is discussed.

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MATERIALS AND METHODS

Animals. Germ-free (termed gnotobiotic after infection with a specific organism) Fischer rats [CDF (344) GN/Crl BR] are bred and maintained in Trexler plastic isolators at the University of Alabama at Birmingham. Weanling rats (age 19 days) to be used in these studies were removed from the isolators and maintained in covered sterile cages in a laminar-flow hood. The rats were provided with autoclaved food (Prolab animal diet RMH 1000; Agway Country Foods, Inc., Syracuse, N.Y.) and water ad libitum. Male and female rats approximately 8 weeks old were used in these experiments. All animal experiments performed in these studies were approved by the University of Alabama at Birmingham Animal Resources Advisory Committee.

Microorganisms. *S. typhimurium* LT-2 and χ4072, a derivative of SR-11, (37) (kindly provided by Roy Curtiss III, Washington University, St. Louis, Mo.), were used in these studies. *S. typhimurium* χ4072 is attenuated as a result of deletions in the genes encoding adenylate cyclase (Δ*cya-1*) and the cyclic AMP receptor protein (Δ*crp-1*). This strain also has a deletion in the gene encoding aspartate β-semialdehyde dehydrogenase (Δ*asdA1*) which renders it deficient in diaminopimelic acid (DAP), an essential component of the cell wall. In the absence of DAP, *S. typhimurium* χ4072 cannot replicate and undergoes DAP-less cell death. In addition, *S. typhimurium* χ4072 lacks the 100-kb *S. typhimurium* SR-11 virulence plasmid, pStR100. Stocks of these strains were stored as frozen cultures in L broth (10% tryptone [Difco Laboratories, Detroit, Mich.], 5% yeast extract [Difco], 5% NaCl [Sigma Chemical Co., St. Louis, Mo.], 1% glucose [Sigma]) containing 15% glycerol (Sigma) at −70°C. Cultures of *S. typhimurium* χ4072 and LT-2 were grown in L broth at 37°C in a rotary shaker for preparation of the challenge inoculum and of whole-cell antigen for use in the enzyme-linked immunosorbent assay (ELISA), respectively (see below).

S. sobrinus 6715 was stored at −70°C in brain heart infusion broth (Difco) containing 50% glycerol. Cultures grown in brain heart infusion broth for 18 h at 37°C in an anaerobic GasPak System (BBL Microbiology Systems, Cockeysville, Md.) were used to inoculate large batch cultures to generate whole-cell and purified antigen preparation (see below).

Reagents. Formalin-killed whole cells of *S. typhimurium* LT-2 and of *S. sobrinus* 6715 were prepared for use in the ELISA. *S. typhimurium* LT-2 was grown in L broth at 37°C with aeration in a rotary shaker for 18 h. The bacteria were harvested by centrifugation (6,000 × *g*), washed three times with sterile saline, and resuspended in 0.1% formalin–saline at a density of 5×10^{10} bacteria per ml. *S. sobrinus* 6715 was cultured anaerobically in brain heart infusion broth at 37°C for 18 h. The bacteria were harvested and washed as described for the preparation of *S. typhimurium* LT-2 whole cells and then resuspended in 0.5% formalin–saline. The formalin-killed whole-cell antigens were stored at 4°C until used.

Native SpaA was prepared from the culture supernatants of *S. sobrinus* 6715 by a modification of the procedures described previously for the purification of *S. mutans* Ag I/II (40). Briefly, *S. sobrinus* was grown at 37°C in chemically defined media for group A streptococci (JRH Biosciences, Lenexa, Kans.) in a 28-liter fermentor. After 18 h of growth, cells were harvested and 24 liters of culture supernatant was concentrated to 2 liters by using a Pelicon filter (Millipore Corp., Marlborough, Mass.) with a 10,000-molecular-weight-cutoff membrane. Ammonium sulfate (520 g/liter; Sigma) was added to the concentrate, and the mixture was stirred overnight at 4°C. The resulting precipitate was collected by centrifugation at 13,700 × *g* for 30 min. The pellet was resuspended and dialyzed against 0.01 M Tris-HCl (pH 8.0; Sigma). SpaA was then purified chromatographically by using DEAE-cellulose (Whatman BioSystems Inc., Clifton, N.J.) and Sephacryl S-300 (Pharmacia Biotech Inc., Piscataway, N.J.). Purity was tested by immunodiffusion initially with rabbit anti-Ag I/II serum (provided by M. W. Russell, University of Alabama at Birmingham) and later with rabbit anti-SpaA serum (see below) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Rabbit anti-SpaA hyperimmune sera were generated by immunizing rabbits with native SpaA purified from *S. sobrinus* 6715. Rabbits were immunized subcutaneously along the back with an alum (0.25% Sigma) precipitate of SpaA (0.5 mg) emulsified in complete Freund's adjuvant (Difco). On days 14 and 28 following the initial immunization, animals were given boosters with an alum (0.25%) precipitate of SpaA (0.5 mg) emulsified in incomplete Freund's adjuvant (Difco).

A portion of the rabbit anti-SpaA hyperimmune sera was biotinylated for use in the capture ELISA to quantitate the amount of SpaA produced by the recombinant *Salmonella* strains. Briefly, the Ig was precipitated with 50% ammonium sulfate by overnight incubation at 4°C with stirring and centrifugation (13,000 × *g* for 20 min). The resulting precipitate was resuspended and dialyzed against 0.1 M Tris-HCl (pH 8.0). The IgG fraction of the serum was purified by fast protein liquid chromatography (Pharmacia Biotech Inc.). The purity of the fraction was assessed by immunodiffusion with goat anti-rabbit IgG (provided by J. Mestecky, University of Alabama at Birmingham). The rabbit IgG anti-SpaA was then biotinylated as described by Goding (16). The antibody was dialyzed against 0.1 M $NaHCO_3$ (pH 8.0) and adjusted to 1.0 mg/ml with the same buffer. Biotin ester (120 μl of a 1.0-mg/ml solution in

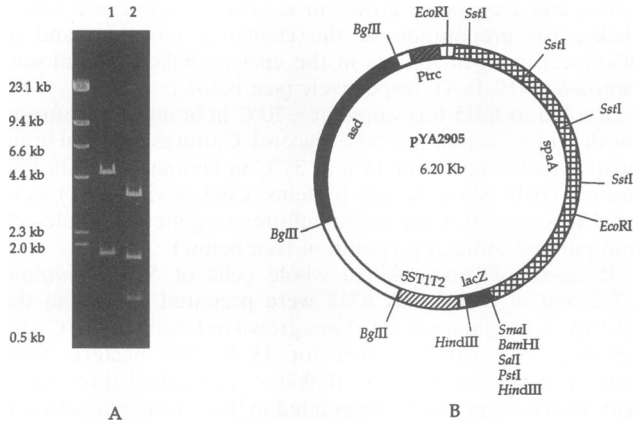

FIG. 1. (A) The recombinant SpaA-expressing plasmid pYA2905 digested with *Eco*RI (lane 1) and *Bgl*II (lane 2). (B) Restriction endonuclease map of pYA2905.

dimethyl sulfoxide [Sigma]) was added to 1.0 mg of antibody per ml. The solution was incubated at room temperature for 2 h. The biotin-conjugated protein was then dialyzed overnight against phosphate-buffered saline (PBS) at 4°C.

Rat bile IgA anti-SpaA or anti-*S. typhimurium* χ4072 was generated as previously described (23). Briefly, 10 to 20 μl of a solution of SpaA (30 μg/ml in sterile saline) or formalin-killed *S. typhimurium* χ4072 (2×10^9 CFU/ml) was injected into seven to nine Peyer's patches of an anesthetized rat (0.05 ml per 100 g of body weight in a solution containing 100 mg of ketamine [Parke-Davis, Morris Plains, N.J.] per ml and 1.5 mg of xylazine [Tranquived VedCo, St. Joseph, Mo.] per ml). After 7 to 10 days, bile was collected via cannulation of the bile duct, clarified by centrifugation (10 min at 13,000 × *g* at 4°C), aliquoted, and stored at −70°C until used.

Restriction endonuclease mapping. Plasmid DNA was isolated from *S. typhimurium* χ4072(pYA2905) (42) and digested with the restriction endonucleases *Eco*RI and *Bgl*II as described by the supplier (International Biotechnologies, Inc., New Haven, Conn.). These enzymes were chosen on the basis of the restriction endonuclease map of pYA2905 (Fig. 1). The digested DNA fragments were separated by electrophoresis through a 0.8% agarose gel in TAE buffer (40 mM Tris-acetate [Sigma], 1 mM EDTA [Sigma]), and visualized by staining with ethidium bromide (0.5 μg/ml; United States Biochemical Corp., Cleveland, Ohio).

Immunoblot analysis and quantification of SpaA. To confirm the production of SpaA by *S. typhimurium* χ4072 (pYA2905), we cultured the bacteria overnight in L broth at 37°C with aeration. The cells were fractionated into cytoplasmic and periplasmic compartments as described by Fischetti et al. (13). Briefly, *S. typhimurium* χ4072(pYA2905) (100 ml) cells were grown at 37°C to an optical density of 0.5 at 600 nm. The cells were centrifuged at 7,000 × *g* for 15 min at 4°C and resuspended in 4.8 ml of TSE buffer (100 mM Tris-HCl [pH 8.0] containing 20% sucrose and 5 mM EDTA). Lysozyme (200 μl) was added to a final concentration of 0.5 mg/ml. Following gentle mixing, the suspension was incubated on ice for 20 min. For the whole-cell control, 0.5 ml of the suspension was removed and lysed by addition of 15 μl Triton X and 100 μl of MD solution (100 mM $MgCl_2$, 100 μg of DNase per ml), freezing and thawing twice, and freezing at −70°C until assayed. $MgCl_2$ was added to the remaining suspension at a final concentration of 50 mM. The suspension was then centrifuged at 7,000 × *g* for 15 min. The resulting supernatant was filtered through an 0.45-μm-pore-size filter, designated SP-1 (the periplasmic fraction), and stored at −70°C until assayed. The pellet (spheroplasts) was washed once with 4.0 ml of TSE buffer and centrifuged at 7,000 × *g*. The supernatant was filtered through a 0.45-μm filter, designated SP-2, and stored at −70°C until assayed. To lyse the spheroplasts, we added 1 ml of MD solution and 3 ml of sterile distilled water to the pellet. The spheroplasts were frozen and thawed twice in a dry ice-ethanol bath and then centrifuged at 50,000 rpm for 2 h in a Sorvall SW type 65 rotor. The supernatant was filtered through an 0.45-μm filter, designated SP-3 (the cytoplasmic fraction), and stored at −70°C until assayed. The protein concentrations of the whole cell and the cytoplasmic and periplasmic fractions were estimated by using the Micro BCA protein assay kit (Pierce, Rockford, Ill.). After being boiled for 5 min, the samples (5 μg of protein per well) were separated by SDS-PAGE (7.5% polyacrylamide). The proteins were transferred from the gel to an Immobilon P membrane (Millipore Corp., Bedford, Mass.) by using a semidry electroblotter (Integrated Separation Systems; Enprotech, Hyde Park, Mass.). The immunoblot was processed as previously described (21). The blot was reacted with rabbit anti-SpaA and alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma) and developed by adding the alkaline phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate (Sigma).

SpaA produced by the recombinant *Salmonella* strain was quantified by a capture ELISA. Individual wells of flat-bottom 96-well plates (ICN Biomedicals, Inc., Horsham, Pa.) were coated by overnight incubation at room temperature with rabbit anti-SpaA serum (5 μg/ml) diluted in borate-buffered saline (pH 8.2). Nonspecific binding sites were blocked with 5% fetal calf serum (Gemini Bioproducts, Calabasas, Calif.) in PBS containing 0.05% Tween 20 (pH 7.4; Fisher Scientific, Fair Lawn, N.J.) for 2 h at room temperature. Five twofold serial dilutions of purified native SpaA (standard) or of *S. typhimurium* χ4072(pYA2905) whole-cell lysate (see above) prepared in PBS containing 1% fetal calf serum and 0.1% Tween 20 were added in duplicate to individual wells. The plates were incubated for 2 h at 37°C and washed, and biotin-conjugated anti-SpaA was added to appropriate wells. The plates were incubated overnight at 4°C and then washed. Streptavidin-alkaline phosphatase (0.4 mg/ml; Southern Biotechnology Associates) was added, and plates were incubated for 30 min at room temperature. The plates were washed, phosphatase substrate (no. 104; Sigma) in diethanolamine buffer (pH 9.8) was added, and color development was recorded at 405 nm in a V_{max} microplate reader (Molecular Devices Corp., Menlo Park, Calif.) interfaced with a Macintosh II computer. The amount of SpaA in the whole-cell lysate was determined by interpolation from the standard curve generated from known concentrations of purified native SpaA by using a four-parameter logistic algorithm (Softmax; Molecular Devices Corp.). Total protein in the whole-cell lysate was determined by using the Micro BCA protein assay kit (Pierce).

Plasmid construction. The plasmid used in these studies, pYA2905, was obtained from Roy Curtiss III, Washington University, St. Louis, Mo. pYA2905 contains three tandem repeats of a 0.48-kb fragment encoding the major SpaA immunodominant determinant fused to the 1.2-kb fragment encoding a minor SpaA immunodominant determinant (18) (Fig. 1). In addition to encoding antigenic determinants of SpaA, pYA2905 contains the *S. typhimurium* gene *asd*, which encodes β-semialdehyde dehydrogenase (14). This enzyme is required for the synthesis of DAP, an essential component of the peptidoglycan in the cell wall of gram-negative organisms.

pYA2905, by encoding β-semialdehyde dehydrogenase, complements the Asd$^-$ phenotype of *S. typhimurium* χ4072 and results in a balanced lethal system, allowing *S. typhimurium* χ4072 to grow in medium lacking DAP.

Experimental design. Overnight cultures of *S. typhimurium* χ4072(pYA2905) were diluted 1:20 in L broth and grown at 37°C with aeration for 4 h. The bacteria were centrifuged for 10 min at 3,000 × *g* and resuspended in PBS. The number of bacteria in the suspension was determined by reading the optical density at 660 nm. The bacteria were then diluted to 4 × 10^9 CFU/ml in intubation medium (26) consisting of 8 parts Hanks balanced saline solution (Life Technologies Inc., Grand Island, N.Y.) and 2 parts sodium bicarbonate (7.5% solution; Mediatech, Washington, D.C.). The bacterial inoculum was then diluted in sterile PBS and plated on bismuth sulfite agar (Difco). After incubation at 37°C for 18 h, the colonies on the plates were enumerated and used to determine the actual immunizing dose of *S. typhimurium* χ4072(pYA2905).

Food was taken away from the rats 4 h prior to immunization. The rats were given *S. typhimurium* χ4072(pYA2905) in intubation medium (0.25 ml; ~10^9 CFU) by gastric intubation via a 21-gauge feeding needle (Popper and Sons Inc., Hyde Park, N.Y.). Food was returned to the rats 30 min following immunization.

Groups of rats were immunized on day 0, on days 0 and 7, or on days 0, 7, and 21. Serum, saliva, and fecal samples were collected at weekly intervals (two rats per time point) and assayed by ELISA for antibody activity to *S. sobrinus* 6715 and *S. typhimurium* LT-2. Following collection of gut secretions, saliva, and serum, the rats were sacrificed. The spleen and all Peyer's patches from each rat were removed and assessed for the number of recoverable *S. typhimurium* χ4072(pYA2905) cells.

Sample collection. Gut secretions were collected and processed as described previously (12). Briefly, rats were given a high-salt lavage solution at 15-min intervals for 1 h prior to being anesthetized with a solution of ketamine and xylazine as described above. They were then given pilocarpine (Sigma; 1 ml of a 75-mg/ml solution per 100 g of body weight). Gut secretion was collected into a petri dish and then centrifuged for 13,000 × *g* for 10 min at 4°C. Saliva was collected with a Pasteur pipette and also centrifuged for 13,000 × *g* for 10 min at 4°C. The clarified gut secretion and saliva were stored at −70°C until assayed for antibody activity. Blood was collected by cardiac puncture while the rats were under anesthesia. The blood was allowed to clot at 4°C, and, after centrifugation at 2,700 × *g*, the serum was collected and stored at −70°C until assayed for antibody activity by ELISA, as described below.

In vivo recovery of *S. typhimurium* χ4072(pYA2905). To determine the persistence of the recombinant *S. typhimurium* χ4072(pYA2905) in rats, we sacrificed the animals at approximately weekly intervals. The spleens and Peyer's patches from immunized animals were removed and placed in sterile PBS (5 ml). Single-cell suspensions were prepared by processing the tissues through a 22-gauge wire mesh. The resulting cell suspensions were then diluted, and aliquots were spread on bismuth sulfite agar. After incubation at 37°C for 24 h, CFU were counted. The data are expressed as the number of CFU per tissue per rat.

Salmonella colonies were isolated from Peyer's patches of individual animals for analysis of SpaA expression. The isolates were grown overnight at 37°C in L broth, and whole-cell lysates were prepared as described by Sambrook et al. (42). Each culture (1 ml) was centrifuged for 30 s at 14,000 rpm in an Eppendorf microcentrifuge (12,000 × *g*). The pellet was resuspended in 0.5 ml of ice-cold 50 mM Tris-HCl (pH 7.4). The bacteria were again pelleted by centrifugation and resuspended in 50 μl of a solution containing 100 mM Tris-HCl (pH 6.8), 4% SDS, 0.2% bromophenol blue, and 200 mM dithiothreitol. The bacteria were boiled at 100°C for 5 min, placed on ice, and sonicated three times for 30 s by using a Heat System Sonicator (Ultasonics, Inc., Plainview, N.Y.) on a setting of 6. The lysate was then centrifuged for 10 min at room temperature in an Eppendorf microcentrifuge (12,000 × *g*). The supernatant was assayed by immunoblot, as described above, for the expression of SpaA.

Antibody responses. Antibody activity to *S. typhimurium* or *S. sobrinus* 6715 was assessed by ELISA. Individual wells of flat-bottom 96-well plates (ICN Biomedicals, Inc.) were coated with 5 × 10^8 formalin-killed *S. sobrinus* 6715 or *S. typhimurium* LT-2 per ml in 0.2 M bicarbonate-carbonate buffer (pH 9.6). Nonspecific binding sites were blocked with 5% fetal calf serum (Gemini Bioproducts) in PBS containing 0.05% Tween 20 (pH 7.4; Fisher Scientific) for 2 h at room temperature. From a starting dilution of serum (1:100), saliva (1:5), and gut secretions (1:5) prepared in PBS containing 1% fetal calf serum and 0.1% Tween 20, five twofold dilutions were added in duplicate to individual wells, and the plates were incubated for 2 h at 37°C. The plates were washed, and biotin-conjugated anti-rat Ig (Southern Biotechnology Associates) or biotin-conjugated anti-rat IgA (Zymed Laboratories, Inc.) was added to appropriate wells. Plates were incubated overnight at 4°C and washed. Streptavidin-alkaline phosphatase (0.4 mg/ml; Southern Biotechnology Associates) was added, and the plates were incubated for 30 min at room temperature. They were then washed again, phosphatase substrate (no. 104; Sigma) in diethanolamine buffer (pH 9.8) was added, and color development was recorded at 405 nm in a V_{max} microplate reader (Molecular Devices Corp.) interfaced with a Macintosh II computer. A standard curve of antibody activity (in ELISA units [EU]) was established by using a pool of rat anti-*S. sobrinus* or anti-*S. typhimurium* hyperimmune sera or bile from immunized animals for an anti-*S. sobrinus* or anti-*S. typhimurium* serum Ig or mucosal IgA standard. The antibody activity in the standard pool was assigned a level of antibody activity in EU per milliliter, whereby 1 EU per milliliter equaled the dilution of the standard giving an optical density reading of 0.1. The level of antibody activity (in EU per milliliter) in serum, saliva, and gut secretion samples run simultaneously with the standard was determined by interpolation from the standard curve by using a four-parameter logistic algorithm (Softmax; Molecular Devices Corp.). In some experiments the levels of salivary and gut secretion IgA antibody are expressed as the optical densities at 405 nm for samples diluted 1:5.

RESULTS

Characterization of pYA2905 and its product in recombinant *S. typhimurium*. pYA2905 is a 6.2-kb plasmid (11); upon digestion with *Eco*RI, 4.6- and 1.6-kb fragments were generated (Fig. 1). Digestion with *Bgl*II yielded three fragments, of 3.3, 1.7, and 1.3 kb, as expected from the restriction endonuclease map. The recombinant SpaA encoded by pYA2905 was expressed in the cytoplasm of the recombinant bacterium, although a trace amount was seen in the periplasmic fraction (Fig. 2). The molecular mass of the recombinant SpaA is approximately 145 kDa, compared with the 210-kDa native SpaA protein (Fig. 2) (11), which is very similar to the 185-kDa Ag I/II (40).

The amount of recombinant SpaA produced by approximately 10^9 *S. typhimurium* χ4072(pYA2905) cells as deter-

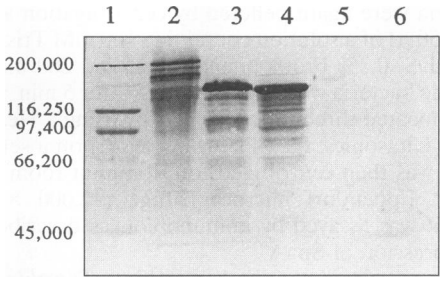

FIG. 2. Immunoblot of the recombinant SpaA expressed in *S. typhimurium* χ4072(pYA2905). Lanes: 1, molecular weight markers; 2, native SpaA; 3, *S. typhimurium* χ4072(pYA2905) whole-cell lysate; 4, *S. typhimurium* χ4072(pYA2905) cytoplasmic compartment; 5, *S. typhimurium* χ4072(pYA2905) periplasmic compartment; 6, whole-cell lysate of *S. typhimurium* χ4072 without pYA2905.

mined by the capture ELISA was 100 ng. Since the total amount of protein in the preparation was 35.1 μg, we have estimated that SpaA represents approximately 0.28% of the total protein.

Persistence and humoral immune response following a single immunization with *S. typhimurium* χ4072(pYA2905). In our initial series of experiments, it was of interest to determine the ability of *S. typhimurium* χ4072(pYA2905) to be taken up by the Peyer's patches and subsequently by the spleens of rats following a single oral immunization with approximately 10^9 bacteria. Large numbers of *S. typhimurium* organisms were detected in the Peyer's patches of immunized rats on day 2 following immunization (Fig. 3). The number of recoverable cells in Peyer's patches decreased and was undetectable after 2 weeks. A twofold-lower level of *S. typhimurium* cells was detected in the spleens of these rats on day 2, and *S. typhimurium* cells were undetectable by day 7. SpaA was expressed by the *S. typhimurium* cells isolated from the Peyer's patches of rats immunized with *S. typhimurium* χ4072(pYA2905), as determined by immunoblot analysis (data not shown).

A serum anti-*Salmonella* antibody response but essentially no anti-*Streptococcus* antibody response was seen in rats given a single oral immunization with *S. typhimurium* χ4072 (pYA2905) (Fig. 4). Even though there was variability, the levels of anti-*Salmonella* antibody in serum peaked on day 21. The level of anti-*Streptococcus* antibody in serum varied but did not increase above the background level obtained in rats used for the day 0 time point in this experiment. In this study, *S. sobrinus* 6715 whole cells were used as coating antigen to measure antibody activity to cloned SpaA. In separate studies, we have found similar levels of antibody activity in serum samples from animals immunized with SpaA when either *S. sobrinus* 6715 whole cells or SpaA was used as the coating antigen. The lack of sufficient quantities of purified SpaA precluded our use of this protein as the coating antigen in this study.

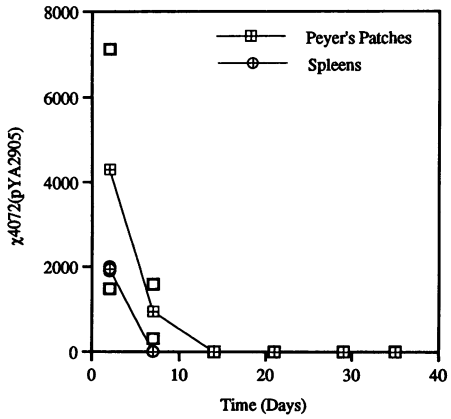

FIG. 3. Persistence of *S. typhimurium* χ4072(pYA2905) in spleens and Peyer's patches of rats given the recombinant bacterium once by gastric intubation. Rats were orally immunized with *S. typhimurium* χ4072(pYA2905) (10^9 CFU) on day 0. Results are expressed as individual (open symbols) and mean (symbols with +) values of the number of *S. typhimurium* χ4072(pYA2905) recovered from the spleen or total Peyer's patches per rat (two animals per time point).

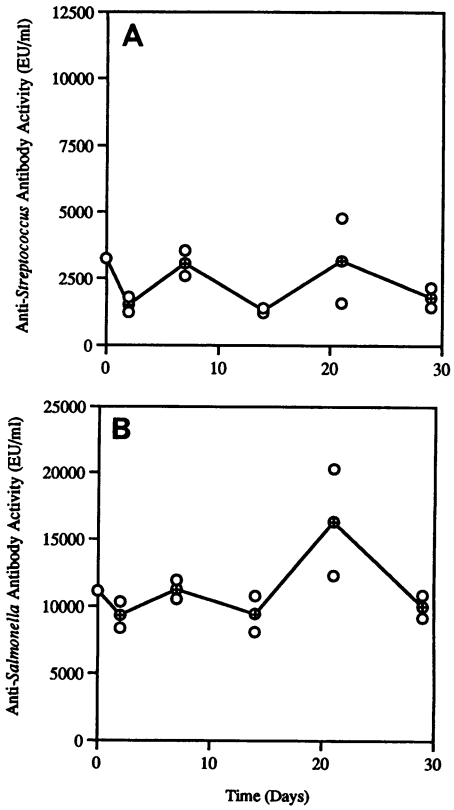

FIG. 4. Total antibody activity in serum to *S. sobrinus* 6715 (A) and *S. typhimurium* (B) in rats given *S. typhimurium* χ4072(pYA2905) once by gastric intubation. Rats were orally immunized with *S. typhimurium* χ4072(pYA2905) (10^9 CFU) on day 0. Results are expressed as the individual (open symbols) and mean (symbols with +) values of the levels of antibody activity in serum (two animals per time point).

Rats immunized with a single dose of *S. typhimurium* χ4072(pYA2905) developed salivary IgA anti-*Streptococcus* and anti-*Salmonella* responses (Fig. 5). The salivary anti-*Streptococcus* responses increased rapidly during the first 2 weeks, remained relatively constant, and then began to increase again on day 28. The salivary anti-*Salmonella* response peaked on day 14 and remained fairly constant throughout the following 14 days. A slight fecal IgA anti-*Salmonella* response, which peaked on day 21, was seen. Essentially no fecal IgA anti-*Streptococcus* antibody was detected throughout the 28 days after immunization.

Effects of multiple immunizations on *S. typhimurium* persistence and antibody responses. The numbers of recombinant

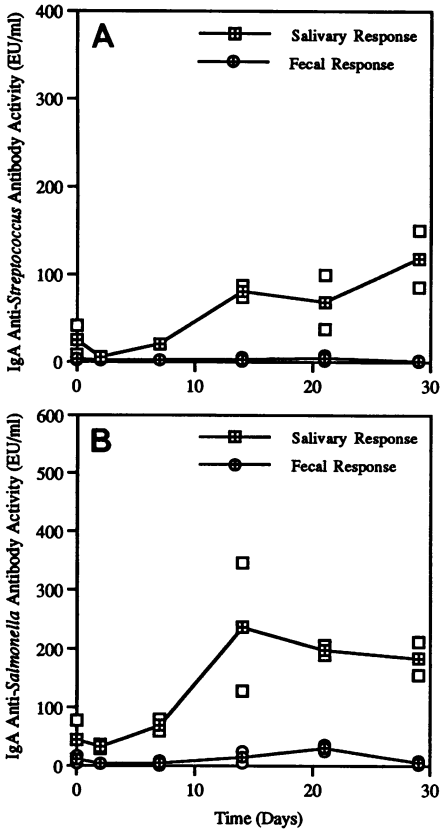

FIG. 5. IgA antibody activity to *S. sobrinus* 6715 (A) and *S. typhimurium* (B) in saliva and gut secretion in rats given *S. typhimurium* χ4072(pYA2905) once by gastric intubation. Rats were orally immunized with *S. typhimurium* χ4072(pYA2905) (10^9 CFU) on day 0. Results are expressed as the individual (open symbols) and mean (symbols with +) values of the levels of IgA antibody activity (two animals per time point).

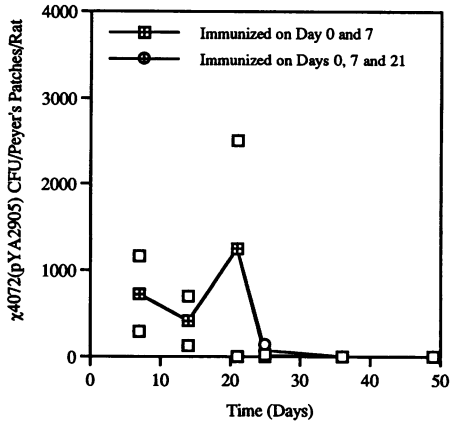

FIG. 6. Persistence of *S. typhimurium* χ4072(pYA2905) in Peyer's patches of rats given the recombinant bacterium two or three times by gastric intubation. Rats were orally immunized with *S. typhimurium* χ4072(pYA2905) (10^9 CFU) on days 0 and 7 or on days 0, 7, and 21. Results are expressed as the individual (open symbols) and mean (symbols with +) values of the number of *S. typhimurium* χ4072(pYA2905) cells isolated from all Peyer's patches per rat (two animals per time point).

Salmonella cells detected in Peyer's patches of animals continued to decrease after the second immunization on day 7 (Fig. 6). On day 21 following the initial immunization, recombinant *Salmonella* cells were recovered from only one of the two rats analyzed. No *Salmonella* cells were detected in Peyer's patches of immunized rats on day 25, 35, or 49. A third immunization on day 21 did not lead to an increase in the number of recoverable *Salmonella* cells in Peyer's patches of immunized animals. *Salmonella* cells were recovered from Peyer's patches on day 25 but not on day 35 or 49. In addition, *Salmonella* cells were not detected in spleens of immunized animals 7 days after the initial immunization (data not shown). SpaA was expressed by the *Salmonella* cells isolated from the rats, as determined by immunoblot analysis (data not shown).

Anti-*Streptococcus* and anti-*Salmonella* antibodies were detected in the serum of animals receiving multiple (two or three) immunizations (Fig. 7). The level of anti-*Streptococcus* activity in serum increased following the first and second immunizations and peaked on day 21 (Fig. 7A). After the third immunization, a slight increase in antibody activity was seen on day 42, which was similar to the level seen on day 21. The serum response in rats given multiple immunizations (Fig. 7) persisted longer than in rats given a single immunization (Fig. 4).

The anti-*Salmonella* activity in serum in animals given three immunizations peaked on day 36 and then, following a slight decrease, began to increase again on day 51 (Fig. 7B). Unlike the anti-*Streptococcus* responses in serum, the level of anti-*Salmonella* activity in serum was augmented in animals receiving three immunizations compared with those receiving two immunizations.

The level of salivary IgA anti-*Streptococcus* antibody activity in animals given multiple immunizations increased during the first week (Fig. 8A). Following a gradual decrease after the second immunization, an increase in activity was seen after day 21. The level of anti-*Streptococcus* activity seen on day 25 in rats immunized on days 0, 7, and 21 was higher than that observed in rats given two immunizations. The salivary anti-*Salmonella* activity was also higher in rats given booster doses than in rats given two immunizations (Fig. 8B). Animals given either two or three immunizations had peak salivary anti-*Salmonella* IgA antibody responses on day 25.

Fecal IgA anti-*Streptococcus* and anti-*Salmonella* responses were also detected (Fig. 9). Following two immunizations, the level of anti-*Streptococcus* activity decreased and then increased on days 25 and 36 (Fig. 9A). This was different from that seen in animals receiving three immunizations, in which the anti-*Streptococcus* response peaked on day 25 and then gradually declined (Fig. 9A). The level of anti-*Salmonella* antibody activity peaked on day 25 in rats given multiple immunizations (Fig. 9B). In addition, the anti-*Salmonella* response in animals receiving three immunizations was more sustained than in those receiving two immunizations (Fig. 9B).

DISCUSSION

In our studies with Fischer rats, the Δ*cya* Δ*crp* *S. typhimurium* mutant χ4072(pYA2905) persisted in the GALT, with peak numbers of recombinant *Salmonella* cells detected within the first 7 days in the Peyer's patches of orally immunized rats. This supports data from previous studies showing that Δ*cya* Δ*crp* *Salmonella* mutants, even though they are avirulent, are capable of invading the GALT and inducing immunity in orally immunized BALB/c mice (6, 8). Similarly, in previous work with BALB/c mice, Δ*cya* Δ*crp* *Salmonella* cells persisted in the GALT, as well as in the spleens, with numbers of recoverable

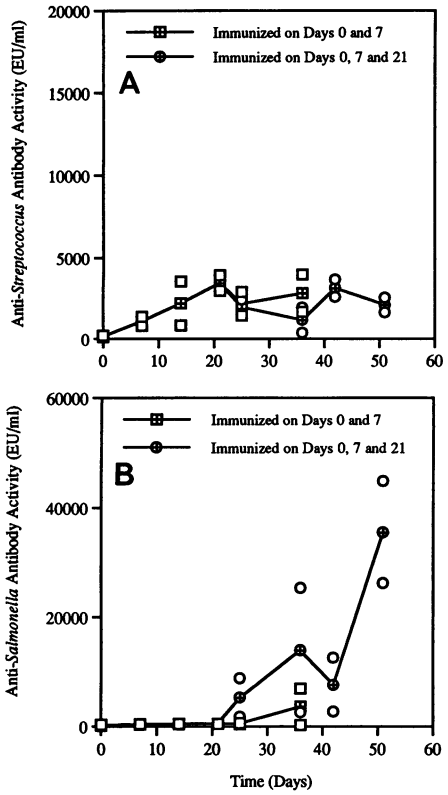

FIG. 7. Total antibody activity in serum to *S. sobrinus* 6715 (A) and *S. typhimurium* (B) in rats given *S. typhimurium* χ4072(pYA2905) two or three times by gastric intubation. Rats were orally immunized with *S. typhimurium* χ4072(pYA2905) (10^9 CFU) on days 0 and 7 or on days 0, 7, and 21. Results are expressed as the individual (open symbols) and mean (symbols with +) values of the levels of antibody activity in serum (two animals per time point).

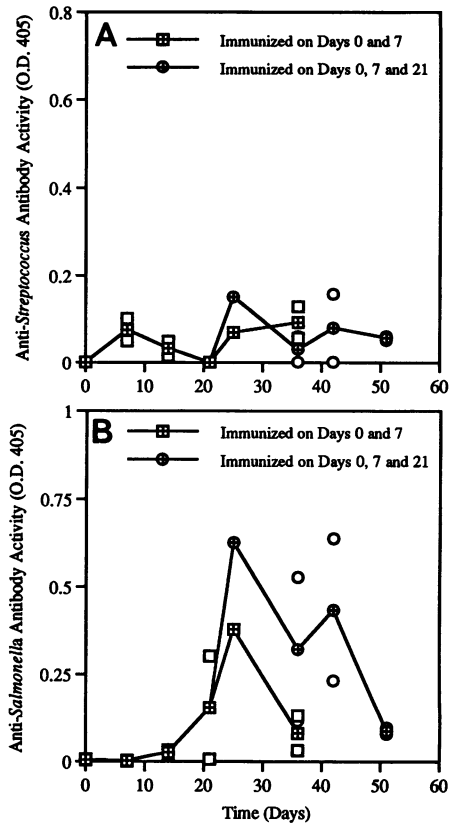

FIG. 8. Salivary IgA antibody activity to *S. sobrinus* 6715 (A) and *S. typhimurium* (B) in rats immunized two or three times with *S. typhimurium* χ4072(pYA2905) by gastric intubation. Rats were orally immunized with *S. typhimurium* χ4072(pYA2905) (10^9 CFU) on days 0 and 7 or on days 0, 7, and 21. Results are expressed as the individual (open symbols) and mean (symbols with +) values of salivary antibody activity in saliva diluted 1/5 and expressed as the optical density (O.D.) at 405 nm (two animals per time point).

Salmonella cells peaking within a week following oral immunization (3, 8, 11). In our studies with Fischer rats, peak numbers of recombinant *Salmonella* cells were detected in the Peyer's patches of orally immunized animals within the first 7 days. However, unlike these previous studies, we detected no *Salmonella* cells in Peyer's patches 14 days following a single oral immunization. This difference could be because *S. typhimurium* is a mouse pathogen. More *Salmonella* cells were detected in Peyer's patches of rats receiving multiple immunizations than in those receiving a single immunization; however, there was no difference in the numbers of recovered *Salmonella* cells in rats receiving two or three immunizations. The recombinant *Salmonella* cells were cleared from the spleens of animals receiving single or multiple immunizations by 7 days, which may be due to an innate resistance of rats to this bacterium.

A single oral immunization of rats with *S. typhimurium* χ4072(pYA2905) induced a serum antibody response against *Salmonella* cells, with the maximum activity occurring on day 21. Anti-*Streptococcus* responses in serum were not above background levels. This absence of anti-*Streptococcus* antibody activity was not due to the lack of expression of the cloned protein but could be due to the actual dose of SpaA. These Δ*cya* Δ*crp* aspartate β-semialdehyde dehydrogenase-deficient *Salmonella* vaccine strains maintain Asd^+ plasmids and express high levels of cloned gene products, e.g., cytoplasmic SpaA (6, 8, 36, 37). We have confirmed the presence of the Asd^+ plasmid pYA2905 (Fig. 1), as well as the high-level expression of the recombinant SpaA protein (Fig. 2) within the cytoplasm of *S. typhimurium* χ4072. We have determined that SpaA represents approximately 0.28% of the total protein of *S. typhimurium* χ4072(pYA2905) and that our immunizing dose of 10^9 recombinant *Salmonella* cells contained 100 ng of SpaA. Since fewer than 2,000 *Salmonella* cells were detected in the spleens of rats 2 days after the single oral immunization with χ4072(pYA2905), it is possible that the amount of SpaA was suboptimal for induction of a systemic response.

An above-background anti-*Streptococcus* antibody response in serum was detected in rats receiving multiple immunizations. In addition, anti-*Salmonella* activity in serum was greater than anti-*Streptococcus* antibody activity in immunized animals. This may be related to the greater number of *Salmonella* epitopes than of *Streptococcus* epitopes expressed by the recombinant *Salmonella* strain. Animals receiving multiple immunizations also had greater anti-*Salmonella* activity in serum than did animals receiving a single immunization. This increased antibody activity in serum may reflect the longer duration of persistence of the recombinant *Salmonella* strain in the Peyer's patches of rats receiving multiple immunizations, as well as repeated exposure to the recombinant protein, compared with the duration of persistence of χ4072(pYA2905) and the dose of SpaA in rats receiving a single immunization.

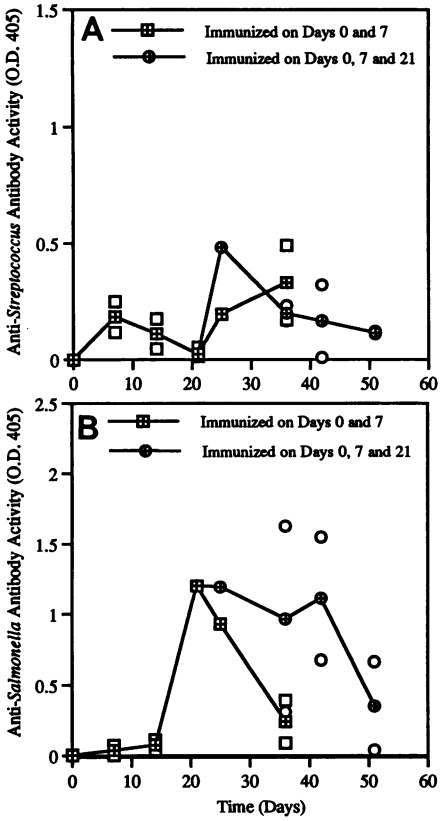

FIG. 9. Gut secretion IgA antibody activity to *S. sobrinus* 6715 (A) and *S. typhimurium* (B) in rats immunized two or three times with *S. typhimurium* χ4072(pYA2905) by gastric intubation. Rats were orally immunized with *S. typhimurium* χ4072(pYA2905) (10^9 CFU) on days 0 and 7 or on days 0, 7, and 21. Results are expressed as the individual (open symbols) and mean (symbols with +) values of fecal antibody activity in gut secretions diluted 1/5 and expressed as the optical density (O.D.) at 405 nm (two animals per time point).

Interestingly, boosting on day 21 augmented anti-*Salmonella* but not anti-*Streptococcus* responses in serum.

In addition to systemic antibody responses, immunized animals developed antigen-specific mucosal antibody responses. In rats receiving a single immunization, the magnitude of the salivary IgA responses was much greater than that of fecal IgA responses. Similar differences between the magnitude of salivary and fecal responses have been found in gnotobiotic rats immunized intranasally with Ag I/II conjugated to the B subunit of cholera toxin (24). This suggests that the common mucosal immune system, in which IgA-secreting cells are induced at one site and act at a distal site (34), may be compartmentalized, as previously suggested (41). In this regard, IgA-secreting cells with different specificities may be localized in different mucosal tissues instead of commonly distributed in mucosal effector sites (22, 38). It may be more beneficial, on an evolutionary level, for orally immunized rats to respond to the cloned antigen of *S. sobrinus* with a salivary instead of fecal antibody response. However, the levels of antigen-specific salivary and fecal antibody in animals receiving multiple immunizations were on the same order of magnitude. Perhaps multiple immunizations, compared with a single immunization, overcome compartmentalization and efficiently induce both salivary and fecal antibody responses. Evidence for this is suggested by the recent finding that BALB/c mice receiving two oral immunizations with *S. typhimurium* χ4072(pYA2905) responded with greater fecal than salivary IgA antibody against SpaA (11).

Interestingly, in our study, boosting on day 21 appeared to delay the peak salivary anti-*Salmonella* and anti-*Streptococcus* antibody responses. Peak responses in animals receiving a single immunization occurred on day 21, compared with day 25 in animals receiving multiple immunizations. In addition, boosting increased salivary anti-*Salmonella* antibody activity nearly twofold and the response persisted longer than in nonboosted animals. This persistence and elevated antibody activity suggest the induction of memory cells in immunized animals (34).

Although boosting on day 21 increased the antibody response, the mucosal IgA anti-SpaA antibody activity was not dramatically augmented. This may be due to the dose of recombinant SpaA and to the nature of the recombinant protein. Because plasmid pYA2905 does not encode the entire SpaA protein, key immunogenic epitopes may be oriented differently on the recombinant protein than on the native protein. This potential difference in epitope architecture may prevent the recombinant protein from inducing an immune response as efficiently as the complete, native SpaA protein does. A similar observation was suggested in a previous study in which BALB/c mice were immunized with a recombinant malaria protein derived from *Plasmodium falciparum* merozoite surface antigen or p190 (45). Following subcutaneous immunizations with the recombinant protein, mice produced serum antibody with lower activity to the native p190 protein than to the recombinant protein. Similarly, rats immunized with a *Salmonella* strain expressing recombinant SpaA may produce antibody less reactive against the native protein than against the recombinant protein.

The objective of oral immunization with recombinant *Salmonella* cells was to induce antigen-specific mucosal IgA antibody responses against the recombinant SpaA protein. However, animals immunized with the recombinant *Salmonella* strain mounted serum antibody responses in addition to mucosal responses. Although very few *Salmonella* cells were detected in the spleens of immunized animals, enough of the recombinant bacteria may have entered the systemic circulation to induce antibody responses in serum. Alternately, activated lymphocytes in Peyer's patches, following exposure to epitopes derived from the recombinant *Salmonella* cells, may have migrated from the Peyer's patches into the systemic circulation, mediating antibody responses in serum. In fact, several studies have been reported in which oral immunizations with recombinant *Salmonella* cells expressing numerous recombinant proteins have resulted in IgG antibody responses in serum (2, 25, 43).

Early work by Lehner et al. (30) provided evidence for a correlation between IgG anti-Ag I/II antibodies in serum and protection against dental caries. Antibody could pass from serum into crevicular fluid and confer a protective effect on the tooth surface. Other studies have provided evidence that salivary IgA anti-Ag I/II is protective against dental caries (35). Since there is much similarity between Ag I/II and SpaA, we are currently investigating, in an experimental Fischer rat model, the effectiveness of the recombinant *S. typhimurium* χ4072(pYA2905) as an oral vaccine for inducing a protective immune response against dental caries.

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