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

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An attenuated, recombinant *Salmonella typhimurium* mutant, χ4072(pYA2905), expressing the surface protein antigen A (SpaA) of *Streptococcus sobrinus* was investigated for its effectiveness in inducing protective immune responses against *S. sobrinus*-induced dental caries in an experimental caries model. Fischer rats were orally immunized with either 10^8 or 10^9 CFU of *S. typhimurium* χ4072(pYA2905). Persistence of salmonellae in Peyer's patches and spleens and the induction of immune responses were determined. Maximum numbers of salmonellae were recovered from Peyer's patches of rats within the first week of immunization, with higher numbers recovered from rats given 10^9 CFU than from those given 10^8 CFU. Serum anti-*Salmonella* and anti-SpaA responses increased more rapidly in rats given 10^9 CFU than in rats given 10^8 CFU. The salivary antibody response to SpaA increased with time, but the response varied in the two groups. In a separate study, rats were orally immunized with the recombinant *Salmonella* mutant and then challenged with cariogenic *S. sobrinus* 6715. The levels of serum and salivary antibody and caries activity were assessed at the termination of the experiment. Higher levels of salivary immunoglobulin A antibody to SpaA and *Salmonella* carrier were detected in rats given 10^9 CFU than in those given 10^8 CFU, and these responses were higher than those in nonimmunized controls. Mandibular molars from immunized rats had lower numbers of recoverable streptococci and less extensive carious lesions than those from nonimmunized, control rats. These data indicate that oral immunization with an attenuated recombinant *S. typhimurium* expressing SpaA of *S. sobrinus* induces the production of antigen-specific mucosal antibody and confers protection against dental caries.

Vaccines against microbial pathogens have significant worldwide public health and economic benefits. An effective vaccine must be safe so as not to cause disease itself, and it must be able to induce long-lasting protection by stimulating the induction of B and T cells. These antigen-specific lymphocytes combat microbial invasion by mediating the elimination and neutralization of a colonizing pathogen. Several types of vaccines against microbial pathogens, including inactivated organisms, live attenuated organisms, and subunit vaccines consisting of immunogenic proteins or virulence factors, have been developed (reviewed in references 6 to 8). Ironically, with the exception of the live, attenuated oral poliovirus and *Salmonella typhi* vaccines, most vaccines are currently administered parenterally and thus induce predominantly serum immunoglobulin G (IgG) responses. However, the majority of microbial pathogens must invade a mucosal barrier in order to cause disease. The development of alternate vaccination routes for the induction of protective mucosal immune responses is, therefore, imperative (reviewed in references 34, 35, and 40). Specifically, oral immunization leads to the production of antigen-specific secretory IgA, the predominant antibody in mucosal secretions, via the common mucosal immune system (reviewed in references 29, 36, and 37).

Because soluble antigens delivered orally are generally taken up inefficiently by the major IgA-inductive sites in the gut, i.e., the gut-associated lymphoid tissue (GALT) or Peyer's patches, different delivery systems are being assessed for use as oral vaccines (reviewed in references 3 and 40). Inert, particulate carriers such as liposomes and microspheres have been used to induce mucosal antibody responses against various microbial antigens, including glucosyltransferase from *Streptococcus mutans* (6) and staphylococcal enterotoxin B derived from *Staphylococcus aureus* (16). In addition, live oral delivery systems under study include viable recombinant vaccinia virus (1) and adenovirus (22) vaccines for rabies virus and respiratory syncytial virus, respectively.

During the past few years, several studies have evaluated the effectiveness of recombinant *Salmonella* strains as oral vaccines (reviewed in references 8, 11, and 40) since these bacteria invade the host through the Peyer's patches of the GALT (4, 23). *Salmonella* vaccine strains have different attenuating mutations and express various heterologous antigens. The *Salmonella typhimurium* Δ*cya*Δ*crp*Δ*asd* mutant χ4072 (44) has been used by several investigators for the expression of heterologous proteins and has been shown to be stable and safe for in vivo use (2, 14, 15, 45, 48). This strain is attenuated as a result of deletions of the genes encoding adenylate cyclase (*cya*) and the cyclic AMP (cAMP) receptor protein (*crp*) (9, 10). Studies in experimental animals have shown that oral immunization with *S. typhimurium* χ4072 expressing cloned gene products of microbial pathogens, such as *S. typhi* (2), *Brucella abortus* (49, 50), *Porphyromonas gingivalis* (15), and *Streptococcus sobrinus* (14, 45), results in the induction of humoral and cellular responses to the *Salmonella* carrier as well as to the cloned gene product.

The oral microbial bacteria *S. mutans* and *S. sobrinus* are members of the mutans streptococci and are the major etio-

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logic agents of human dental caries (33). The disease process involves an initial sucrose-independent adherence of the bacteria to the salivary pellicle covering the tooth surface, which leads to further colonization by mutans streptococci in a sucrose-dependent manner (18, 21). The production of acid by the established mutans streptococci causes demineralization of the tooth surface. The initial adherence to the tooth surface is believed to be mediated by SpaA for *S. sobrinus* (12) and antigen I/II (Ag I/II) for *S. mutans* (30, 46). SpaA and Ag I/II have 66% amino acid homology within their middle regions (31, 51).

Previous studies have shown that oral or intranasal immunization of rats with Ag I/II vaccines result in salivary anti-Ag I/II antibody responses (13, 47) and protection against *S. mutans*-induced dental caries (25, 39). The presence of IgG antibodies specific for Ag I/II in serum and gingival crevicular fluid also correlated with protection against caries formation in rhesus monkeys immunized subcutaneously with Ag I/II in Freund's incomplete adjuvant (32). In other studies, Δ*cya*Δ*crp* Δ*asd S. typhimurium* χ4072(pYA2905), which expresses SpaA of *S. sobrinus* 6715 (serotype *g*), has been used as an oral vaccine to determine its effectiveness in inducing systemic and mucosal responses (14, 45). Dogget et al. (14) showed the induction of a salivary and systemic antibody response against the recombinant SpaA in BALB/c mice orally immunized with *S. typhimurium* χ4072(pYA2905). We have demonstrated the induction of serum and salivary antibody against the recombinant protein as well as against the *Salmonella* carrier in Fischer rats orally immunized with this bivalent vaccine (45).

In this study, we examined the effect of the dose of orally administered live, recombinant *S. typhimurium* χ4072(pYA2905) on the induction of salivary and serum antibody responses to SpaA as well as to *Salmonella* carrier. Evidence is presented relating the induction of salivary anti-SpaA antibody in immunized animals with protection against *S. sobrinus*-induced dental caries. The possible mechanisms which could account for the observed protection are 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) used in these studies were removed from the isolators and maintained in covered sterile cages in a laminar-flow hood. Rats were provided autoclaved food (Agway Prolab animal diet RMH 1000 [Agway Country Foods, Inc., Syracuse, N.Y.]) for the antibody response study or the caries-promoting diet 305 for the protection study (42) and given water ad libitum. Male and female rats approximately 8 weeks old were used in the initial dose-response study, whereas 19- to 21-day-old weanling rats were used in the protection study. All animal experiments were approved by the University of Alabama at Birmingham Animal Resources Advisory Committee.

Microorganisms. *S. typhimurium* χ4072, a derivative of SR-11 (44) (kindly provided by Roy Curtiss III, Washington University, St. Louis, Mo.), was 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 cAMP 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 peptidoglycan of the cell wall of gram-negative organisms. In the absence of DAP, the *Salmonella* mutant cannot replicate and undergoes DAP-less cell death. 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% dextrose [Sigma]) containing 15% glycerol (Sigma) at −70°C. Cultures of the *S. typhimurium* χ4072(pYA2905), used for the challenge inoculum, and LT-2, used as whole-cell antigen in enzyme-linked immunosorbent assay (ELISA) (see below), were grown in L broth at 37°C in a rotary shaker.

S. sobrinus 6715 was stored in brain heart infusion (BHI) broth (Difco) containing 50% glycerol at −70°C. Cultures grown in BHI 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 preparations (see below) and to orally challenge weanling rats.

Plasmid construction. The plasmid used in these studies, pYA2905, was obtained from Roy Curtiss III. 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 (19). In addition to encoding antigenic determinants of SpaA, pYA2905 also contains the *S. typhimurium asd* gene encoding β-semialdehyde dehydrogenase (17), which complements the Asd$^-$ phenotype of *S. typhimurium* χ4072 and results in a balanced-lethal system allowing the *Salmonella* mutant to grow in medium lacking DAP (11).

Reagents. Formalin-killed whole cells of *S. typhimurium* χ4072 and *S. sobrinus* 6715 were prepared for use in the ELISA as previously described (45). *S. typhimurium* χ4072 was grown in L broth for 18 h, harvested by centrifugation (6,000 × *g*), washed three times with sterile saline (0.9% NaCl), and resuspended in 0.1% formalin-saline at a density of 5×10^{10} bacteria per ml. *S. sobrinus* 6715 was cultured anaerobically in BHI broth for 18 h, harvested, washed, and resuspended in 0.5% formalin-saline at a density of 10^{11} bacteria per ml. The formalin-killed whole-cell antigens were stored at 4°C until used.

Native SpaA was prepared from *S. sobrinus* 6715 grown in a 28-liter culture of a streptococcal defined medium (JRH Biosciences, Lenexa, Kans.) as previously reported (45). The culture supernatant was concentrated by using a Pelicon filter (Millipore Corp., Marlborough, Mass.) with a 10,000-Da-molecular-mass cutoff membrane and then precipitated with ammonium sulfate (Sigma) at 75% saturation. The resulting precipitate was resuspended and dialyzed against 0.01 M Tris-HCl (Sigma), pH 8.0. SpaA was then purified chromatographically by using DEAE-cellulose (Whatman BioSystems Inc., Clifton, N.J.) and Sephacryl S-300 (Pharmacia Biotec Inc., Piscataway, N.J.). Purity was tested by immunodiffusion using rabbit anti-SpaA serum (45) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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 (10 min at 3,000 × *g*) and resuspended in phosphate-buffered saline (PBS). The number of bacteria in the suspension was determined by reading the optical density at 660 nm and extrapolating from a growth curve. The bacteria were then diluted to 4×10^9 or 4×10^8 CFU/ml in intubation medium (28) consisting of 8 parts Hanks' balanced salt solution (Life Technologies Inc., Grand Island, N.Y.) and 2 parts sodium bicarbonate (7.5% solution; Mediatech, Washington, D.C.). The bacterial inoculum was 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.

For the initial immunization study, rats (five to six per group) were immunized on days 0, 4, and 6. Serum and saliva were collected at weekly intervals and assayed for antibody activity to SpaA and *S. typhimurium* χ4072 by ELISA. In the protection study, rats (five to seven per group) were immunized on days 0, 2, and 4, and the immunized and control animals were then orally challenged by using sterile cotton swabs on days 1, 2, and 3 with fresh 18-h cultures of *S. sobrinus* 6715 grown anaerobically in BHI broth under anaerobic conditions (45). At the termination of the experiment (7 weeks after the initial immunization), individual serum and saliva samples were collected. Rats were then sacrificed, and their mandibles were aseptically removed and assessed for the number of *S. sobrinus* 6715 organisms present and for the level of caries activity on the molar surfaces (41). Briefly, the right mandible from each rat was transferred to a tube containing 3 ml of sterile phosphate (Sigma) buffer (0.067 M, pH 7.2). The plaque was disrupted from the molar surfaces by sonication (Branson Instruments Co., Plainview, N.Y.), and the numbers of bacteria in plaque were determined by culturing known dilutions of samples, using a Spiral Plater model D (Spiral Systems, Inc., Cincinnati, Ohio), on mitis salivarius agar (Difco) for *S. sobrinus* 6715 counts and on blood agar (tryptose blood agar base, dehydrated; Difco) for total bacteria counts. The right and left mandibles were then cleaned and stained with murexide (0.4% in 70% ethanol; Sigma), allowing optimal visualization of carious lesions. The mandibles were then hemisectioned, and the buccal, sulcal, and proximal molar caries were scored by the procedure of Keyes (26).

Sample collection. Prior to sample collection, rats were anesthetized by intramuscular injection (0.05 ml/100 g of body weight) of a solution consisting of 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. To facilitate saliva collection, rats were given carbacol (carbamylcholine chloride; Sigma) (10 to 15 μl of a 100-μg/ml solution per 100 g of body weight) by intraperitoneal injection. Saliva was collected with a Pasteur pipette and clarified by centrifugation (13,000 × *g*, 10 min, 4°C). Blood was collected by cardiac puncture while rats remained anesthetized. The blood was allowed to clot at 4°C, and after centrifugation (2,700 × *g*), the serum was collected. Serum and saliva samples were stored at −70°C until assayed for antibody activity by ELISA (as described below).

In vivo recovery of *S. typhimurium* χ4072(pYA2905). After gastric intubation of the rats with the recombinant *S. typhimurium* on days 0, 2, and 4, the animals

(two rats per group per time point) were sacrificed on days 5, 7, and 9 to determine the persistence of recombinant *S. typhimurium*. The spleens and Peyer's patches from individual animals were removed, and each was placed in sterile PBS (5 ml). Single-cell suspensions were prepared by processing the tissues through a 22-gauge stainless steel wire mesh. The resulting cell suspensions were then diluted, and aliquots were spread on bismuth sulfite agar for CFU determination following incubation at 37°C for 24 h. The data are expressed as the number of CFU per tissue per rat.

Antibody responses. Antibody activity to *S. typhimurium* or *S. sobrinus* 6715 was assessed by ELISA as previously described (45). Individual wells of flat-bottom 96-well plates (Dynatech Laboratories, Chantilly, Va.) were coated with 5×10^8 CFU of formalin-killed *S. typhimurium* LT-2 per ml, 5 μg of purified native SpaA per ml, or anti-rat α μ, γ heavy-chain antibody in 0.2 M 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 (Fisher Scientific, Fair Lawn, N.J.) (pH 7.4) for 2 h at room temperature. From starting dilutions of serum (1:100) and saliva (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. In the initial assays, a pool of Fischer rat sera was used as a standard, and serial twofold dilutions were added to the anti-rat Ig-coated wells in each plate. Plates were incubated 2 h at 37°C and washed, and biotin-conjugated anti-rat Ig (Southern Biotechnology Associates, Birmingham, Ala.) was added to each well. Plates were incubated overnight (4°C) and washed, and streptavidin-alkaline phosphatase (0.4 mg/ml) (Southern Biotechnology Associates) was added (30 min at room temperature). Plates were washed, phosphatase substrate (Sigma 104) in diethanolamine buffer (1 M diethanolamine [Fisher Scientific], 0.2 mM $MgCl_2 \cdot 6H_2O$ [Fisher Scientific] [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 (Apple Computer, Inc., Cupertino, Calif.). Anti-SpaA and anti-*Salmonella* activities were determined by interpolating from the standard curve of total Ig activity established by using the pool of Fischer rat sera and expressed as ELISA units (EU) per milliliter, whereby 1 EU/ml equaled the dilution of the standard giving an optical density reading of 0.1. The level of antibody activity (EU per milliliter) in serum and saliva samples run simultaneously with the standard was determined by interpolating from the standard curve, using a four-parameter logistic algorithm (Softmax; Molecular Devices Corp.).

In subsequent assays, the levels of total IgG, IgM, and IgA, as well as isotype-specific anti-SpaA and anti-*Salmonella* activities, were determined by using a standard curve generated by using a calibrated pool of Fischer rat sera with known concentrations of IgG, IgM, and IgA. These assays were processed the same as the assays for determining EU of antibody activity per milliliter. However, instead of being coated with anti-rat Ig, the individual wells used to generate the standard curve were coated with anti-rat α, μ, γ heavy-chain antibody. The equivalent concentrations (nanograms per milliliter) of IgG, IgM, and IgA anti-SpaA and anti-*Salmonella* activities and the total levels of Ig isotypes were determined by running the samples simultaneously with the calibrated pool of Fischer rat sera and interpolating from the standard curve by using a four-parameter algorithm. The calibrated rat serum used in the ELISA was prepared from a pool of Fischer rat sera. The amount of IgG, IgM, and IgA in the rat sera pool was determined by using a standard curve generated by using a known concentration of purified rat IgG, IgM, of IgA (UAB Immunochemical Core Facility, Birmingham, Ala.).

In the initial experiment, the levels of salivary IgA activity were determined by recording optical densities at 405 nm for samples diluted 1:5. In later assays, the equivalent concentrations (nanograms per milliliter) of total salivary IgA and salivary IgA anti-SpaA and anti-*Salmonella* activities were determined. Salivary anti-SpaA and anti-*Salmonella* activities were expressed as percentages of total salivary IgA.

Statistics. The significance of differences in the mean antibody levels, numbers of *S. sobrinus* or total bacteria, and caries activities between groups was determined by analysis of variance with StatView 4.0 software (Abacus Concepts, Inc., Berkeley, Calif.). A P value less than 0.05 was considered statistically significant.

RESULTS

Persistence following multiple immunizations with different doses of *S. typhimurium* χ4072(pYA2905). To help explain the effectiveness of the recombinant *Salmonella* vaccine in inducing antibody responses, the persistence of salmonellae in Peyer's patches and spleens of animals immunized on days 0, 2, and 4 with 10^8 or 10^9 CFU of *S. typhimurium* χ4072(pYA2905) was determined. More salmonellae were recovered from tissues of animals immunized with 10^9 viable recombinant *Salmonella* vaccine than from animals immunized with 10^8 CFU (Fig. 1). On day 5 following oral immunization, approximately threefold-higher numbers of salmonellae were detected in the Peyer's patches of rats given 10^9 CFU than in those of rats given 10^8 CFU (Fig. 1A). The number of recoverable salmonellae dropped slightly on day 7. By day 9 following immunization, 1.97×10^4 CFU was recovered from rats given 10^9 *S. typhimurium* χ4072(pYA2905) whereas only 2.05×10^3 CFU was recovered from rats given 10^8 *S. typhimurium* χ4072(pYA2905). Higher numbers of salmonellae were also recovered from the spleens of rats given 10^9 CFU than from those of rats given 10^8 CFU (Fig. 1B). On day 7, one rat given 10^9 *S. typhimurium* χ4072(pYA2905) had 2.55×10^3 CFU in its

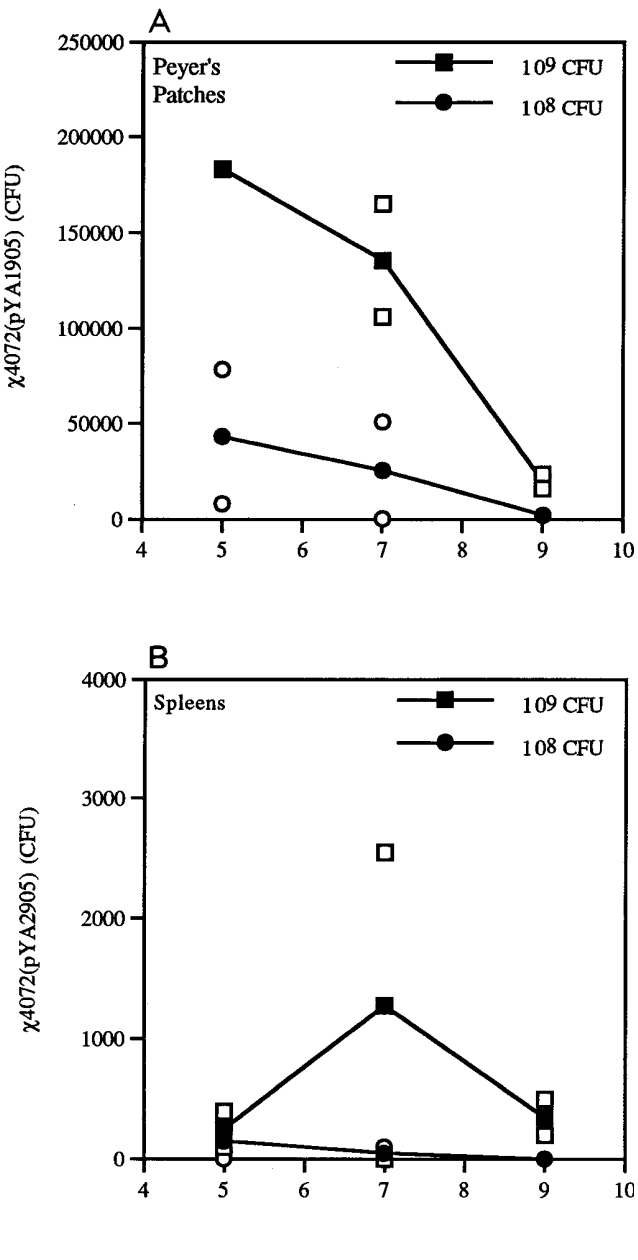

FIG. 1. Persistence of *S. typhimurium* χ4072(pYA2905) in Peyer's patches (A) and spleens (B) of rats given the recombinant bacteria orally by gastric intubation. Rats were immunized with *S. typhimurium* χ4072(pYA2905) (10^9 or 10^8 CFU) on days 0, 2, and 4. Results are expressed as individual (open symbols) and mean (closed symbols) values of the number of *S. typhimurium* χ4072(pYA2905) recovered from the spleen or Peyer's patches of each rat (two animals per time point).

spleen, compared with a mean of 100 CFU in the spleens of rats given 10^8 *S. typhimurium* χ4072(pYA2905). By 9 days after immunization, salmonellae were cleared from the spleens of rats immunized with 10^8 organisms. However, at this time, 350 CFU was detected in the spleens of rats orally immunized with 10^9 recombinant *S. typhimurium* χ4072(pYA2905) vaccine.

Effect of dose on induction of humoral immune responses against SpaA and the *Salmonella* carrier. Serum Ig responses to SpaA and *Salmonella* carrier were detected in animals orally immunized with 10^8 or 10^9 CFU of *S. typhimurium* χ4072 (pYA2905) on days 0, 4, and 6 (Fig. 2). Animals immunized with 10^9 recombinant *S. typhimurium* χ4072(pYA2905) had higher antibody responses to both SpaA and *Salmonella* carrier than animals immunized with 10^8 CFU throughout most of the experiment. The difference in the anti-*Salmonella* antibody responses on days 21, 28, 42, and 49 between animals given 10^9 or 10^8 CFU was significant ($P < 0.05$) (Fig. 2A). Maximum anti-*Salmonella* antibody activity occurred about 70 days after immunization in animals receiving either dose. The maximum serum anti-SpaA response in animals immunized with 10^9 recombinant *S. typhimurium* χ4072(pYA2905) was detected on day 35, and following a decline, the level of activity gradually increased throughout the experiment (Fig. 2B). Animals immunized with 10^8 CFU had maximum serum anti-SpaA activity about 70 days after immunization.

Salivary IgA responses to the *Salmonella* carrier and SpaA were detected in rats receiving either 10^8 or 10^9 CFU of *S. typhimurium* χ4072(pYA2905) (Fig. 3). Although variability in the responses was seen, rats immunized with either dose had maximum salivary anti-SpaA responses about 84 days following immunization. Anti-SpaA responses were similar in rats given 10^8 or 10^9 CFU; however, rats given 10^8 CFU had significantly greater anti-SpaA activity on days 49 and 70 than did rats immunized with 10^9 CFU. Similarly, salivary anti-*Salmonella* responses were induced but differences in responses were not significant (data not shown).

Induction of protective immune responses against *S. sobrinus* induced dental caries. In a second set of experiments, the effectiveness of the induced immune response on conferring protection against dental caries was evaluated. In this study, rats were immunized intragastrically with 10^8 or 10^9 CFU of *S. typhimurium* χ4072(pYA2905) and then challenged with cariogenic *S. sobrinus* 6715. Animals immunized with 10^9 CFU had levels of serum antibody against SpaA and the *Salmonella* carrier on day 49 which were similar to those in rats immunized with 10^8 CFU (Table 1). Animals receiving either 10^8 or 10^9 CFU had significantly ($P < 0.05$) higher serum IgG and approximately sixfold-higher serum IgA anti-*Salmonella* responses than nonimmunized animals. The levels of serum IgG anti-SpaA in the immunized rats and of serum IgG anti-SpaA in rats immunized with 10^9 CFU were slightly higher than those seen in nonimmunized rats; however, the differences were not significant. No IgM responses were induced with immunization.

Mucosal IgA anti-SpaA responses detected on day 49 in saliva of immunized animals were nearly twofold greater than those seen in nonimmunized animals; however, they are not statistically different (Table 2). Similarly, the salivary IgA anti-*Salmonella* responses were greater in immunized than in nonimmunized rats. The salivary IgA anti-SpaA and anti-*Salmonella* responses were higher in rats given 10^9 than in rats given 10^8 CFU.

Animals immunized with the recombinant *Salmonella* vaccine expressing SpaA were protected against *S. sobrinus*-induced dental caries (Table 3). Lower caries activity was seen on the smooth buccal and proximal molar surfaces of immunized compared with nonimmunized, infected control animals. The levels of caries activity on the sulcal molar surfaces (pits and fissures) were significantly lower ($P < 0.05$) in immunized than in nonimmunized, infected control rats. Furthermore, animals immunized with the recombinant *Salmonella* vaccine had fewer numbers of *S. sobrinus* and total bacteria on molar surfaces than infected controls. No notable difference was seen in the levels of caries activity or in the number of recoverable

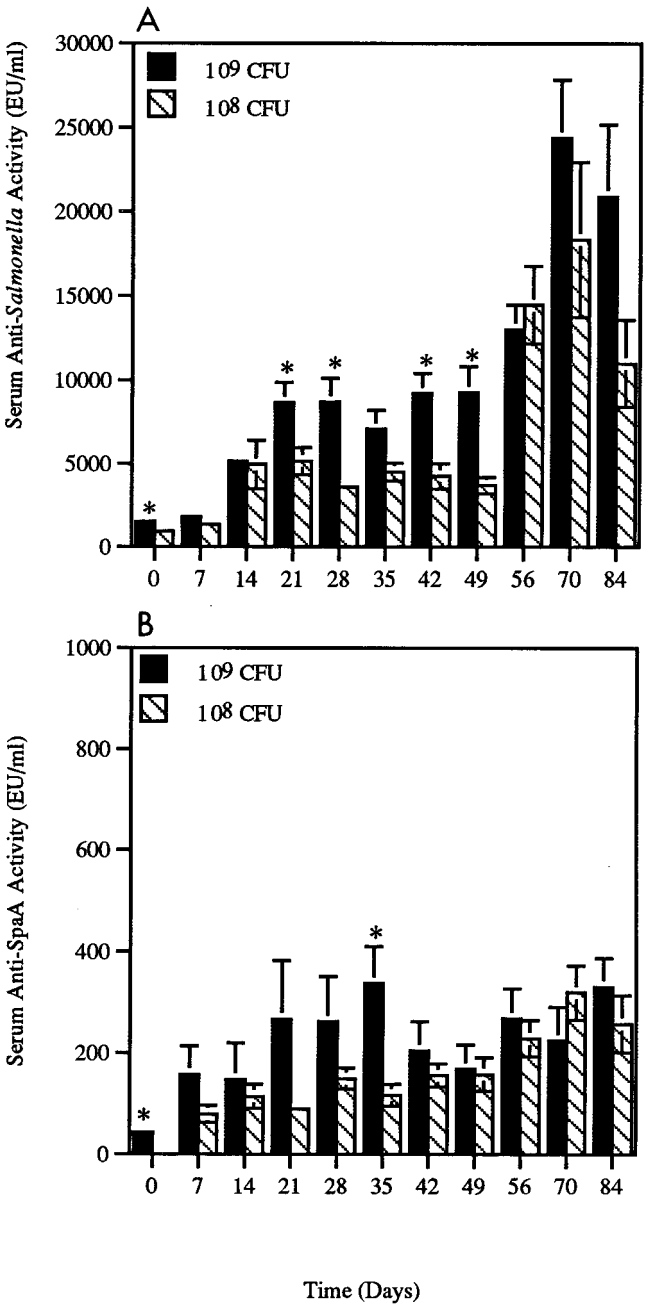

FIG. 2. Serum Ig activity to *S. typhimurium* (A) and SpaA (B) in rats given *S. typhimurium* χ4072(pYA2905) three times by gastric intubation. Rats were orally immunized with *S. typhimurium* χ4072(pYA2905) (10^9 or 10^8 CFU) on days 0, 4, and 6. Results are expressed as the means of the levels of serum antibody activity (five to six animals per time point). A significant difference ($P < 0.05$) in activity in animals receiving an immunization dose of 10^9 CFU compared with 10^8 CFU is indicated by an asterisk.

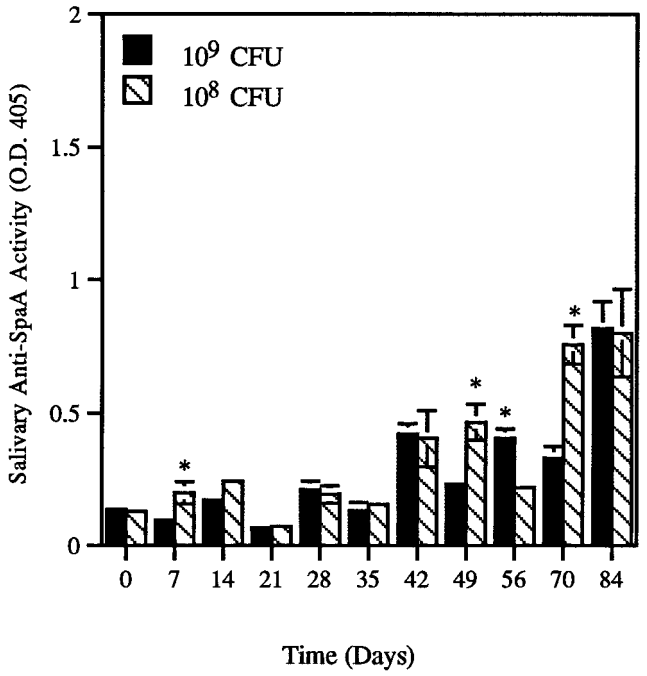

FIG. 3. Salivary IgA antibody activity to SpaA in rats given *S. typhimurium* χ4072(pYA2905) three times by gastric intubation. Rats were orally immunized with *S. typhimurium* χ4072(pYA2905) (10^9 or 10^8 CFU) on days 0, 4, and 6. Results are expressed as the mean optical density (O.D.) values at 405 nm of the levels of antibody activity in saliva diluted 1:5 (five to six animals per time point). A significant difference ($P < 0.05$) in activity in animals receiving an immunization dose of 10^9 CFU compared with 10^8 CFU is indicated by an asterisk.

bacteria on molar surfaces between the two groups of immunized rats. No difference was seen in the mean body weights of rats in these groups.

DISCUSSION

The finding that salmonellae penetrate the host via the Peyer's patches of the GALT prior to dissemination to systemic tissues has led investigators to determine the effectiveness of recombinant *Salmonella* strains as delivery systems for inducing mucosal and systemic immune responses to heterologous protein antigens (3, 11, 40). The persistence of the *Salmonella* vaccine in the GALT is an important factor influencing the induction of a mucosal immune response. However, the precise relationship between persistence and induction of responses has not been defined. Persistence of an attenuated Δ*cya*Δ*crp*Δ*asd* recombinant *Salmonella* strain in Peyer's patches and spleens of BALB/c mice given this organism by gastric intubation has been previously demonstrated (2). The numbers of recoverable salmonellae peaked within the first week and were detected for 3 weeks following immunization. In the present study, *S. typhimurium* χ4072(pYA2905) persisted as long as 9 days in the Peyer's patches and spleens of Fischer rats given 10^9 CFU (Fig. 1). Rats immunized with 10^8 CFU had fewer recoverable salmonellae which were cleared more quickly from their Peyer's patches and spleens than seen in animals given 10^9 CFU.

The difference in the persistence of *S. typhimurium* χ4072(pYA2905) in rats immunized with either 10^8 or 10^9 CFU was reflected in the humoral antibody responses to the recombinant SpaA as well as to the *Salmonella* carrier. Serum and salivary anti-*Salmonella* and anti-SpaA responses were induced in the immunized animals. Significantly greater serum antibody responses to *Salmonella* carrier were seen during the 7 weeks following immunization in animals given 10^9 CFU than in those given 10^8 CFU (Fig. 2A). Similarly, the serum anti-SpaA response (Fig. 2B) was greater in animals given 10^9 CFU than in those given 10^8 CFU. The serum anti-SpaA responses in rats given 10^9 CFU reached maximum activity by day 35. It is not known why the serum anti-*Salmonella* and anti-SpaA responses and the salivary anti-SpaA antibody responses continued to increase through days 70 to 84 of the experiment.

Although variation was seen in the serum and salivary responses to the recombinant SpaA, the kinetics of the serum antibody response to the recombinant SpaA were similar to the kinetics of serum antibody responses to a recombinant hemagglutinin (HagB) of *P. gingivalis* (15). Dusek et al. (15) used *S. typhimurium* χ4072 as the delivery system for inducing immune responses to the recombinant HagB protein in BALB/c VAF/Plus mice following intragastric immunization on days 1, 3, and 5 with 10^9 CFU of the *Salmonella* vaccine. They reported that serum antibody activity to HagB reached and maintained maximum activity 21 days after immunization. These investigators also showed the induction of a salivary IgA response to HagB which remained constant during the first 4 weeks and peaked 5 weeks, the final time point of the experiment, after immunization. In the present study, a salivary IgA anti-SpaA response was initially detected 6 weeks after intragastric immunization in rats given either 10^8 or 10^9 CFU on days 1, 3, and 5. The salivary anti-SpaA activity continued to increase throughout the 10 weeks following immunization. Differences in antibody responses to the HagB and SpaA recombinant proteins may be due to differences in the mouse and rat systems or to differences in the two cloned proteins, since the delivery system and immunization regimen were the same in both studies. The induction of an antibody response to a cloned protein delivered by a recombinant *Salmonella* vaccine may be less effective

TABLE 1. Serum antibody responses on day 49 in rats orally immunized with *S. typhimurium* χ4072(pYA2905) and challenged with *S. sobrinus* 6715[a]

Immunizing dose (CFU)	Mean concn (μg/ml) ± SEM						Total Ig (μg/ml)		
	Anti-SpaA			Anti-*Salmonella*					
	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA
~10^9	1.25 ± 0.23	0.10 ± 0.04	1.10 ± 0.36	7.35* ± 0.89	0	7.11* ± 1.06	6,453.00	242.38	62.29
~10^8	1.28 ± 0.20	0.24 ± 0.07	0.54 ± 0.26	7.49* ± 1.14	0	7.40* ± 1.31	6,441.00	337.44	96.09
Nonimmunized, infected	0.94 ± 0.17	0.39 ± 0.07	0.73 ± 0.18	0.94 ± 0.14	0.07 ± 0.01	1.28 ± 0.14	4,208.00	438.70	73.23

[a] Animals were immunized on days 0, 2, and 4 with *S. typhimurium* χ4072(pYA2905) and infected on days 1, 2, and 3 with *S. sobrinus* 6715. Sera were collected 7 weeks following the initial immunization. The numbers of animals per group were four to six for anti-SpaA and anti-*Salmonella* responses. Groups for total IgG, IgM, and IgA contained two animals. *, Significant difference from nonimmunized, infected rats ($P < 0.05$).

TABLE 2. Salivary antibody responses on day 49 in rats orally immunized with *S. typhimurium* χ4072(pYA2905) and challenged with *S. sobrinus* 6715[a]

Immunizing dose (CFU)	% Anti-SpaA of total IgA (mean ± SEM)	% Anti-*Salmonella* of total IgA (mean ± SEM)
$\sim 10^9$	1.60 ± 0.76	4.13 ± 2.74
$\sim 10^8$	1.13 ± 0.51	2.02 ± 0.91
Nonimmunized, infected	1.01 ± 0.41	0.57 ± 0.19

[a] Animals were immunized on days 0, 2, and 4 with *S. typhimurium* χ4072(pYA2905) and infected on days 1, 2, and 3 with *S. sobrinus* 6715. Saliva samples were collected 7 weeks following the initial immunization. The numbers of animals per group were four to five.

in the rat than in the mouse, since *S. typhimurium* is a murine pathogen.

An important consideration in these studies is whether the immune response induced in the immunized rats was protective. We have shown that animals immunized with either 10^8 or 10^9 CFU of *S. typhimurium* χ4072(pYA2905) and challenged with *S. sobrinus* 6715 had greater anti-SpaA antibody responses than nonimmunized, infected animals (Tables 1 and 2). These antibody responses were detected long after clearance of the recombinant *S. typhimurium* (Fig. 1 and 2). The induction of immune responses corresponded with protection against *S. sobrinus* infection and caries. Rats orally immunized with *S. typhimurium* χ4072(pYA2905) expressing recombinant SpaA from *S. sobrinus* 6715 had lower numbers of recoverable *S. sobrinus* from molar surfaces and less caries, especially at the pit and fissure (sulcal) surfaces, than nonimmunized, infected rats (Table 3). These data indicate that oral immunization with the recombinant *Salmonella* vaccine was effective in inducing protection and that prolonged persistence of recombinant *S. typhimurium* in the Peyer's patches or spleens was not required for induction of this protective immune response. Killar and Eisenstein (27) reported that the persistence of an AroA$^-$-attenuated mutant of *S. typhimurium* was not essential for protection from subsequent challenge with virulent *S. typhimurium*. This work suggests that the immune cells responsible for mediating protection are long-lived.

Evidence from studies using rhesus monkey (32) and Fischer rat (24, 43) animal models has shown that protection from dental caries in immunized animals is antibody mediated. In our experimental caries model, the rats were challenged with cariogenic *S. sobrinus* prior to 25 days of age (42), i.e., the period of susceptibility to infection. During this time, the molars are erupting and undergoing mineralization and maturation, making them increasingly resistant to infection and caries formation. Oral immunization with *S. typhimurium* χ4072 (pYA2905) expressing recombinant SpaA prior to and during challenge with cariogenic *S. sobrinus* (as in this study) resulted in the induction of protective salivary antibody. These anti-SpaA antibodies, presumably present before complete mineralization of the susceptible molars, could block the initial attachment of *S. sobrinus* to the salivary pellicle coating the tooth surface (38). Antibody-agglutinated bacteria would then be blocked from adhering and unable to colonize the tooth surface. This view is supported by studies in which IgA antibody specific for Ag I/II, an *S. mutans* protein analogous to SpaA of *S. sobrinus* (51), inhibited the binding of *S. mutans* to saliva-coated hydroxyapatite (20). Previous protection studies have been done with Ag I/II in which Fischer rats were immunized intranasally with Ag I/II coupled to the B subunit of cholera toxin and then challenged with cariogenic *S. mutans* (24). As in our study, multiple immunization resulted in the induction of a protective salivary immune response. Furthermore, the induction of an anti-Ag I/II salivary antibody was associated with reduced numbers of recoverable *S. mutans* as well as total oral bacteria in addition to decreased dental caries.

In this study, immunized rats were exposed to native SpaA during challenge with *S. sobrinus* as well as the recombinant SpaA during immunization with the *Salmonella* vaccine. The nonimmunized rats, however, were exposed only to native SpaA as a result of infection with *S. sobrinus*. Of importance was the demonstration that the specific antibody induced in immunized rats was sufficient to confer protection from *S. sobrinus*-induced dental caries. The protective effect of the anti-SpaA antibody induced in immunized animals may be due to the antibody's specificity to a key SpaA epitope. For example, the pYA2905-encoded recombinant SpaA has three tandem repeats of an immunodominant region of the native SpaA, as defined by Goldschmidt et al. (19). Perhaps the induction of antibodies to this and not another epitope on the protein accounts for the greater protection against dental caries in immunized animals. It is also possible that differences in antibody avidity can influence antibody effector function (5). Perhaps in our model, animals orally immunized with *S. typhimurium* χ4072(pYA2905) produced anti-SpaA antibodies of higher avidity than those antibodies induced in rats as a result of infection with *S. sobrinus*. This could also account for the protection observed in immunized compared with nonimmunized rats. Finally, the induction of protective anti-SpaA antibody may require exposure to a threshold amount of antigen. Such a threshold amount of antigen, and thus the induction of

TABLE 3. Effect of oral immunization with *S. typhimurium* χ4072(pYA2905) on protection against *S. sobrinus* 6715-induced dental caries[a]

Immunizing dose (CFU)	Body wt (g)	10^6 CFU/ml		Mean molar caries score[b]					
				Buccal		Sulcal		Proximal	
		S. sobrinus[c]	Total bacteria[d]	Dentinal slight	Dentinal extensive	Dentinal slight	Dentinal extensive	Enamel	Dentinal slight
$\sim 10^9$	167 ± 10	1.79* ± 1.47	3.25* ± 1.90	9.4 ± 1.7	3.8 ± 1.3	8.6† ± 1.6	0.8† ± 0.4	2.6 ± 0.6	0*
$\sim 10^8$	166 ± 11	2.47 ± 1.76	4.22* ± 2.95	9.2 ± 1.6	2.6 ± 1.2	8.8† ± 1.5	0.4† ± 0.4	3.6 ± 1.0	0.8 ± 0.5
Nonimmunized, infected	170 ± 8	10.3 ± 1.95	18.9 ± 6.87	11.2 ± 1.3	5.8 ± 0.7	18.0 ± 1.5	5.4 ± 1.6	4.0 ± 1.1	2.4 ± 1.2

[a] Animals were immunized on days 0, 2, and 4 with *S. typhimurium* χ4072(pYA2905) and infected on days 1, 2, and 3 with *S. sobrinus* 6715. Animals were sacrificed 7 weeks following the initial immunization, and mandibles were removed. The numbers of rats per group were five to seven. Values are the means ± standard errors of the means. *, Significant difference from nonimmunized, infected rats ($P < 0.05$); †, significant difference from nonimmunized, infected rats ($P < 0.01$).

[b] Determined by the method of Keyes (26).

[c] Total number of *S. sobrinus* 6715 cells in plaque as determined by growth and morphology on mitis salivarius agar plates.

[d] Total number of bacteria in plaque as determined by growth on blood agar plates.

a protective response, was attained by oral immunization with either 10^9 or 10^8 CFU of the recombinant *Salmonella* vaccine.

Here we demonstrate the successful use of an attenuated strain of *S. typhimurium* expressing SpaA of *S. sobrinus* as an oral vaccine for dental caries. Salivary and serum anti-SpaA responses were induced and corresponded with protection against *S. sobrinus* infection and dental caries. We are currently investigating the effectiveness of different *Salmonella* strains as bivalent delivery systems for the induction of protective mucosal immune responses.

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