Effect of Attenuated *Salmonella enterica* Serovar Typhimurium Expressing a *Streptococcus mutans* Antigen on Secondary Responses to the Cloned Protein

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Attenuated *Salmonella enterica* serovar Typhimurium has been used for targeted delivery of recombinant antigens to gut- and nose-associated lymphoid tissues. Contradictory reports have described the effect of preexisting immunity to the antigen delivery vehicle. We decided to examine this discrepancy by studying the effect of immunizing mice by the intranasal (i.n.) route with *Salmonella* expressing an insoluble protein and to study the ability to augment recall responses by boosting with either *Salmonella*-expressed protein or purified soluble protein alone. The glucan-binding domain (GLU) of the enzyme glucosyltransferase (GTF), which is an important virulence factor of *Streptococcus mutans*, was recombinantly expressed in the insoluble phase in *S. enterica* serovar Typhimurium, and the immunogenicity of this construct was studied in mice. We examined the induction of primary immune responses by insoluble GLU polypeptide delivered in *Salmonella* at week 1 (groups 1 and 2) and recall responses after a week 15 boost with either *Salmonella* expressing GLU (group 1) or purified GLU polypeptide (groups 2 and 3). Group 4 served as the control and received phosphate-buffered saline alone by the i.n. route. Significant anti-GLU serum immunoglobulin G (IgG) levels were seen in groups 1, 2, and 3 at week 18 ($P < 0.001$), i.e., 3 weeks after the booster immunization. Mice in group 2, who received *Salmonella* followed by GLU, had the highest GLU-specific IgG levels among all groups. The serum IgG levels persisted in all responding groups for at least 7 weeks after the boost (week 22). The IgG2a/IgG1 subclass ratio of serum anti-GLU antibodies in group 1 significantly increased after the boost. These results support the induction of a type 1-like immune response to GLU after primary and booster immunizations with *Salmonella* expressing GLU. On the other hand, group 2 mice, which received *Salmonella* expressing GLU as the primary dose and soluble protein as the booster dose, exhibited a shift from a type 1-like to a more type 2-like immune response to GLU following the boost. These results indicate that *S. enterica* serovar Typhimurium is an excellent delivery vehicle for the insoluble and recombinantly expressed GLU of GTF and that this construct was especially effective in priming the host for a secondary response to soluble GLU polypeptide.

Glucosyltransferases (GTFs) are extracellular enzymes of *Streptococcus mutans*, a principal etiological agent of human dental caries (23). The GTFs use sucrose to synthesize glucans, which are involved in the attachment and accumulation of *S. mutans* on the tooth surface. GTF has two functional domains, i.e., the N-terminal catalytic sucrose-binding domain, involved in sucrose hydrolysis, and the C-terminal glucan-binding domain (GLU), involved in binding of the synthesized glucan polymer and presumably chain extension of the growing glucan polymers (19, 25, 26, 43). It has been shown that antibodies directed towards GTF or its functional domains are capable of inhibiting glucan synthesis (5, 6, 17, 22, 33, 34). Furthermore, secretory immunoglobulin A (IgA) antibodies in saliva to peptide fragments or polypeptides derived from the two distinct functional domains are protective against the development of caries (18, 38). One could presume that induction of substan-

tial salivary IgA levels in humans by a mucosal subunit vaccine representing the functional domains of GTF would inhibit the activity of this virulence factor and thereby reduce *S. mutans*-induced dental caries. Although not life threatening, dental caries is a very costly disease, and a mucosal vaccine preventing *S. mutans* infection would indeed be beneficial (10).

Due to the fact that many soluble proteins are poor mucosal immunogens and may induce oral tolerance when administered orally (24), we decided to investigate the potential of using particulate delivery systems, such as attenuated *Salmonella* strains in combination with purified protein. Previously, attenuated *Salmonella* strains had been shown to be very effective in the delivery of a variety of antigens to mucosa-associated lymphoid tissue, resulting in the induction of antigen-specific antibody responses (4, 20, 27, 41). Interestingly, attenuated *Salmonella enterica* serovar Typhimurium BRD509, a vaccine strain with *aroA aroD* attenuations causing an inability to produce or obtain essential metabolites in mammalian hosts (36), has been used for targeted delivery of recombinantly expressed *S. mutans* antigens to gut- and nose-associated lymphoid tissues in mice (11, 14). Specifically, high levels of antibodies against the cloned heterologous antigen were demonstrated in serum and mucosal secretions after oral or intranasal (i.n.) immunization (11, 15).

There have been contradictory reports describing the effect

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of preexisting immunity to homologous serotypes of the antigen delivery bacteria. It has been shown that prior immunological experience with the delivery vehicle potentiates the subsequent antibody response following oral immunization with recombinant *Salmonella* (2). Also, it was demonstrated that mice primed with a carrier strain 3 to 6 months prior to intraperitoneal administration of the same strain carrying a model antigen actually enhanced the immune response to the foreign antigen (42). In addition, antibody responses against antigens delivered through *Salmonella* vectors can be boosted by subcutaneous injections of purified protein (1, 41). In contrast, preexisting immunity to *Salmonella* can lower the serum IgG recall response, depending on when mice were boosted with *Salmonella* expressing a bacterial virulence factor (21). Furthermore, preexisting immunity to *S. enterica* serovar Typhimurium had a major negative effect on the immune response to a bacterial antigen in mice orally immunized with *Salmonella* expressing the antigen (31). Both reduced serum antibody levels and a lack of protection against infection were seen compared to mice with no preexisting immunity.

In the present study, we investigated the effect of immunizing mice by the i.n. route with *Salmonella* expressing the GLU of GTF (17) and the ability of either purified GLU or *Salmonella* expressing GLU to augment recall responses. The magnitudes of the serum and mucosal antibody responses were assessed after primary and secondary i.n. immunizations of mice to determine the effect of the vaccine on the resulting responses.

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MATERIALS AND METHODS

Bacterial strains and genetic construction. The construction of the *S. enterica* serovar Typhimurium BRD509[pGP1-2, pET20b(+)-GLU] strain used in this study was previously described in detail (17). Briefly, the GLU of GTF-I (amino acids 1183 to 1473 [32]) was inserted into the expression vector pET20b(+) (Novagen, Madison, Wis.). This construct was electroporated into *S. enterica* serovar Typhimurium BRD509 along with pGP1-2, which provides a source of T7 RNA polymerase under the control of the λP_L promoter, which is regulated by a temperature-inducible λ repressor (37).

Western blot analysis. An overnight culture of *S. enterica* serovar Typhimurium BRD509(pGP1-2) containing pET20b(+)-GLU was diluted 1:100 in Luria-Bertani broth containing 50 μ g of carbenicillin/ml [selection for pET20b(+)-GLU] and 50 μ g of kanamycin/ml (selection for pGP1-2). The inoculated culture was grown to mid-log phase (approximately 4 h) at 30°C before recombinant protein expression was induced by a temperature shift of the cells from 30 to 37°C for 30 min. The cells were grown for an additional 3 h at 30°C and then harvested by centrifugation. The pelleted cells were solubilized in TTE buffer (0.05 M Tris-HCl [pH 8.0], 0.1% Triton X-100, 2 mM EDTA) and sonicated on ice, and the insoluble proteins were recovered by centrifugation. The pellet was solubilized in urea buffer (8 M urea, 50 mM Tris-HCl [pH 7.9], 0.5 M NaCl, 1 mM EDTA, 30 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonylfluoride). The soluble and insoluble fractions were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (4 to 15% gradient gel) using protein amounts corresponding to that expressed by 1.2×10^6 cells. The SDS-PAGE was followed by Western blotting, and the blot was probed with biotinylated rabbit anti-GLU specific antibodies in order to determine the location and solubility of the *Salmonella*-expressed GLU polypeptide (17).

Protein purification. The purification of the GLU polypeptide was obtained by nickel column affinity purification as previously described (18, 20). Briefly, an overnight culture of *Escherichia coli* BL21(DE3) containing pET20b(+)-GLU was grown to mid-log phase at 30°C, induced by 1 mM IPTG (isopropyl- β -D-thiogalactopyranoside), and grown for an additional 3-h period. The main proportion of GLU was recovered from inclusion bodies through solubilization of

TABLE 1. Experimental groups used in this study

Group	Immunization ^a	
	Primary	Booster
1	<i>Salmonella</i> expressing GLU ^b	<i>Salmonella</i> expressing GLU
2	<i>Salmonella</i> expressing GLU	GLU ^c
3	Buffer ^d	GLU
4	Buffer	Buffer

^a Animals received primary and booster immunizations by the nasal route at weeks 0 and 15, respectively.

^b Groups of six mice (8 to 10 weeks of age) received i.n. immunizations with 10^9 CFU of *S. enterica* serovar Typhimurium BRD509[pGP1-2, pET20b(+)-GLU].

^c Groups of mice received i.n. booster immunizations with 50 μ g of affinity-purified GLU polypeptide.

^d Groups of mice were sham-immunized i.n. with PBS.

the insoluble protein fraction in 6 M guanidine-HCl-0.1 M NaH_2PO_4 -1 mM Tris-HCl (pH 8.0) by stirring the solution at room temperature for 4 h. The lysate was sonicated, clarified by centrifugation, and loaded on a precharged and equilibrated His-Bind Resin column (Novagen) at 4°C overnight. The unbound protein was passed through the column by gravity, and the column-bound protein was washed with 5 column volumes of 8 M urea-0.1 M NaH_2PO_4 -1 mM Tris-HCl (pH 8.0), followed by 3 column volumes of 8 M urea-0.1 M NaH_2PO_4 -1 mM Tris-HCl (pH 6.3). The protein was refolded by gradually lowering the initial urea content in 1 M steps of the refolding buffer (8 M urea, 0.5 M NaCl, 10 mM Tris-HCl, 20% glycerol, pH 7.4) and then eluted with 0.25 M imidazole in refolding buffer (without urea). Finally, the recovered protein was dialyzed against 50 mM Tris-HCl (pH 7.9)-0.5 M NaCl-10% glycerol before being used for immunization.

Mouse i.n. immunizations. Groups of six female BALB/c mice (8 to 10 weeks of age) received on day zero an i.n. immunization with either 10^9 CFU of *S. enterica* serovar Typhimurium BRD509[pGP1-2, pET20b(+)-GLU] (groups 1 and 2) or phosphate-buffered saline (PBS) only (groups 3 and 4) (Table 1). The cells were grown to mid-log phase, washed, and resuspended in PBS before being slowly applied to each mouse nostril in a total volume of 20 μ l. The animals were boosted at week 15 with either *S. enterica* serovar Typhimurium BRD509[pGP1-2, pET20b(+)-GLU] (group 1), 50 μ g of purified GLU polypeptide (groups 2 and 3), or buffer only (group 4). Blood samples were taken at weeks 0, 3, 7, 11, 15, 18, and 22, whereas saliva and vaginal-wash samples were collected at week 22 as previously described (18). The animal experiments were performed according to National Institutes of Health guidelines, and the protocols were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

Enzyme-linked immunosorbent assay. The amount of GLU-specific antibodies in samples was determined by enzyme-linked immunosorbent assay on Maxi-sorp microtiter plates (Nunc, Roskilde, Denmark) coated with purified GLU polypeptide (1 μ g/ml) as previously described (18). The amount of specific antibodies to *Salmonella* was determined on plates coated with 5×10^8 CFU of formalin-killed *S. enterica* serovar Typhimurium BRD509 containing pGP1-2 (a plasmid encoding only T7 polymerase) (19, 25, 26, 30, 43). Plates examined for IgG and IgA antibodies specific for GLU were developed with peroxidase-labeled antibodies to mouse IgG and IgA, respectively. Plates used for determination of IgG antibodies specific to *Salmonella* were blocked for 2 h at room temperature with 5% fetal calf serum in 0.01 M phosphate buffer (pH 7.2) containing 0.5 M NaCl and 0.15% Tween 20. Plates used for detection of GLU- or *Salmonella*-specific IgG2a or IgG1 antibodies were blocked with 1% bovine serum albumin in 0.01 M phosphate buffer (pH 7.2) containing 0.5 M NaCl and 0.15% Tween 20 for 2 h at room temperature. Detection was done using peroxidase-labeled antibodies to mouse IgG2a or IgG1. Total levels of IgA in secretions were measured in plates coated with an optimal concentration of antibodies to mouse IgA. Peroxidase-labeled antibodies to mouse IgA or IgG were used as detection reagents, followed by *o*-phenylenediamine substrate with H_2O_2 . The detecting and coating antibodies used in this study were purchased from Southern Biotechnology Associates, Inc., Birmingham, Ala. The levels of antibody in samples were logarithmically transformed, and statistical analyses (Student's *t* test) of differences between groups were performed by using the InStat program (GraphPad Software, San Diego, Calif.).

FIG. 1. Western blot of soluble (lane 1) and insoluble (lane 2) protein extracts of approximately 1.2×10^6 cells of *S. enterica* serovar Typhimurium[pGP1-2, pET20b(+)-GLU]. The blot was probed with biotinylated anti-GLU antibodies and developed with alkaline phosphatase-conjugated streptavidin.

RESULTS

Protein expression. Fractionation of soluble and insoluble protein extracts from approximately 1.2×10^6 cells of *S. enterica* serovar Typhimurium[pGP1-2, pET20b(+)-GLU] on SDS-PAGE, followed by immunoblotting and probing with GLU-specific antibodies, illustrates that the GLU polypeptide is predominantly expressed in the insoluble phase (Fig. 1). The number of *Salmonella* cells used for preparation of the West-

ern blot lysate was equal to 0.125% of the *Salmonella* cells used for immunization. The insoluble nature of GLU makes it difficult to determine the amount of GLU polypeptide expressed by *Salmonella*; however, we normally recover approximately $10 \mu\text{g}$ of GLU/ 10^9 CFU by nickel column affinity purification.

Serum IgG antibody responses. Mice receiving *Salmonella* expressing GLU as both the primary and booster immunizations (group 1) reached specific anti-GLU serum IgG levels which were significantly different ($P < 0.05$) from those of the control group of mice 7 weeks after the primary immunization (Fig. 2A). The GLU-specific antibody level was enhanced (approximately 3.5-fold) following the boost to a mean value of approximately $24 \mu\text{g/ml}$ at week 18. The anti-GLU IgG levels were significantly different from those of the control group of mice at both weeks 18 ($P < 0.001$) and 22 ($P < 0.01$). Mice primed with *Salmonella* expressing GLU and boosted with purified GLU polypeptide alone (group 2) showed slightly lower levels of GLU-specific IgG in serum before the boost than did group 1. However, the specific anti-GLU serum IgG response in group 2 reached levels higher than those seen in all other groups at weeks 18 and 22. The GLU-specific IgG levels were enhanced approximately 100-fold, from a mean value of 1.2 to a mean value of $114 \mu\text{g/ml}$, at week 18. The anti-GLU IgG levels were significantly different from those of control (group 4) ($P < 0.001$) and group 1 ($P < 0.05$) mice at both week 18 and 22 time points, in addition to a difference from group 3 at week 18 ($P < 0.05$). Mice receiving only a single immunization with soluble protein (group 3) exhibited a serum IgG anti-GLU response at week 18 which was similar in magnitude to that seen in group 1 mice and significantly different ($P < 0.001$) from that of the control group at weeks 18 and 22. Essentially no anti-GLU activity was detected in sera from the control group of unimmunized animals (group 4) throughout the duration of this experiment. Overall, groups receiving booster immunizations with either *Salmonella* expressing GLU or purified GLU polypeptide exhibited significant levels of serum IgG anti-GLU antibodies after the boost. The group receiving the soluble protein showed the greatest recall response.

FIG. 2. Serum IgG anti-GLU (A) and anti-*Salmonella* (B) antibody levels in mice during the time course of the experiment. The results are shown as the geometric mean $\times$ standard deviation for six mice. Specific antibody levels were significantly different from those of the control group at $P < 0.05$ (*), $P < 0.01$ (**), or $P < 0.001$ (***). Arrow, time of immunization.

Mice immunized and boosted with *Salmonella* expressing GLU (group 1) displayed a significant increase ($P < 0.01$) in the level of *Salmonella*-specific IgG antibodies in serum (Fig. 2B). Specifically, the mean preboost serum IgG anti-*Salmonella* level increased approximately 2.4-fold, from 45 to 107 $\mu\text{g/ml}$. The level of serum IgG anti-*Salmonella* antibody activity in mice immunized but not boosted with *Salmonella* (group 2) peaked between weeks 3 and 7 and then decreased slightly throughout the remainder of the experiment. Animals that did not receive *Salmonella*-based immunizations (groups 3 and 4) exhibited a slight increase in the level of antibodies specific for or cross-reacting with *Salmonella* throughout the study.

Serum IgG subclass response to GLU. The levels of serum IgG1 anti-GLU antibodies in group 1 were significantly different ($P < 0.05$) from the levels seen in group 3 at weeks 3 and 7 (Fig. 3A). Group 2 mice also showed a slight increase in the GLU-specific IgG1 response after the primary immunization, which was significantly different ($P < 0.05$) from that of group 3 at week 7. Mice immunized with purified GLU polypeptide at week 15 (groups 2 and 3) exhibited an IgG1 anti-GLU response. Specifically, groups 2 and 3 showed 210- and 208-fold increases, respectively, in their postboost GLU-specific responses compared to preboost levels. The IgG1 response to GLU in group 1 mice showed only a 2.3-fold increase after the boost. The responses in group 2 and 3 mice were significantly different from those seen in group 1 at week 18 ($P < 0.01$ and $P < 0.05$, respectively) and at week 22 ($P < 0.001$ and $P < 0.01$, respectively).

The levels of serum IgG2a anti-GLU antibodies in group 1 mice were significantly different ($P < 0.05$, $P < 0.001$, and $P < 0.01$, respectively) from the levels seen in group 3 at weeks 3, 7, and 11 (Fig. 3B). Group 2 mice showed a similar increase in the GLU-specific IgG2a level after the primary immunization, which was significantly different ($P < 0.01$ and $P < 0.05$, respectively) from group 3 at weeks 7 and 11. All groups showed increases in GLU-specific IgG2a levels upon receiving booster immunizations. Groups 1 and 2 showed 158- and 811-fold increases, respectively, in their postboost GLU-specific IgG2a responses compared to preboost levels. The IgG2a response to GLU in group 3 showed only a 23-fold increase after the boost. The levels in group 1 and 2 mice were significantly different ($P < 0.01$) from those seen in group 3 at week 18, whereas only group 2 mice were different at the week 22 time point ($P < 0.01$).

The ratio of anti-GLU specific serum IgG2a to IgG1 for group 1 was significantly different from the ratio for group 3 at several time points, including both pre- and postboost ratios (Fig. 3C). Specifically, the IgG2a/IgG1 ratio for group 1 showed an increase after the primary immunization, with significant differences compared to group 3 at weeks 3, 7, and 11 ($P < 0.05$, $P < 0.05$, and $P < 0.01$, respectively). Group 2 mice, which were primed with *Salmonella*, showed a similar initial enhancement of the IgG2a/IgG1 ratio postpriming which was significantly different ($P < 0.01$) at week 11 from that of group 3. The IgG2a/IgG1 ratio for group 1 was augmented by a factor of 74 after the boost, to a mean ratio of 74, whereas the IgG subclass ratios for groups 2 and 3 were unchanged (≈ 1.8) and decreased (< 1), respectively. The IgG2a/IgG1 ratios for group 1 were significantly different from those of groups 2 ($P < 0.05$) and 3 ($P < 0.001$) at weeks 18 and 22.

FIG. 3. Serum levels of anti-GLU IgG1 (A) and IgG2a (B) and IgG2a/IgG1 ratios (C) in mice during the time course of the experiment. The results are shown as the geometric mean $\times$ standard deviation for six mice. Specific antibody levels and ratios were significantly different from those of group 3 at $P < 0.05$ (*), $P < 0.01$ (**), or $P < 0.001$ (***). Open squares, *Salmonella* plus *Salmonella*; solid circles, *Salmonella* plus GLU; open circles, buffer plus GLU; arrow, time of immunization.

Anti-*Salmonella* serum IgG1 and IgG2a subclass responses.

Anti-*Salmonella* serum IgG1 levels were constant throughout the duration of the experiment and were not significantly augmented after the boost (Fig. 4A). In contrast, serum IgG2a levels specific for *Salmonella* seemed to increase in all groups during the experiment (Fig. 4B). The anti-*Salmonella* serum IgG2a levels in groups 1 and 2 were significantly higher ($P < 0.05$) than those seen in group 3 at various time points after the

FIG. 4. Serum levels of anti-*Salmonella* IgG1 (A) and IgG2a (B) in mice during the time course of the experiment. The results are shown as the geometric mean $\times$ standard deviation for six mice. Specific antibody levels and ratios were significantly different from those of group 3 at $P < 0.05$ (*), $P < 0.01$ (**), or $P < 0.001$ (***). Arrow, time of immunization.

initial immunization. The mean level of IgG2a antibodies in group 1 was significantly different from the mean levels in group 2 ($P < 0.05$) and group 3 ($P < 0.001$) mice at the week 18 and 22 time points. A predominant serum IgG2a (compared to IgG1) anti-*Salmonella* antibody response was seen in mice immunized with the *Salmonella* vector.

GLU-specific IgA in serum, saliva, and vaginal washes. Only low levels of GLU-specific IgA were detected in pooled samples in serum and secretions at time points prior to week 22,

which were not above background level (data not shown). In week 22 samples, the percentage of GLU-specific IgA out of total IgA in saliva from group 1 or 2 mice was significantly different from that seen in group 3 ($P < 0.01$) or 4 ($P < 0.01$ and $P < 0.05$, respectively) mice (Fig. 5A). The percentage of GLU-specific IgA out of total IgA in vaginal-wash samples exhibited a similar pattern, with elevated levels of GLU-specific IgA in groups 1 and 2 (Fig. 5B). However, only the levels in group 2 mice were significantly different from those in group

FIG. 5. Salivary IgA (A), vaginal IgA (B), and serum IgA (C) responses to GLU polypeptide at week 22. The results are shown as geometric means of either the percentage of specific IgA response out of total IgA (A and B) or the actual level of specific IgA (C) $\times$ standard deviations for six mice. The specific antibody levels were significantly different from those of control mice at $P < 0.05$ (*) or $P < 0.01$ (**).

3 and the control group 4 ($P < 0.05$). A similar trend was seen in serum IgA activity, in that elevated levels of GLU-specific IgA were seen in sera from group 1 and 2 animals which were significantly different from that of group 4 ($P < 0.05$) at week 22 (Fig. 5C).

DISCUSSION

We have investigated the effect of immunizing mice by the i.n. route with *S. enterica* serovar Typhimurium expressing an insoluble protein and the influence of the *Salmonella* carrier on the ability of the host to respond to a secondary immunization with the purified soluble protein. It has been shown that *S. enterica* serovar Typhimurium can gain entry into the host via the nasal mucosal tissue (7, 15). It has also been shown that immunization by the nasal route with a nonvirulent *Salmonella* strain expressing a cloned antigen results in the induction of large amounts of specific IgA antibodies in serum and secretions, e.g., saliva and vaginal washes (11, 13, 15). The nasal route of immunization appears to be more efficient than the oral route in inducing systemic as well as mucosal immune responses to the vector and cloned antigens (11, 13, 15, 28, 41).

In our study, mice primed with *Salmonella* expressing GLU and boosted with purified GLU polypeptide alone had the highest level of GLU-specific serum IgG among all groups tested. This could reflect a lack of antigenic competition, as the presence of a competing antigen can regulate the response to an unrelated antigen, here, the *Salmonella* carrier and GLU, respectively (39). The magnitude of the response to GLU induced in mice immunized with *Salmonella* expressing GLU was similar to the level of specific antibody previously reported in mice immunized with three doses of purified soluble GLU polypeptide (18) or in mice immunized with a recombinant *Salmonella* strain expressing another *Streptococcal* virulence factor, the saliva-binding region (SBR) (11). On the other hand, the magnitude of specific IgA in mucosal secretions was less than that seen previously (11, 18). There are several possible explanations to account for this difference. It has to be considered that the GLU polypeptide is mainly produced in an insoluble cytoplasmic form by *Salmonella*, whereas the SBR is produced as a soluble polypeptide (8). It is unknown to what extent the inclusion bodies within *Salmonella* are accessible to degradation by macrophages, though it was previously shown that antigen processing of a viral nucleoprotein (NP) expressed as inclusion bodies in *Salmonella* required at least 6 h after bacterial infection before macrophages could present NP motifs effectively (3). In comparison, soluble NP only required 2 to 4 h before motifs were presented on macrophage cell surfaces. This difference could be due to the greater stability of a polypeptide in insoluble aggregates compared to that of a soluble protein by reason of resistance to degradative enzymes present in phagolysosomes. Furthermore, it was previously demonstrated that mice receiving multiple primary immunizations followed by a single boost with *Salmonella* expressing SBR displayed higher levels of antigen-specific IgA in mucosal secretions than animals receiving only a single primary immunization (11). It is possible that a similar primary immunization regimen using *Salmonella* expressing GLU would result in a higher mucosal immune response after the boost.

It is well known that *Salmonella* clones carrying the T7

promoter produce a large amount of protein in vitro when transferred from 30 to 37°C (8). This level of production of recombinant protein leads to plasmid instability, and the toxic effect of the foreign antigen leads to death of the bacteria within 24 h both in vivo and in vitro (14). Although the T7 *Salmonella* clones used in our study may have been eliminated quickly, they were efficient in inducing strong serum IgG responses as well as immunological memory. The memory induction is clearly illustrated by the difference in magnitude of the recall responses seen in mice primed with *Salmonella* expressing GLU in comparison to those of mice primed with PBS after administration of a single booster dose of purified GLU.

It is still unclear what is the most favorable way to deliver antigen. Several possibilities exist, including (i) aggregates in T7 *Salmonella* clones, which would prolong the exposure of immunocompetent cells to antigen due to slower protease-induced degradation of insoluble protein; (ii) soluble protein in T7 *Salmonella* clones (it is possible that soluble protein would be more accessible to antigen-presenting cells during the short exposure and would therefore maximize uptake [3]); (iii) other, more persistent *Salmonella* strains under the expression of in vivo-inducible promoters, like that of *nirB*, which is activated in anaerobic environments expressing nontoxic levels of cloned foreign immunogen and thus is not cleared as fast as T7 clones (14). It is extremely complex to predict the most efficient way to deliver antigen, and the question has to be resolved by actually performing comparative experiments.

In the murine system, T helper type 1 (Th1) and Th2 cells specifically induce antigen-specific B cells to secrete IgG2a or IgG1, respectively (35). The actual amounts and ratios of IgG2a and IgG1 are therefore an indication of the nature of the response, i.e., Th1 cells represent a cell-mediated response, whereas Th2 cells delineate an antibody response induced by more efficient B-cell activation. It is well known that *Salmonella* generally induces a Th1 response with elevated levels of IgG2a (12, 40). Furthermore, while entry of bacteria into macrophages is likely to be critical for the generation of gamma-interferon-dominant Th1 cellular immune responses, their persistence is not (29). A strong type 1-like response could therefore be anticipated even if the *Salmonella* strain is rapidly cleared. A mixed IgG2a-IgG1 pattern of response to SBR was previously demonstrated for mice immunized with *Salmonella* expressing SBR (11), whereas a predominant IgG1 response was demonstrated in mice immunized with SBR (9), indicating that the *Salmonella* vector induced a shift towards the IgG2a subclass. Various vehicle delivery systems may influence the induction of distinct T helper cell subsets to a specific antigen (16), and in this study we demonstrate the ability to shift a *Salmonella*-primed type 1-like immune response in mice to a more type 2-like response following boosting with soluble GLU polypeptide protein. The shift to a more type 2-like response with efficient B-cell activation and antibody production would be advantageous for the development of a salivary IgA antibody vaccine against dental caries.

In conclusion, we have shown that *S. enterica* serovar Typhimurium is an excellent delivery vehicle for the recombinantly expressed insoluble GLU of GTF. This construct was effective in priming the host for a secondary response to either soluble GLU polypeptide or *Salmonella* expressing GLU, and preexisting immunity to the *Salmonella* vector did not inhibit recall

responses. The *Salmonella* vector was demonstrated to be important for establishing memory but was not required for the induction of recall responses. The lack of antigenic competition and avoidance of amplification of unnecessary secondary responses, in addition to the shift from a type 1-like immune response to a more type 2-like response following a secondary immunization with soluble protein, appears to be very beneficial in attaining the highest specific anti-GLU serum IgG response among the different experimental groups, and this immunization strategy seems very promising. It would be of great interest to investigate the feasibility of inducing recall responses after even longer periods in addition to determining the nature of the later recall immune responses. These studies are ongoing in our laboratory.

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