Influence of Promoter, Gene Copy Number, and Preexisting Immunity on Humoral and Cellular Responses to a Vectored Antigen Delivered by a *Salmonella enterica* Vaccine\(^\dagger\)

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Attenuated *Salmonella* strains are currently in production as vaccines for protection of animals against salmonellosis. Such commercial strains offer the potential to deliver heterologous antigen to protect animals against other diseases. One vaccine strain, attenuated *Salmonella enterica* serovar Typhimurium (STM-1), was tested for the ability to deliver ovalbumin and to induce immune responses in mice. Two vaccine trials were performed testing the influence of promoter choice, the location of the encoding DNA (plasmid or chromosome), and the effect of preexisting homologous or heterologous immunity. The results demonstrated that humoral and T-cell responses were induced from either of two promoters, from either the plasmid or the chromosome, and that preexposure to the empty homologous vector, STM-1, or the heterologous vector, *S. enterica* serovar Enteritidis, had no detrimental effect on subsequent antigen-specific responses. In the case of homologous preexposure, responses were generally greater, and this was correlated with an increased uptake of *Salmonella* by macrophages in vitro after opsonization with immune sera.

*Salmonella enterica* strains have been well characterized for the delivery of heterologous vaccine antigens in several animal models (4, 5, 8, 11, 22, 39, 43, 50). The potential advantages of live attenuated vaccines, such as the simple mode of inoculation and generation of strong immune responses, make them ideal candidates for the delivery of antigens (38–40, 57). Infection by *Salmonella* induces both humoral and cell-mediated responses, not only against homologous antigens, but also against the heterologous antigen for which they act as a carrier (2, 16, 32, 36, 41, 43).

STM-1 is an *S. enterica* serovar Typhimurium mutant developed by RMIT and harbors a mutation in the *aroA* gene region that renders it attenuated (M. Saxena, P. Smooker, and P. Coloe, submitted for publication). It is a commercial vaccine strain currently in use to protect livestock against *Salmonella* infection (1). It is delivered to the immune system of chickens by spraying or administration via drinking water and therefore enters via mucosal tissues. It has been previously demonstrated that STM-1 is capable of eliciting immune responses in mice to model plasmid-borne antigens (8) and from inserts integrated at a chromosomal location (55). We have extended these studies to further analyze the capacity of STM-1 in three ways: first, a comparison between plasmid- and chromosome-borne delivery routes using two different promoters; second, an analysis of the T-cell responses induced in mice, in addition to humoral responses; and last, an experiment designed to investigate the influence of preexisting immunity to the vaccine vector. Emphasis was given to an analysis of expression from the chromosomal location, since in order to progress to any commercial application, the use of plasmid-borne antigens should be eliminated. There are two main reasons for this: (i) to ensure the stability of antigen expression in the absence of plasmid selection and (ii) to eliminate the possibility of environmental contamination of resident bacterial flora by horizontal transmission of plasmids.

The efficiencies of two in vivo-inducible promoters, *pagC* and *nirB*, were studied. The *nirB* promoter was isolated from *Escherichia coli*, in which it directs the expression of an operon that includes the nitrate reductase gene (34) and is regulated by nitrate and by the changes in the oxygen tension of the environment, becoming active under anaerobic conditions (23). *pagC* is a macrophage-inducible promoter isolated from *Salmonella* (33) and is controlled by the PhoP/PhoQ two-component regulatory system, which activates the transcription of genes following phagocytosis by macrophages. Previous studies have shown that the expression of antigenic protein under the control of the either the *pagC* promoter (21, 22, 28, 33) or the *nirB* promoter (20, 42) provides stable, high-level expression of heterologous antigen in *Salmonella* from a multicopy plasmid and stimulates greater immune responses than the expression of the same antigens from the same strain under the control of a constitutive promoter.

A major consideration in the proposed use of *Salmonella* as a vector to deliver heterologous antigens is the consequence of repeated use of the carrier or environmental exposure to related strains. The possibility that prior exposure of animals to homologous or related strains might compromise the efficacy of a *Salmonella* vector in delivering foreign antigen has long been considered (26). Bao and Clements (9) first reported the consequences of prior exposure of animals to the vector strain and showed that both serum and mucosal antibody responses against the foreign antigen were upregulated after the animals were first exposed to the vector or a heterologous strain. Furthermore, they showed that the increase in mucosal antibody was greater after homologous rather than heterologous prim-
ing. Whittle and Verma (61) reported similar findings in which mice that were immunized intraperitoneally with an *S. enterica* serovar Dublin *aroA* mutant expressing heterologous antigen after being exposed to the same vector showed a greater humoral response to the vectored antigen.

Other studies (35, 37) have also indicated the upregulation of immune responses after animals have been exposed to either homologous or related strains of the vector before the delivery of heterologous antigen in the vector. A recent study conducted by Metzger et al. (44) on human volunteers using *S. enterica* serovar Typhi as a vector suggested that there was no change in the immune responses against the heterologous antigen in human volunteers who were exposed to empty vector in comparison to the volunteers who did not have prior immunological experience with the vector strain. Taken together, these results indicate that preexisting immunity may not compromise the immunogenicity of vectored antigen in a subsequent vaccination.

However, there are conflicting reports. Attridge et al. (6) reported that a preexisting immune response against the bacterial vector can downregulate humoral responses in mice against a subsequently vectored antigenic protein. Furthermore, they demonstrated that the hyporesponsiveness could be reduced by delivery of the vectored antigen in a strain of a different serotype than that used as the priming dose. Similar results were obtained in a later study (46, 59), which reported that prior exposure of animals to homologous or related strains reduced the subsequent immune response in mice against the vectored foreign antigen. Finally, Attridge and Vindurampulle found that the hyporesponsiveness could be largely eliminated by exposing animals to the foreign antigen prior to vector priming (7). Although this strategy is not practical for routine immunization purposes, it illustrates that epitope suppression caused by the dominance of vector-derived antigens is largely responsible for the hyporesponsiveness. In a similar way, Roberts et al. (46) found that antibody responses to vectored fragment C were reduced in the presence of homologous-vector preimmunity.

There is evidence that T-cell responses are also affected by prior immunity. A series of experiments conducted by Sevil Domenech and colleagues demonstrated that preexisting immunity against the vector limited the induction of CD8 responses against a vectored *Listeria* antigen. This could be circumvented by using different *Salmonella* serovars for the prime and boost (25, 48, 49).

Attempts were made to explain these inconsistent results by noting the differences in the protocols employed, in particular, the route of immunization. However, in the experiments performed by Attridge and Vindurampulle, the effects of prior immunity were tested using both intraperitoneal (i.p.) and oral delivery of the second dose, and it was found that hyporesponsiveness occurred irrespective of the route (7).

These studies indicate that the prior exposure of animals to a bacterial vector can certainly influence the immune response against the heterologous antigenic protein in animal models. However, there are a number of factors, such as the *Salmonella* carrier strain used and the nature of the foreign antigen, that may influence the outcome (58). In this study, we used STM-1 and *S. enterica* serovar Enteritidis as a model for preexisting immunity to either a homologous or a related, but not identical, vector and analyzed the resulting humoral and T-cell responses. The influence of opsonizing antibodies induced by the priming dose was also studied in vitro.

### MATERIALS AND METHODS

**Growth media, supplements, and strains.** All *E. coli* and *Salmonella* strains were grown on solid microbiological media under aerobic conditions at 37°C for 16 h. In instances where broth cultures were used, the strains were grown aerobically at 37°C for 16 h on a Ratek orbital shaker at 120 to 150 rpm. Antibiotic supplements were added to an appropriate concentration when required. 37742 cells were routinely cultured in Dulbecco's modified Eagle's medium.

**Strains used in this study.** The following strains were used: *Escherichia coli* DH5α [K- 6880lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17(r~K- m~K- ); supE44 thi-1 gyrA relA1]; a standard laboratory strain used for DNA manipulation; *S. enterica* serovar Typhimurium STM-1 (*aroA* Δ*aroC*), an attenuated vaccine strain (1); *S. enterica* serovar Enteritidis SEM-2 (*aroA* Δ*aroD*), an attenuated strain; and 37742, a murine macrophage cell line used in *Salmonella* uptake experiments.

**Creation of pagC-Ova and nirB-Ova plasmid-based constructs.** All PCRs were performed using Expand long-template polymerase (Promega) to ensure proofreading activity. The constructs were initially cloned into the pCR2.1 plasmid backbone (Invitrogen). The plasmids used in this study are listed in Table 1. The *nirB* and *pagC* promoters were PCR amplified using a forward primer with a BamHI restriction site and a reverse primer with an MboI restriction site (Table 1).
The chicken ovalbumin gene was amplified from the sOVA-C1 plasmid supplied by J. S. Boyle and colleagues (14) using the Ova forward primer and the Ova reverse primer. The primers used for the amplification of the chicken ovalbumin gene carried an EcoRI restriction site.

The PCR products were ligated into the pCR2.1 plasmid and transformed into E. coli TOP10F<sup>®</sup>. The pCR2.1 plasmid carrying the pagC promoter was termed pCRpagC, the plasmid carrying the nirB promoter was called pCRnirB, and the chicken ovalbumin gene was termed pCROva (Table 1). The pCROva plasmid was digested with EcoRI, and the insert was purified and ligated into the pCRpagC and pCRnirB plasmids at the MfeI restriction sites (as MfeI and EcoRI have compatible ends) to yield pCRpagCOva and pCRnirBOva plasmids. The integrity of each of the constructs was analyzed by restriction digestion and DNA sequence analysis.

The medium-copy-number pMW2 vector was chosen for the delivery of heterologous vaccine antigen from the plasmid location. The constructs were digested from the holding pCR2.1 plasmid with KpnI and XhoI, and the inserts were ligated into the pMW2 vector.

Integration of constructs into the araO gene region of the STM-1 chromosome using the Lambda Red System. The Lambda Red System, supplied by the E. coli Genetic Stock Center (courtesy of B. L. Wanner), was used for the integration of constructs into the araO gene region of STM-1. The system takes advantage of phage lambda recombination, which allows the integration of linear PCR products with very short regions of identity (24).

The pKD13 plasmid (24) was utilized as the template for the generation of PCR products by ligating the constructs described above at the BamHI restriction site of the plasmid. Primers with 5′ tails comprising 40 bp of identity with the araO gene of STM-1 were used to generate the PCR product. The PCR mixture was digested with DpnI to remove any residual plasmid template, and 50 to 100 ng of purified PCR product was used to transform fresh STM-1/pKD46 competent cells by electroporation. Transformed STM-1 was selected on Mueller-Hinton plates containing 50 μg/ml kanamycin. Transformants were confirmed by colony PCR with the araO forward primer and the araO reverse primer.

Immunization of mice. Two immunization experiments were designed. All animal experimentation was approved by the RMIT Animal Ethics Committee.

In the first experiment, the utility of STM-1 as a vehicle for the induction of immune responses against chicken ovalbumin antigen expressed from both plasmid and chromosomal locations was evaluated. Also, the efficacies of pagC and nirB promoters were compared in vivo. In this experiment, groups of five female BALB/c mice (6 to 8 weeks old) were vaccinated three times at intervals of 2 weeks with STM-1 expressing heterologous antigen from either plasmid or chromosomal, with empty STM-1 vector and STM-1 vector harboring empty plasmid as controls. Vaccination was administered either orally or by i.p. injection. Oral vaccination was performed by inoculation of 5×10<sup>8</sup> CFU, and i.p. vaccination was with 1×10<sup>8</sup> CFU. Serum samples were collected 3 weeks after the third vaccination to analyze humoral immune responses. Mice were sacrificed 3 weeks after the third vaccination, and spleenocytes were collected for enzyme-linked immunosorbent assay (ELISpot) assays. Table 3 shows the groups, vaccine constructs, and modes of inoculation for the evaluation of ovalbumin-specific immune responses.

The second experiment was designed to test the effect of preexisting immunity on ovalbumin-specific responses. The format for this trial is shown in Table 4. Groups of mice were vaccinated orally either once or twice at 3- or 12-week intervals. Some groups were given a first vaccination with STM-1 or S. enterica serovar Enteriditis to mimic preexisting immunity, followed by a second vaccination with the vectored antigen. Sera were sampled at week 6 and at week 15, which was also when spleenocytes were collected.

Analysis of immune responses. For the analysis of humoral immune responses, enzyme-linked immunosorbent assay was performed essentially as previously described (8). Purified antigen was diluted to 10 μg/ml in coating buffer (35 mM NaHCO<sub>3</sub>, 11 mM Na<sub>2</sub>CO<sub>3</sub>, pH 9.6), and 100 μl was added to 96-well plates (Nunc, Denmark) and incubated overnight at 4°C. The plates were washed and blocked, and serum was serially diluted and incubated for 2 h at 37°C. After the washing, detection antibody [mouse anti-immunoglobulin G(H+L) supplied by Bio-Rad Laboratories] was added to each well and incubated for 1 h. Substrate was added, and after development, the absorbance at 450 nm was measured. The titer was defined as the dilution at which the absorbance reading reached 0.2.

T-cell responses were evaluated as previously described (8). Briefly, spleens were crushed and cells were isolated, depleted of red blood cells, and washed as described above. The ELISpot plate (Millipore) was prepared by wetting the membrane with 100% methanol for 5 min, followed by three washes in sterile PBS. Capture antibody (100 μl) was diluted in ELISpot coating buffer (35 mM NaHCO<sub>3</sub>, 11 mM Na<sub>2</sub>CO<sub>3</sub>, pH 9.6) and added to the wells (0.5 μg/ml interleukin 4 [IL-4] and 1 μg/ml gamma interferon [IFN-γ] supplied by BD Pharamingen), and the plates were incubated overnight at 4°C. The capture antibody was

| Group | Vaccination | Mode of inoculation |
|-------|-------------|---------------------|
| 1     | STM-1 only  | i.p.                |
| 2     | STM-1 only  | Oral                |
| 3     | STM-1 harboring empty plasmid | i.p. |
| 4     | STM-1 harboring empty plasmid | Oral |
| 5     | STM-1 expressing ovalbumin antigen under the control of the pagC promoter from plasmid | i.p. |
| 6     | STM-1 expressing ovalbumin antigen under the control of the pagC promoter from chromosomal location | i.p. |
| 7     | STM-1 expressing ovalbumin antigen under the control of the nirB promoter from plasmid | i.p. |
| 8     | STM-1 expressing ovalbumin antigen under the control of the nirB promoter from chromosomal location | Oral |
| 9     | STM-1 expressing ovalbumin antigen under the control of the nirB promoter from plasmid | i.p. |
| 10    | STM-1 expressing ovalbumin antigen under the control of the nirB promoter from chromosomal location | Oral |
| 11    | STM-1 expressing ovalbumin antigen under the control of the nirB promoter from chromosomal location | Oral |
| 12    | STM-1 expressing ovalbumin antigen under the control of the nirB promoter from chromosomal location | Oral |

* Restriction sites are underlined.

**TABLE 2.** Oligonucleotide primers<sup>a</sup>

| Primer name | Sequence (5′ → 3′) | Binding site | Enzyme | Reference |
|-------------|--------------------|--------------|--------|-----------|
| OVA FWD     | GGAATTCGAGTTCACAT | 5′ Ova       | EcoRI  | This study |
| OVA REV     | GGAATTCGAGTTCACAT | 3′ Ova       | EcoRI  | This study |
| PAGFWD      | CTCGAGATATTCAGTTTGC | 5′ pagC gene promoter | BamHI | 28 |
| PAGCREV     | AATAGATCCATTGCAACTCC | 3′ pagC gene promoter | MfeI | 28 |
| NIRBFWD     | GCCGGATCTTTGAACTTG | 5′ nirB gene promoter | BamHI | 28 |
| NIRSREV     | AATAGATCCATTGCAACTCC | 3′ nirB gene promoter | MfeI | 28 |
| M13 REV     | CAGGAAGACGTCATGAC  | Sequencing primer | Sequencing primer | |
| M13-20      | GATAAAAACGCGGCAC  | Sequencing primer | Sequencing primer | |

* Restriction sites are underlined.
TABLE 4. Influence of preexisting immunity

| Group | Vaccination | Week 0 | Week 3 | Week 12 |
|-------|-------------|--------|--------|---------|
| 1     | None        | STM-1  | None   | None    |
| 2     | None        | STM-1/pKKOva | None   | None    |
| 3     | STM-1       | STM-1/pKKOva | None   | None    |
| 4     | S. enterica serovar Enteritidis | STM-1/pKKOva | None   | None    |
| 5     | None        | None   | STM-1  | None    |
| 6     | None        | None   | STM-1/pKKOva | None    |
| 7     | STM-1       | STM-1/pKKOva | None   | None    |
| 8     | S. enterica serovar Enteritidis | None   | STM-1/pKKOva | None    |

* Vaccine trial plan showing the groups, vaccine constructs, and schedule to test the effect of preexisting immunity on antigen delivery using *Salmonella* as a vector.

RESULTS

Analysis of differential uptake of STM-1 by macrophages. An in vitro assay was undertaken to analyze the differential uptake of STM-1 by the J774 macrophage cell line. Briefly, 4 × 10^7 J774 cells in Dulbecco’s modified Eagle’s medium containing 5% NCS were allowed to adhere to a 24-well plate. To each well, 1 × 10^6 STM-1 cells opsonized with naïve serum, STM-1-specific serum, or *S. enterica* serovar Enteritidis-specific serum (1 μl serum for a 30-μl volume of STM-1 in PBS) were added. The sera were taken from the mice at week 3 after a single vaccination with either STM-1 or *S. enterica* serovar Enteritidis. Gentamicin was added to each well at a final concentration of 100 μg/ml at different time intervals, and the plates were incubated for 1 h to kill noninternalized bacterial cells. The wells were sampled at time intervals of 0, 15, 30, 45, 60, and 75 min. After three washes with PBS, the macrophages were lysed with 0.25% Triton X-100 and appropriate dilutions were enumerated on LB agar plates. All parameters were evaluated for significance using Student’s t test.

Induction of immune responses against the vectored ovalbumin antigen. In an induction experiment, the ability of the *Salmonella* STM-1 mutant to raise an immune response against chicken ovalbumin expressed from both the plasmid and chromosome was evaluated. Also, the relative efficacies of the *pagC* and *nirB* promoters for the optimal expression of heterologous antigen, inferred from immune responses, were compared in vivo.

The distribution of groups and the modes of inoculation are shown in Table 3. Groups 1 to 4 were negative controls; group 1 and 2 mice were vaccinated with STM-1 only, and group 3 and 4 mice were vaccinated with STM-1 harboring empty plasmid. The evaluations of humoral and cell-mediated immune responses from other groups were compared with those of groups 1 to 4. Constructs with chromosomal integration were compared to groups 1 and 2, and constructs containing plasmids were compared to groups 3 and 4.

Humoral responses to ovalbumin were analyzed by enzyme-linked immunosorbsent assay to confirm that STM-1 could deliver the antigen in vivo, activating humoral responses. As the ovalbumin is expressed within the cell and is not secreted, humoral-response levels are low. A preliminary test with pooled sera showed that responses were induced in groups 5 to 12 (data not shown). Figure 1A shows induction of significant humoral responses in mice vaccinated with STM-1 expressing ovalbumin either from a plasmid or from the chromosome compared with the corresponding negative control (*P* < 0.001). There was no significant difference observed in humoral responses when the expression from the plasmid location was compared with expression from the chromosomal location. Also, no significant difference in humoral responses was observed when the antigen was delivered from either the *pagC* or the *nirB* promoter.

It was established in the above-mentioned experiments that the delivery of ovalbumin from both plasmid and chromosomal locations in STM-1 can induce humoral immune responses. To infer activation of T-cell responses, secretion of IL-4 and IFN-γ by stimulated splenocytes was measured by the ELISPOT assay. Splenocytes that had been primed by vaccination responded to the antigen. Figure 1B shows that mice vaccinated with STM-1 expressing ovalbumin under the control of the *pagC* promoter from the plasmid (i.e., inoculation only) and from the chromosomal location resulted in significantly increased numbers of activated splenocytes secreting IL-4 (*P* < 0.001). Significant increases were also observed from mice vaccinated with STM-1 expressing ovalbumin under the control of the *nirB* promoter from both the plasmid and the chromosomal locations (*P* < 0.05).

A significant increase in the number of cells secreting IFN-γ was observed in stimulated splenocytes isolated from mice vaccinated with STM-1 expressing ovalbumin under the control of the *nirB* promoter from the chromosome (Fig. 1C) (*P* < 0.001). Also, splenocytes from mice vaccinated with STM-1 expressing ovalbumin antigen under the control of the *pagC* promoter from the plasmid and from the chromosomal locations (i.e., inoculation) showed a significant increase (*P* < 0.001).

Generally, cellular immune responses induced after i.p. vaccination are higher than after oral vaccination. This is presumed to be due to the high numbers of macrophages present in the peritoneal cavity, which can efficiently take up STM-1, process it, and present antigen. It should be noted that the numbers of cells used for i.p. vaccination are also considerably lower than those required to induce comparable responses after oral inoculation.
The influence of preexisting immunity on immune responses induced to vectored antigen. The second experiment was designed to simulate the influence of preexisting immunity to the vaccine vector and the influence it has on the development of subsequent immune responses to vectored antigen. Analysis of responses was performed identically to experiment 1, albeit at different time points. The experimental design is shown in Table 4, and Fig. 2 and 3 illustrate the results. In this experiment, groups of mice received only one vaccination with vectored ovalbumin (groups 2 to 4 and 6 to 8). Groups 1 and 5 received only STM-1. Humoral responses were analyzed in
sera taken at week 6 (groups 1 to 4) and week 15 (all groups). Week 6 was 3 weeks after the vaccination of groups 2 to 4 with STM-1 carrying the pKKOva plasmid. A significant difference was observed at the 99% confidence level between mice vaccinated with STM-1 expressing ovalbumin from the pKKOva plasmid (groups 2 to 4) and the control group, which was vaccinated with nonrecombinant STM-1 (group 1) (Fig. 2A). Also, a significant difference was observed between a group preexposed to the vector strain (group 3) and the unexposed group (group 2) (Fig. 2A) ($P < 0.05$). However, no significant difference was observed between the group that was exposed to the related strain (group 4) and the unexposed group (group 2).

Serum samples from all mice were collected at week 15, which was 3 weeks after the vaccination of groups 6 to 8 with STM-1 carrying the pKKOva plasmid. All groups of mice vac...
vaccinated with vectored ovalbumin at either week 3 or week 12 yielded titers significantly higher than those of the control groups (groups 1 and 5). There was no difference between the titers of animals vaccinated at week 3 with ovalbumin, irrespective of whether there was no vaccination at week 0 or vaccination with STM-1 or S. enterica serovar Enteritidis. Therefore, the modest boost seen after prior vaccination with STM-1 (Fig. 2A, group 3 versus group 2) was not observed. However, such boosting was observed in groups in which the second vaccination was at week 12 (Fig. 2B, groups 7 and 8 versus group 6; P < 0.05). These results indicate that there is no reduction in the development of antigen-specific humoral responses in animals that have preexisting immunity and that there may be modest boosting in animals that have been exposed to the homologous vector.

Splenocytes were prepared at week 15 for the analysis of T-cell responses using the ELISPOT assay. In each group vaccinated with ovalbumin, there was an increase in cytokine-secreting cells compared to control groups (groups 1 and 5). Furthermore, compared to unexposed groups, there was an increase in IL-4- and IFN-γ-secreting cells in groups of mice that were preexposed to either STM-1 or S. enterica serovar Enteritidis, with the exception of group 4, IL-4 secretion. Again, these data indicate that there is no reduction in immune responses in animals that have prior immunity to the vector, and in most cases, the magnitude of the responses is increased.

**Uptake of opsonized STM-1 by the J774 macrophage cell line.** An in vitro assay to analyze the differential uptake of STM-1 was undertaken to analyze the role of opsonizing antibodies in the early uptake of the STM-1 mutant. Sera used for these experiments were pooled sera from group 1, 3, or 4 in trial 2, taken at week 6. Therefore, this experiment tested the ability of sera from mice primed with either STM-1 or S. enterica serovar Enteritidis to opsonize STM-1 and to promote uptake by macrophages. The results are depicted in Fig. 4 and show that opsonization with either STM-1- or S. enterica serovar Enteritidis-specific serum resulted in an increase in the uptake rate of STM-1 by macrophages. It was observed that uptake of STM-1 opsonized with sera from mice primed with STM-1 was significantly higher at all the time points, not only compared with STM-1 opsonized with naïve sera from unvaccinated mice, but also compared with STM-1 cells opsonized with sera from mice primed with S. enterica serovar Enteritidis. This experiment was repeated using only STM-1- and S. enterica serovar Enteritidis-specific sera to confirm this result, and the same pattern was observed (data not shown).

**DISCUSSION**

S. enterica strains have long been considered suitable vectors for the delivery of heterologous vaccine antigens of bacterial, viral, and eukaryotic origin (11–13, 15, 17, 19, 30, 31, 53). The choice of antigen to deliver is one aspect of creating a successful vaccine, but also of great importance is designing the construct to ensure optimal expression of foreign antigens to induce robust immune responses. The majority of experimental studies are performed with antigens delivered from plasmid-borne genes (3, 4, 8, 10, 27), which generally allows higher expression of antigenic protein due to increased gene copy numbers. However, this method of antigen delivery is unsuitable in the field due to issues of plasmid instability in the absence of antibiotic selection, metabolic burden due to plasmid replication, and gene expression from the multicopy plasmid (18, 27, 51) and the possibility of gene transfer to other organisms in the environment. The issue of metabolic burden due to high levels of protein expression from plasmid-borne genes can be addressed by using in vivo-inducible promoters (28, 29, 45, 55); however, the maintenance of plasmids in vivo in the absence of antibiotics may be an issue. Chromosomal integration of genes encoding antigenic protein is an elegant solution for maintaining stable expression of the antigenic protein (47, 52, 54); however, this may result in a reduction in the production of antigenic protein and, hence, reduced immune responses.

Experiments performed in this study indicated that the induction of humoral and T-cell responses to antigen were not significantly different in plasmid- and chromosome-borne constructs. In each case, antibody was produced and memory spleen cells secreting either IL-4 or IFN-γ were induced. Therefore, using STM-1, the gene copy number is not a limiting factor. The plasmid used here was a medium-copy-number plasmid, and it may be that using a high-copy-number plasmid would increase protein expression. However, this would be at the expense of increased metabolic load, which would render the plasmid more unstable in the absence of selection. This may in fact decrease, rather than increase, immune responses. There was also no significant difference in the immune responses induced against ovalbumin from either the pagC or nirB promoter, which indicates similar expression efficiencies of the two in vivo-inducible promoters.

The results of this study indicate that the STM-1 mutant can be used effectively as a carrier to deliver the heterologous...
vaccine antigen. The expression of heterologous vaccine antigen under the control of in vivo-inducible promoters, such as pagC and nirB, can induce immune responses from both plasmid and chromosomal locations. For ultimate effectiveness in the field, use in animals that have preexisting immunity to related pathogens must be considered (60, 62). Also, the consequences of repeated use of the same vector to deliver different foreign antigens need to be considered. Several studies have shown conflicting results regarding the impact of vector priming and its effect on the efficiency of generating a protective immune response against the heterologous antigen (6, 9, 44, 46).

Trial 2 (Table 4) was designed to mimic the effect of prior exposure of the animal host to the vector strain or a related strain and to determine the influence this exposure has on the induction of both humoral and cellular immune responses against the passenger antigen (ovalbumin). Two parallel experiments were performed, one with a 3-week gap between exposure and vaccination and one with a 12-week gap. The results indicate that the prior exposure of animals to the vector strain or a related strain 3 or 12 weeks prior to vaccination does not reduce the subsequent induction of ovalbumin-specific humoral responses. In fact, in the case of prior exposure to STM-1, humoral responses were significantly increased ($P < 0.05$) compared to groups with no exposure when the sera were analyzed 3 weeks after ovalbumin vaccination (Fig. 2A, compare groups 2 and 3). When sera were analyzed at week 15 (12 weeks after the antigen vaccination), this modest increase was not maintained, although it was evident in the groups that had been vaccinated at week 12 (Fig. 2B, compare groups 2 and 3 and groups 7 and 8).

For T-cell responses, the situation was more complex, and preexisting immunity appears to have different effects on IL-4-secreting and IFN-γ-secreting cells. As shown in Fig. 3A, preexposure to the homologous vector increased the number of IL-4-secreting splenocytes, while preexposure to the related strain did not have such a large impact (as was seen with humoral responses). Conversely, the increase in IFN-γ-secreting cells occurred irrespective of the strain to which the mice were preexposed (Fig. 3B). Therefore, IFN-γ-secreting T cells can be potentiated by a priming dose from either a homologous or a heterologous carrier. This may reflect the nature of the ovalbumin antigen, in which there is a dominant CD8 epitope (SIINFEKL) that is processed and presented readily. It has been demonstrated that attenuated araA Salmonella strains expressing this epitope can readily deliver it to the class I presentation pathway of macrophages (18a), and we have previously shown that splenocytes from mice vaccinated with STM-1-vectored ovalbumin can be restimulated with SIINFEKL (8).

The greater immune responses against antigens carried in mice that were preexposed to either the homologous (STM-1) or heterologous (S. enterica serovar Enteritidis) empty vector may be partly due to the presence of opsonizing antibodies against Salmonella. It has been shown that the presence of opsonizing antibodies facilitates the early uptake of Salmonella by phagocytic cells (43, 56). The presence of opsonizing antibodies facilitates early uptake of Salmonella araA mutants expressing heterologous antigens, and this in turn leads to increased processing of these antigens. As shown by the results depicted in Fig. 4, the addition of immune sera increases the uptake of Salmonella by macrophages, and this increase is greater when homologous immune sera are used rather than heterologous immune sera. This may explain why prior vaccination with the homologous vector generally gives boosting of subsequent vectored-antigen responses better than that after heterologous-vector priming, as the antibodies produced are largely specific for the homologous strain, although there are surface epitopes that are cross-reactive between the two S. enterica serovars.

The effects of this increased uptake appear to be a modest short-term increase in humoral responses and a generalized increase in cellular responses. The increase in cellular responses is unsurprising, as increased uptake into cells should result in higher levels of antigen processing and presentation.

These results suggest that ovalbumin-specific humoral and cellular immune responses can be upregulated when the animal host has immunological memory against the vector (particularly) or a related strain, indicating that STM-1 strains may be effective carriers for the delivery of heterologous antigen in the field. Immunological memory against the bacterial vector or related strains in target hosts will not jeopardize the use of STM-1 as a vector to deliver heterologous antigens, and in fact, the responses may be enhanced. This suggests that even in flocks or herds previously infected with wild-type Salmonella, an STM-1-vectored vaccine is likely to be effective in inducing both cellular and humoral immune responses to the heterologous antigen(s) carried by the vaccine.

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