Macrophage-inducible expression of a model antigen in *Salmonella typhimurium* enhances immunogenicity

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ABSTRACT Attenuated *Salmonella* are useful oral vaccine vectors capable of carrying multiple heterologous antigen genes, but optimal expression of foreign antigens has not yet been achieved. We hypothesized that *Salmonella phoP*-activated genes, which are transcriptionally activated within antigen-processing macrophages, could prove useful for delivery of heterologous antigens to the immune system. We have created a suicide vector that allows the stable chromosomal insertion of heterologous antigen genes within the *phoP*-activated gene C (*pagC*) of *Salmonella* and permits the expression of heterologous antigens as fusion proteins between the first 84 amino acids of PagC and the chosen antigen. The *Escherichia coli phoA* gene encoding alkaline phosphatase was cloned into this vector; the resultant plasmid was used to construct *Salmonella typhimurium* strains that express PagC-alkaline phosphatase fusion proteins from a single chromosomal gene copy. Such strains were administered orally and i.p. as vaccines to BALB/c mice and compared with control strains expressing alkaline phosphatase constitutively. After 3 weeks, mouse sera were analyzed for IgG responses to *S. typhimurium* lipopolysaccharide and alkaline phosphatase. Remarkably, though all mice had comparable antibody responses to lipopolysaccharide, only mice immunized with strains bearing *phoP*-activated fusion genes had antibody responses to the heterologous antigen. We conclude that expression of a heterologous antigen from an *S. typhimurium* *in vivo*-induced promoter that is activated within macrophages markedly enhances the immunogenicity of a model antigen expressed from a single chromosomal gene copy.

Universal immunization would be greatly facilitated by the creation of multivalent oral vaccines capable of protecting individuals against multiple pathogens. Attenuated *Salmonella* have been widely investigated as live oral vaccine vectors capable of stimulating humoral, cell-mediated, and mucosal immunity in mammalian hosts (1), but optimal expression of heterologous antigens within *Salmonella* has not yet been achieved (2). In most vaccine strains, foreign immunogens have been constitutively expressed from plasmid-encoded antigen genes (3–6). Difficulties associated with these vaccines have included the instability of plasmids within mammalian hosts and toxic effects of overexpressed heterologous proteins on bacterial vectors, leading to poor immunogenicity despite high levels of antigen expression. To circumvent these problems, heterologous antigen genes have been inserted into specific attenuating loci within the *Salmonella* chromosome (2, 7–9). Such strains rely upon the expression of a single gene copy rather than multicopy plasmid-encoded antigen genes. Although cytotoxic T-cell responses to such constructs have been demonstrated (8), single chromosomal copies of heterologous antigens in *Salmonella* have not elicited significant humoral immune responses to date.

Bacterial genes expressed *in vivo* have been postulated to encode virulence determinants that may be important immunogens (10). The ability of *Salmonella* to survive within mammalian macrophages is a prominent feature of *Salmonella* pathogenesis and correlates with virulence (11–13), suggesting that essential bacterial survival strategies are operative within this antigen-processing cell. We hypothesized that expression of heterologous antigens from a *Salmonella* promoter specifically induced within macrophages and previously shown to control a *Salmonella typhimurium* virulence factor could provide an ideal means of *in vivo* expression and attenuation of a potential vaccine strain. Intracellular transcriptional activation of a chromosomal gene fusion between a virulence factor and a foreign antigen may lead to high levels of antigen expression in a manner temporally and physically ideal for the generation of an immune response. The *Salmonella* PhoP/PhoQ regulon is a bacterial two-component regulatory locus (11, 12), similar to a family of histidine kinases (PhoQ) and transcriptional regulators (PhoP). This locus regulates multiple unlinked *phoP*-activated genes (the *pag* genes) (11, 13, 14) and *phoP*-repressed genes (the *prg* genes) (15). PhoP-null *S. typhimurium* mutants are markedly attenuated for virulence in BALB/c mice (16, 17), animals that serve as a useful model for the development of *Salmonella*-vectored vaccines. One important phenotype contributing to this attenuation is the impaired survival of PhoP-null organisms within mammalian macrophages (11, 16, 18). We have demonstrated (19) that *phoP*-activated genes are specifically transcriptionally activated by >100-fold after phagocytosis by murine bone marrow-derived macrophages, but not by epithelial cells. In addition, *phoP*-regulated gene products are among the most prominent bacterial products synthesized upon entry into mammalian macrophages (20). *pagC* encodes a 188-amino acid outer membrane protein essential for full virulence of *S. typhimurium* in BALB/c mice, and *pagC* mutant *S. typhimurium* survive poorly within mouse macrophages (13). We created a suicide vector that allows chromosomal insertion of heterologous antigen genes within the *pagC* locus in such a way that fusion proteins are generated between the first 84 amino acids of the PagC protein and the heterologous antigen of interest. We demonstrate that expression of a model antigen gene from a single chromosomal copy under control of *phoP/phoQ* is both attenuating and highly immunogenic.

MATERIALS AND METHODS

Bacterial Strains and Genetics. *S. typhimurium* CS019 [ATCC 14020 *phoN2xx::6251Tn10d-Cm* (11)] was utilized as a wild-type mouse-virulent strain. *S. typhimurium* SL3261 (21) (the gift of B. Stocker, Stanford University, Palo Alto, CA) was used as an attenuated parent strain. Strain SM10 Δ pir (*thi-1 thr-1 leuB6 supE44 tonA21 lacY1 recA::RP4-2-Tc::Mu*) was utilized as an *Escherichia coli* host. Bacteria were grown in Luria broth, supplemented when necessary with an aromatic

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Abbreviations: LPS, lipopolysaccharide; cfu, colony forming unit(s); SLT-IB, Shiga-like toxin IB.

amino acid mixture (21). *pir*-dependent suicide vectors were mobilized to the chromosome of *Salmonella* by conjugal transfer (22). Agar plates for selection of *S. typhimurium* strains that had eliminated suicide vector sequences were modified Luria broth plates containing 10% (wt/vol) sucrose and no NaCl (23). P22HTint bacteriophage transductions (24) were utilized to transfer some chromosomal genetic elements from wild-type to *aroA*-deleted *Salmonella*. *PhoP*-null strains were created (15) by using bacteriophage lysates grown on *S. typhimurium* AD154 (*phoP12purB1744::Tn10*) (the gift of E. Eisenstadt, Naval Medical Center, Bethesda).

Molecular Biology. DNA manipulations were performed as described (25). PCRs used *Taq* polymerase (GeneAmp, Perkin-Elmer/Cetus). DNA hybridization analyses (26) employed ³²P-radiolabeled DNA probes prepared by the random-primer method (27).

Mouse Studies. Female BALB/c mice (18–24 g, Charles River Breeding Laboratories) were used. Oral and i.p. inoculations were performed as described (15), by using four mice per group for LD₅₀ determinations. Inoculum doses were confirmed by serial dilution and plating for colony forming units (cfu). Mice were bled via tail veins 3 weeks after immunization.

Western Blot Analysis and ELISAs. Whole bacterial protein lysates were prepared and separated by SDS/PAGE (28). Western immunoblot analysis was performed (29) with a polyclonal rabbit antibody directed against alkaline phosphatase (gift of John Mekalanos, Harvard Medical School, Boston) and goat anti-rabbit secondary antibody conjugated to alkaline phosphatase (Sigma). Dot blots utilized a peroxidase-labeled goat anti-mouse antiserum and an enhanced chemiluminescent detection system (Amersham). ELISAs were performed (29) by using 96-well plates (Dynatech) coated with *S. typhimurium* lipopolysaccharide (LPS, Sigma) or purified *E. coli* alkaline phosphatase (Sigma). Phosphate-buffered saline containing 0.05% Tween 20 (Sigma) and 5% (wt/vol) dried milk was used as a blocking agent. Goat anti-mouse IgG conjugated to peroxidase and tetramethylbenzidine dihydrochloride substrate (Sigma) were used for detection. Reactions were stopped with 1 M H₂SO₄ and plates were read at 450 nm. IgG subclass determinations were performed similarly by using secondary antibodies to IgG1 or IgG2a subclasses (Boehringer Mannheim).

RESULTS

Construction of a Suicide Vector pVAC468 for Chromosomal Insertion of Heterologous Antigen Genes Under *phoP/phoQ* Regulation. pVAC468 is a derivative of pGP704, an RP4 replicon that requires the *pir*-encoded protein for replication (30). pGP704 was modified to include an ~1.5-kb segment of *S. typhimurium* DNA containing the entire *pagC* locus. Subsequently, a 490-bp internal region of *pagC* flanked by *Cla* I and *Bgl* II restriction endonuclease sites was replaced with a synthetic oligonucleotide polylinker, resulting in deletion of the DNA encoding the 104 C-terminal amino acids of the PagC protein. To allow positive selection for the loss of plasmid sequences by homologous recombination, the *sacB* gene of *Bacillus subtilis*, which encodes levan sucrase, was then cloned into this intermediary vector, yielding pVAC468 (Fig. 1).

E. coli alkaline phosphatase was chosen as the initial antigen studied because it is easily detected enzymatically. Use of this antigen also allowed comparison of the engineered PagC-alkaline phosphatase fusion antigen with fusions to other *phoP*-activated genes generated by another less time consuming method, *TnphoA* mutagenesis (31). Enzymatically active alkaline phosphatase fusions are located extracytoplasmically in Gram-negative bacilli (32), ensuring similar localization within the bacterial cell for the different constructs compared in this study. The *E. coli* alkaline phosphatase gene was amplified

FIG. 1. Suicide vector pVAC468. The plasmid is an RP4 derivative that requires the *Pir* protein for replication. It contains the *pagC* locus, minus the region encoding the C-terminal 104 amino acids of PagC (*pagCΔ*), and ~1 kb of flanking *S. typhimurium* DNA. *bla*, β -Lactamase gene; *sacB*, levan sucrase gene; *mob*, mobilization function gene; *ori*, origin of replication. Several restriction endonuclease sites are also noted.

by PCR from pRT291 (31) by using PCR primers engineered to create flanking *EcoRV* restriction endonuclease sites and, subsequently, cloned into the *EcoRV* site of pVAC468, yielding pVAC468-*phoA*. pVAC468-*phoA* was mobilized via conjugation into *S. typhimurium* CS019. Strains with homologous recombination of pVAC468-*phoA* at the *pagC* locus were recognized by plating on medium containing ampicillin. Subsequently, activation of the *sacB* gene at 30°C on low salt/high sucrose agar plates (23, 33) allowed recognition of strains that had spontaneously deleted plasmid sequences via a second homologous recombination event; such strains either reconstituted the wild-type *pagC* locus or had undergone replacement of the native locus with the *pagCΔ490-phoA* allele. Blot hybridization analysis of bacterial chromosomal DNA was used to differentiate between these two possibilities, and one strain of CS019 carrying the *pagCΔ490-phoA* fusion gene was designated strain LH491.

Strain LH491 bearing the engineered *pagC-phoA* fusion gene was evaluated as a prototype vaccine vector in BALB/c mice. LH491 was markedly attenuated for virulence in BALB/c mice when compared with the isogenic wild-type parent CS019 (intraperitoneal LD₅₀ for CS019, <10 cfu per mouse; LD₅₀ for LH491, 3.5×10^5 cfu per mouse). LH491 was demonstrated to contain an enzymatically active fusion protein, on indicator agar plates containing the substrate 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and in liquid-phase assays (21). Expression of the fusion antigen was stable as measured by these techniques after 30 passages *in vitro* and 5 passages in mice (data not shown). The expression of the PagC-alkaline phosphatase antigen was *phoP*-regulated as measured by enzymatic activity (Fig. 2C) and Western blot analysis (Fig. 3)—i.e., virtually undetectable in the presence of a *PhoP*-null allele. The PagC-alkaline phosphatase fusion protein was demonstrated to be expressed *in vitro* in response to low pH and starvation, conditions previously demonstrated to induce the expression of *phoP*-regulated genes (data not shown).

Immunogenicity of the *phoP*-Activated Model Antigen. LH491 was administered orally (10^8 cfu per mouse) or i.p. (10^5 cfu per mouse) as a vector for delivery of the model antigen to BALB/c mice and animals were subsequently bled. Several immunoassays were used to demonstrate a specific humoral response to the heterologous antigen and to the *S. typhimurium* vector. Mice immunized i.p. with a single dose of

FIG. 2. Alkaline phosphatase activity and immunogenicity of vaccine strains carrying either *phoP*-regulated or constitutively expressed alkaline phosphatase fusion antigens. (C) Alkaline phosphatase (AP) activity of four alkaline phosphatase fusion antigens, in the presence (PhoP⁺) and absence (PhoP⁻) of an intact *phoP/phoQ* locus. Alkaline phosphatase assays were performed on bacterial suspensions grown to midlogarithmic phase as described (34) with pNPP as a substrate. Mice were immunized i.p. with comparable single doses (cfu per mouse) of each strain in the presence of a wild-type *phoP/phoQ* allele (PhoP⁺) and bled 3 weeks later. Sera were diluted 1:100 and applied in parallel to ELISA plates coated with *S. typhimurium* LPS (B) or purified alkaline phosphatase (A). ELISA plates were developed with a secondary antibody-peroxidase conjugate directed against total mouse IgG and results are presented as ELISA OD units at 450 nm. MNS, mouse normal serum.

LH491 developed significant IgG responses to the heterologous antigen and vector (Fig. 2 B and C), as measured by ELISAs utilizing purified alkaline phosphatase and *S. typhimurium* LPS as antigen. IgG responses were also measurable by a dot blot immunoassay in which immune sera were incubated with nitrocellulose dot blots spotted with alkaline phosphatase. A positive reaction was detected at serum dilutions of at least 1:10,000 in all animals boosted i.p. 2–3 weeks after the primary immunization (Fig. 4). Sera obtained 3 weeks after single i.p. immunizations of 2×10^4 to 2×10^5 cfu per mouse were positive at dilutions of 1:500 to 1:5000 in this sensitive assay (data not shown). Reverse dot blot assays, in which immune sera were applied to nitrocellulose dots and developed with the addition of purified alkaline phosphatase and enzyme substrate, demonstrated analogous results (data not shown). Single-dose oral immunization was also successful in generating an IgG response to the model heterologous

FIG. 3. Western blot of whole bacterial cell lysates of experimental *S. typhimurium* vaccine strains containing *phoP*-regulated and constitutively expressed alkaline phosphatase fusion protein antigens. Alkaline phosphatase fusion antigens were expressed in the presence of an intact *phoP/phoQ* locus (P⁺; wild type) or disrupted *phoP/phoQ* allele (P⁻). Late logarithmic phase bacterial cultures were pelleted and suspended in SDS/PAGE loading buffer, and equal amounts of total protein (0.6 mg) were electrophoresed on a 12.5% polyacrylamide gel, blotted to nitrocellulose, and probed with an antibody to alkaline phosphatase. PagC-alkaline phosphatase and PagD-alkaline phosphatase fusion proteins of ≈ 55 kDa are significantly decreased as a result of introducing a *phoP*-null allele; constitutively expressed alkaline phosphatase fusion proteins in strains LH610 (≈ 100 kDa) and LH617 (≈ 55 kDa) are unaltered by the introduction of a *phoP*-null allele. Densitometric scanning of this image (NIH IMAGE 1.54) yielded the following density unit values (integrated over the entire lane) for the respective PhoP⁺ strains: WT, 1.26; PagC, 4.3; LH610, 8.9; LH617, 4.2; PagD, 12.

antigen as measured by ELISAs. ELISAs were performed by applying immunized mouse sera in parallel to microtiter plates coated with alkaline phosphatase and developing these plates with secondary antibodies to total mouse IgG, IgG1, IgG2a, and IgG2b. IgG directed against alkaline phosphatase was primarily of the IgG2a and IgG2b subclasses; OD values for wells developed with anti-IgG2b were not significantly higher than control wells (data not shown). A representative experiment in which IgG2a was measured is shown in Fig. 5.

To determine whether the apparent immunogenicity of the PagC-alkaline phosphatase fusion antigen was generalizable to other *phoP*-regulated genes, alkaline phosphatase fusions to other recently identified *phoP*-activated genes, *pagD* and *pagJ* (14), were studied. In this case, alkaline phosphatase fusions created by *TnphoA* mutagenesis in *S. typhimurium* CS019 were utilized. *In vivo* attenuation of these strains was comparable to that of LH491 (CS019*pagD*::*TnphoA* i.p. LD₅₀, 3×10^5 cfu per mouse; CS019*pagJ*::*TnphoA* i.p. LD₅₀, 4×10^3 cfu per mouse). *S. typhimurium* strains expressing fusion proteins between PagD (Fig. 2A) or PagJ (data not shown) and alkaline phosphatase were also able to induce an IgG response to alkaline phosphatase. Additionally, a previously isolated CS019 strain bearing a *pagC-phoA* gene fusion demonstrated to encode only the initial 11 amino acids of PagC (rather than the 84 N-terminal amino acids included in the engineered fusion protein carried by LH491) was also immunogenic. These data suggest that transcriptional acti-

FIG. 4. Western dot blots demonstrating mouse IgG response to alkaline phosphatase under *phoP* regulation. BALB/c mice were immunized i.p. with 2×10^4 cfu per mouse of strain LH491, given a booster injection 3 weeks later with 1×10^5 cfu per mouse, and bled 2 weeks later. Immune sera (mouse 1 through mouse 4) and a normal serum control were diluted 1:10,000 and incubated with nitrocellulose dot blots previously spotted with 10 ng of purified *E. coli* alkaline phosphatase. Detection was performed with a chemiluminescent peroxidase substrate and autoradiography. This is a representative experiment of four performed.

tissue was an important variable affecting immunogenicity (7); minimal or no antibody responses were achieved to heterologous antigens expressed from single chromosomal copies (2, 7–9). Here we have shown that foreign antigen expression driven by macrophage-inducible promoters leads to a dramatic humoral response to an antigen expressed from a single chromosomal copy. Our results utilizing Pag-alkaline phosphatase fusion antigens are comparable to previously published murine data for multicopy plasmid-encoded heterologous antigens (3–5) and superior to those for single chromosomal copies of malarial and bacterial antigens (7–9). In our experiments, even 3- to 10-fold greater constitutive expression was unable to induce humoral responses approaching those induced by *phoP*-regulated expression. Further study will be needed to determine whether cell-mediated and mucosal immunity are as effectively stimulated by this method of antigen expression. The exact reason for this enhanced humoral immune response is not yet clear. It is possible that our expression system may dramatically increase the effective antigen concentration *in vivo*, perhaps by prolonged or enhanced transcriptional activation. We hypothesize that the intracellular location and timing of antigen expression may be critical variables that overcome the need for large amounts of constitutively expressed antigen. Extension of this *phoP*-regulated antigen expression system to relevant bacterial and viral antigens should provide efficacious vaccines. Furthermore, our findings validate the emerging concept that bacterial genes expressed within mammalian cells are optimal targets for development of drugs and vaccines.

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