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Oral Vaccination with *Salmonella* Simultaneously Expressing *Yersinia pestis* F1 and V Antigens Protects against Bubonic and Pneumonic Plague

Xinghong Yang,* B. Joseph Hinnebusch,† Theresa Trunkle,* Catharine M. Bosio,‡ Zhiyong Suo,§ Mike Tighe,* Ann Harmsen,* Todd Becker,* Kathryn Crist,* Nancy Walters,* Recep Avcı,§ and David W. Pascual* *

The gut provides a large area for immunization enabling the development of mucosal and systemic Ab responses. To test whether the protective Ags to *Yersinia pestis* can be orally delivered, the *Y. pestis* caf1 operon, encoding the F1-Ag and virulence Ag (V-Ag) were cloned into attenuated *Salmonella* vaccine vectors. F1-Ag expression was controlled under a promoter from the caf1 operon; two different promoters (P), PreA in pV3, PphoP in pV4, as well as a chimera of the two in pV55 were tested. F1-Ag was amply expressed; the chimera in the pV55 showed the best V-Ag expression. Oral immunization with *Salmonella*-F1 elicited elevated secretory (S)-IgA and serum IgG titers, and *Salmonella*-V-Ag(pV55) elicited much greater S-IgA and serum IgG Ab titers than *Salmonella*-V-Ag(pV3) or *Salmonella*-V-Ag(pV4). Hence, a new *Salmonella* vaccine, *Salmonella*-(F1+V)Ags, made with a single plasmid containing the caf1 operon and the chimeric promoter for V-Ag allowed the simultaneous expression of F1 capsule and V-Ag. *Salmonella*-(F1+V)Ags elicited elevated Ab titers similar to their monotypic derivatives. For bubonic plague, mice dosed with *Salmonella*-(F1+V)Ags and *Salmonella*-F1-Ag showed similar efficacy (>83% survival) against ~1000 LD₅₀ *Y. pestis*. For pneumonic plague, immunized mice required immunity to both F1- and V-Ags because the mice vaccinated with *Salmonella*-(F1+V)Ags protected against 100 LD₅₀ *Y. pestis*. These results show that a single *Salmonella* vaccine can deliver both F1- and V-Ags to effect both systemic and mucosal immune protection against *Y. pestis*. The *Journal of Immunology*, 2007, 178: 1059–1067.

*Y. pestis* is the causative agent of both bubonic and pneumonic plague, and this zoonotic disease is generally transmitted via the bite of an infected flea (1), which can also give rise to septicemic plague (2). Plague still remains a serious public health threat in some regions of the world, accounts for the deaths of 200 million people throughout recorded history, and is endemic to Africa, India, and the southwestern states of the United States (1, 3). Because plague is highly infectious and can readily spread by aerosolization, it poses as a bioterrorist threat (4).

Two *Y. pestis* Ags have been shown to effectively protect against both pneumonic and bubonic plague (5–7). F1-Ag is encoded by a large, 100-kb plasmid unique to *Y. pestis* and is the major protein component of the capsule encompassing *Y. pestis* bacilli (8). It is only expressed at 37°C and is believed to help avoid phagocytosis (9, 10). Elevated anti-F1 Ab titers have been correlated with animal survival following plague infection (11). Virulence Ag (V-Ag) is a 37-kDa protein encoded by the lcrV gene on the conserved plasmid pCD1 and plays a multifunctional role in *Y. pestis* virulence. V-Ag serves as a positive regulator for expression of low calcium response virulence genes (12) and is involved in the translocation of effector proteins into eukaryotic cells via the type III secretion system (13, 14). In addition, it has been suggested that V-Ag can act as an immunosuppressive agent, alter host cytokine production (15), and inhibit neutrophil chemotaxis (16). Based on these observations, perhaps the protective effect of anti-V-Ag Abs is due to their ability to neutralize V-Ag-induced immunosuppression (17).

Previous studies have shown that V-Ag expressed by attenuated *Salmonella enterica* serovar Typhimurium (S. Typhimurium) (18) or as a F1- and V-Ag fusion protein (19) can stimulate Ab responses to V-Ag and confer protection against the wild-type *Y. pestis* challenge. However, the plasmids used for expressing these passenger Ags are dependent on antibiotic selection, which may lessen their stability in vivo. Unstable plasmid maintenance can result in low gene expression. To achieve sufficient protective Ab titers, up to five doses need to be administered (18). To avoid plasmid segregation, mice must be dosed with the antibiotics to force selection, which achieves partial success (19). Alternatively, when F1- and V-Ags are expressed as a fusion protein in *Salmonella* (19) and administered parenterally, Ag

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*This work was supported by Public Health Service Grant AI-56286 and was supported in part by Montana Agricultural Station and U.S. Department of Agriculture Formula Funds and also supported in part by National Aeronautics and Space Administration-Experimental Program to Stimulate Competitive Research under Grant NCC5-579. The Veterinary Molecular Biology BSL-3 facility was in part supported by Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, and the Rocky Mountain Research Center of Excellence, National Institutes of Health Grant U54 AI06537.

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Received for publication July 28, 2006. Accepted for publication November 11, 2006.

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3 Abbreviations used in this paper: V-Ag, virulence Ag; BHI, brain-heart infusion; CFC, cytokine-forming cell; LB, Luria-Bertani; P, promoter; sPBS, sterile PBS.
stabilization again is problematic, but protection can be achieved. In this present study, to avoid immunizing with two different Salmonella vaccines, one expressing F1-Ag and another V-Ag, we investigated whether both protective Ags could be expressed simultaneously using a balanced-lethal vaccine vector ΔaroA, Δasd S. Typhimurium mutant, thus, avoiding dependency upon antibiotic selection for passenger Ag expression.

Materials and Methods

Bacterial strains, media, plasmids, primers, and growth conditions

Bacterial strains and plasmids used in this study are depicted in Table I. Escherichia coli H681, a Δasd mutant strain, and S. Typhimurium H683, a ΔaroA and Δasd mutant strain (20), were used as recipient strains for transformation of the recombinant asd′ plasmid carrying the F1- and/or V-Ag(s). For control, the S. Typhimurium strain H647, which is H683 harboring asd′ vector pJRD184-asd′ (20), lacked in any Ag expression. All strains were cultured with Luria-Bertani (LB) medium without antibiotics. The host strains H681 and H683 were grown in LB medium supplemented with diaminopimelic acid (50 μg/ml). Neither H681 nor H683 will grow in LB agar plates without diaminopimelic acid supplementation. The F1- and V-Ag expression in Salmonella were confirmed by Western blot analysis. F1-Ag expression was also confirmed by the encapsulated appearance of Salmonella-F1-Ag as compared with the Salmonella vector strain, H647.

Immunofluorescence and Western blot analyses

The expression of F1- and V-Ags on the Salmonella cell surface was investigated using the immunofluorescence assay as follows: After overnight culture in liquid LB medium, Salmonella-F1-Ag (pV3), Salmonella-V-Ag (pV55), and Salmonella-(F1 + V)Ags and H647 were collected by centrifugation, and cell pellets were resuspended in sterile PBS (pH 7.4) in the presence of the primary Ab. To detect F1-Ag, a mouse anti-F1-Ag mAb (1 μg/ml; Fitzgerald Industries International Inc., Concord, MA) was used with Salmonella-F1-Ag, Salmonella-(F1 + V)Ags, and H647; to detect V-Ag, a rabbit polyclonal anti-V-Ag Ab (1:1000 dilution; produced in-house) was used for Salmonella-V-Ag (pV3), Salmonella-V-Ag (pV4), Salmonella-(F1 + V)Ags, and H647. They were incubated at room temperature for 30 min and then washed in PBS thrice by centrifugation to remove any residual Abs. For F1-Ag detection, bacilli were then resuspended with the secondary Ab, fluorescein (FITC)-conjugated, rabbit anti-mouse AffiniPure F(ab′)2 (Jackson ImmunoResearch Laboratories) or for V-Ag detection, goat anti-rabbit IgG (H+L) Ab (Southern Biotechnology Associates). The mixture was incubated at room temperature for 30 min, washed in PBS thrice, and resuspended in PBS. Subsequently, the cells were viewed under a fluorescent microscope.

Comparative SDS-PAGE and immunoblot analyses of V-Ag from Salmonella-V-Ag (pV3), Salmonella-V-Ag (pV55), and Salmonella-(F1 + V)Ags were performed by using standard procedures. For Western blot analysis, proteins were transferred from the SDS-PAGE (12% (w/v) polyacrylamide) gel to 0.2-μm-pore-size nitrocellulose membranes (Bio-Rad). The membranes were probed first with the rabbit polyclonal V-Ag antiserum and then with HRP-conjugated goat anti-rabbit IgG (H+L) Ab (Southern Biotechnology Associates). Detection of V-Ag was achieved upon development with the substrate 4-chloro-1-naphthol chromogen and H2O2 (Sigma-Aldrich).

V-Ag is a membrane protein in Y. pestis (13, 22), but where it is expressed within Salmonella is unknown. This can be further complicated when both F1- and V-Ags are coexpressed in Salmonella-(F1 + V)Ags. F1-Ag is readily identified by capsule formation. To help discern V-Ag's
location within the Salmonella-(F1 + V)Ags construct, a subcellular fractionation study was performed. The PeriPreps Periplasmic kit (Epicentre) was used to separate cellular fractions for subsequent analysis. Salmonella-(F1 + V)Ags cells were grown in liquid LB medium at 37°C and harvested from a 200 rpm shaking incubator at 3, 12, and 24 h postinoculation. At each time point, 3 × 10⁹ CFU were used for cell fraction separation. Per manufacturer’s protocols, the bacilli were separated into three fractions: spheroplast, periplasmic, and outer membrane plus cell wall. These fractions were examined by Western blot analysis, as described above.

Assessment of copro-IgA and serum IgG titers by ELISA

Salmonella-F1-Ag, Salmonella-V-Ag(pV3), Salmonella-V-Ag(pV4), Salmonella-V-Ag(pV55), Salmonella-(F1 + V)Ags, or H647 were grown on LB plates for 24 h at 37°C. Bacteria were harvested from the plates in 5 ml of PBS. Bacterial suspensions were centrifuged at 10,000 × g for 4 min, and the bacterial pellets were resuspended in 1.0 ml of PBS. The density of the bacteria was adjusted by OD measurement at 600 nm and confirmed by serial dilutions on LB agar plates. Groups of BALB/c mice (five per group) were pretreated with a 50% saturated sodium bicarbonate solution, followed by an oral dose of 2 × 10⁶ CFU (contained in 0.2 ml) of recombiant Salmonella vaccines, and then 4–6 wk later, these were boosted with 3 × 10⁶ CFU.

Serum and fecal Ab titers were determined by an ELISA. Briefly, malsynovia-microtiter-plaque-forming protein fusion (1.0 μg/ml) or recombiant E. coli F1-Ag in sPBS (pH 7.2) were used to coat Maxisorp Immunoplate II microtiter plates (Nunc) at 50 μl/well. Following overnight incubation, microtiter wells were blocked for 1 h with PBS + 1% BSA. Various dilutions of immune mouse serum or fecal extracts were diluted in ELISA buffer (PBS, 0.5% BSA, 0.05% Tween 20) and incubated overnight at 4°C. Specificities of the Inc-F1- and V-Ag, respectively, were determined using HRP conjugates of the following detecting Abs (1.0 μg/ml): goat anti-mouse IgG, IgA, IgG1, IgG2a, IgG2b, or IgG3 Abs (Southern Biotechnology Associates). Following 90 min of incubation at 37°C and washing, the specific reactivity was determined by the addition of an enzyme substrate, ABTS (Moss) at 50 μl/well, and absorbance was measured at 415 nm on a Kinetics Reader model EL312 (Bio-Tek). Endpoint titers were expressed as the reciprocal log₂ of the last sample dilution, giving an absorbance of 0.1 OD units above negative controls 1 h of incubation.

Cytokine ELISPOT

Groups of BALB/c mice (5–10/group) were euthanized 3 wk after the last immunization to collect spleens. Total splenic mononuclear cells (5 × 10⁶/ml) were resuspended in complete medium: RPMI 1640 (Invitrogen Life Technologies) plus 10% FBS (Atlanta Biologicals) plus 10 mM HEPES buffer plus 10 mM nonessential amino acids plus 10 mM streptomycin. Lymphocytes were restimulated with 10 μg/ml recombinant GST-V-Ag, F1-Ag, or media in the presence of 10 U/ml human IL-2 (PeproTech) for 2 days at 37°C. Cells were washed and resuspended in complete medium. Stimulated lymphocytes were then evaluated by IFN-γ-, IL-4-, IL-10-, and IL-13-specific ELISPOT assays, precisely as described previously (23, 24). Coating and detecting Ab concentrations used to detect IL-13 cytokine-forming cells (CFC) were identical to that previously described for IL-13 ELISA (24).

Challenge studies

For bubonic plague challenges, Y. pestis 195/P was grown in liquid brain-heart infusion (BHI) medium at 21°C for 16 h without aeration. The number of bacteria per ml was determined by using a Petroff-Hauser bacterial counting chamber. The concentration was adjusted to 5.0 × 10⁹/ml by serial dilution in PBS, and after being anesthetized, vaccinated BALB/c mice were injected sc with 1 × 10⁹ CFU. All challenges adhered to Biosecurity Level 3 practices. Mice were monitored four times daily and euthanized when it became clearly evident that they showed signs of terminal plague, such as recumbency, ruffled fur, and reluctance to move. Peripheral blood and/or spleens were collected from euthanized mice to verify plague. Dilutions of blood and splenic homogenates were plated on Yersinia selective agar (Difco) and incubated at 28°C for 48 h to determine Y. pestis CFU. All experiments were approved by the National Institutes of Health, NIAID, and RML Biosafety and Animal Care and Use Committees in accordance with National Institutes of Health guidelines.

For nasal challenges (pneumonic plague), the Y. pestis Madagascar (MG05) strain, a member of the biowar Orientalis as is strain 195/P, was used (20). This recent clinical isolate obtained from a bubo of an infected male in Madagascar and shares similar virulence properties with Y. pestis CO92 strain. The bacterium was cultured in BHI supplemented with 2 mM CaCl₂ broth at 26°C with constant shaking overnight. A 100-μl aliquot was then transferred to fresh BHI and grown overnight at 37°C with constant shaking. Bacteria were diluted to achieve an inoculum of ~1 × 10⁶/ml in sPBS immediately before infection of the mice. The inoculum was titered by enumerating viable bacteria from serial dilutions plated on BHI agar plates. Vaccinated BALB/c mice were infected with Y. pestis MG05 intranasally. Briefly, mice were anesthetized i.p. with 200 μl of a 2.5% solution of Avertin (Sigma-Aldrich). Forty microliters (4 × 10⁴ or 100 LD₅₀s) of freshly grown and diluted Y. pestis MG05 were administered to the nares of each mouse. This dose routinely results in 100% lethality within 2.5 days of infection. For mice surviving challenge, lungs, spleens, livers, and mediastinal lymph nodes were collected and homogenized in PBS using a stomacher (Tekmar). CFU levels in each organ were determined by plating serial 10-fold dilutions of organ homogenate on BHI agar and incubating the plates at 37°C for 48 h.

Statistical analysis

An ANOVA, followed by Tukey’s method, was used to evaluate differences between variations in Ab titers and CFC responses; these were discerned to the 95% confidence interval. The Kaplan-Meier method (GraphPad Prism; GraphPad Software) was applied to obtain the survival fractions following bubonic or nasal Y. pestis challenges of orally immunized mice. Using the Mantel-Haenszel log-rank test, the p-value for statistical differences between surviving plague challenges and Salmonella-vaccinated groups or oral PBS was discerned at the 95% confidence interval.

Results

Oral immunization with Salmonella-F1-Ag elicits elevated systemic and mucosal Abs to F1-Ag

To avert antibiotic stabilization, the caf₁ operon for F1-Ag expression was cloned into a balanced-lethal Δarol, Δasd S. typhimurium vaccine vector H683 (Table I). This system has been previously shown to stably express fimbriae from enterotoxigenic E. coli (20, 25). To validate expression, an immunofluorescence assay showed that F1-Ag was being expressed by the Salmonella-F1-Ag construct (Fig. 1A), and no reactivity was obtained with similarly treated Salmonella vector strain, H647 (Fig. 1B). F1-Ag was successfully transported to the bacterial cell surface, as evidenced by

FIGURE 1. F1- and V-Ags’ expression in S. Typhimurium verified by immunofluorescence and Western blot analysis. Salmonella-F1-Ag (A) and H647 (B) were incubated with mouse anti-F1-Ag mAb and Salmonella-V-Ag(pV3) (C), Salmonella-V-Ag(pV4) (D), Salmonella-V-Ag(pV55) (E), and H647 (F) were incubated with rabbit polyclonal anti-V-Ag Ab. All of the recombinant cells, except H647, fluoresced showing that the F1- and V-Ags are expressed by the Salmonella vector vaccine. To detect F1- and V-Ag expression, 1 × 10⁶ whole cells from each strain were lysed using a polyvalent anti-V-Ag rabbit Ab (H). G, lane 1, Salmonella-F1-Ag; lane 2, Salmonella-(F1 + V)Ags; lane 3, H647. H, lane 1, Salmonella-V-Ag(pV3); lane 2, Salmonella-V-Ag(pV4); lane 3, Salmonella-V-Ag(pV55); lane 4, Salmonella-(F1 + V)Ags; lane 5, H647.
Ab titers are representative of three experiments. Arrows indicate time of titers were all elevated. Values are the means of eight mice samples were analyzed for IgG subclass biases. IgG1, IgG2a, and IgG2b contained resulting in only slight increases in serum IgG Ab titers.

mice orally immunized with $2 \times 10^9$ CFU of Salmonella-V-Ag showed the expected molecular mass of 16 kDa when examined by Western blot analysis using a mAb specific for F1-Ag (Fig. 1G, lane 1).

To test the immunogenicity of the Salmonella-V-Ag vaccine, mice orally immunized with $2 \times 10^9$ CFU showed, by week 4, elevated serum IgG endpoint titers of $2^{17}$ and copro-IgA Ab titers of $2^{10}$ (Fig. 2A). To determine whether this response can be enhanced, an oral boost ($3 \times 10^9$ CFU) was given at week 6, which stimulated a 4.3-fold increase in serum IgG ($p < 0.001$) by week 8, but did not augment mucosal IgA Ab responses against F1-Ag (Fig. 2A). IgG subclass responses were analyzed showing equivalent elevations in IgG1, IgG2a, and IgG2b titers (Fig. 2B) similar to fimbriated Salmonella vaccines (23, 25). Thus, these data show that Salmonella can readily express F1-Ag from the cloned caf1 operon to stimulate mucosal and serum Abs to F1-Ag, and additional manipulation of its promoters/regulators is unnecessary.

Oral immunization with Salmonella-V-Ag constructs elicits variable systemic and mucosal Ab responses

The amount of vaccine produced by Salmonella vaccine vectors in vivo can influence host immunity to the heterologous Ag. Recent studies have also shown that enhancing the in vivo heterologous Ag expression by means of various kinds of promoters, especially the macrophage-inducible promoter (26–28), enhanced the vaccine’s immunogenicity. Previous attempts to express V-Ag expressed in Salmonella produced weak Ab responses requiring multiple immunizations to achieve sufficient Ab titers (18). To ensure that our Salmonella vaccine could induce elevated anti-V-Ag Ab titers, we tested two promoters and their chimeras to determine the degree to which the V-Ag expression can be enhanced. Henceforth, the plasmids pV3, pV4, and pV55, in which lcrV are respectively regulated by PretA (20), PphoP (21), and a chimeric promoter of PretA and PphoP were constructed.

The V-Ag expression of Salmonella-V-Ag(pV3), Salmonella-V-Ag(pV4), and Salmonella-V-Ag (pV55) was verified by immunofluorescence microscopy (Fig. 1, C–E) with the H647 as control (Fig. 1F). Their expression levels were compared by Western blot analysis. The results showed that all of these strains present a $\sim 37$-kDa band (Fig. 1H, lanes 1–3), but their expression levels differ: Salmonella-V-Ag(pV4) showed 2-fold greater V-Ag expression than Salmonella-V-Ag(pV3), and Salmonella-V-Ag (pV55) was 2-fold greater than Salmonella-V-Ag(pV4). This result indicates that PphoP is stronger than PretA in vitro. Also, it shows that the chimeric promoter of PretA and PphoP is stronger than the single promoters to drive V-Ag gene expression.

To investigate whether these constructs differ in eliciting immune responses to V-Ag, Salmonella-V-Ag(pV3), Salmonella-V-Ag(pV4), and Salmonella-V-Ag(pV55) were orally applied to BALB/c mice with $5 \times 10^7$ CFU and boosted with $2 \times 10^7$ CFU 6 wk later. Three weeks post immunization, Salmonella-V-Ag(pV55) induced significantly higher IgA and IgG anti-V-Ag titers than either Salmonella-V-Ag(pV3) or Salmonella-V-Ag(pV4) (Fig. 3, A and B). Ten weeks postimmunization, Salmonella-V-Ag(pV55) was still greater than the other two vaccines.

Since Salmonella-V-Ag(pV55) produced elevated serum IgG and mucosal IgA responses (Fig. 3A and B), this construct was subsequently tested using a higher primary vaccine dose. A group of BALB/c mice was orally immunized with $2 \times 10^9$ CFU of Salmonella-V-Ag(pV55). Four weeks later, serum IgG Ab titers were $2^{15.9}$, while copro-IgA reached up to $2^{10.4}$, Moreover, a boost with $3 \times 10^8$ CFU given at week 6 did not significantly augment serum IgG or mucosal IgA endpoint Ab titers (Fig. 4A), but these Ab titers were maintained throughout the tested period. IgG subclass responses were analyzed and showed equivalent elevations in IgG1, IgG2a, and IgG2b titers at week 10 (Fig. 4B).
F1- and V-Ags can be expressed simultaneously without loss of their immunogenicity

The results from the studies of Salmonella-F1-Ag, Salmonella-V-Ag(pV3), Salmonella-V-Ag(pV4) and Salmonella-V-Ag(pV55) confirmed that F1- and V-Ags can be stably expressed without dependence on antibiotic selection to eventually stimulate elevated Ab responses. We next questioned whether both F1- and V-Ags can be simultaneously expressed by Salmonella vaccine vector. Previous attempts (19) to successfully express both of these protective Ags were preempted with difficulties such as antibiotic marker leakage and maintaining vaccine strain stability of F1- and V-Ag fusion plasmids.

In the present study, we designed and constructed a single Salmonella vaccine expressing both F1- and V-Ags to mimic the native expression of these Y. pestis Ags to similar structures in Salmonella.

The caf1 operon was subcloned from pF1 to pV55, termed pV55F (Fig. 5A and Table I). In pV55F, the caf1 operon expression remains independent of V-Ag expression since caf1 expression is driven by its own promoter upstream of caf1M and controlled by its own regulator caf1R. V-Ag expression continues to be regulated by chimeric promoter of PetA and PphoP. The expression of F1-Ag and V-Ag was verified by immunofluorescence assay, showing that these are expressed by Salmonella (Fig. 5, B and D). To assess if their expression levels are affected by coexpression, Western blot analysis was performed. The Salmonella-(F1+V)-Ags present a similar band to the Salmonella-F1-Ag (Fig. 1G, lane 2 vs 1) when evaluated by anti-F1-Ag mAb. For V-Ag expression, Salmonella-(F1+V)-Ags also presents a similar band to the Salmonella-V-Ag(pV55) (Fig. 1H, lane 4 vs 3) when evaluated by a polyclonal anti-V-Ag Ab. These results demonstrate that Salmonella-(F1+V)-Ags successfully express F1-Ag and V-Ag, and the expression levels correlate to the levels produced when individually expressed.

V-Ag is a known secreted protein that is present on Y. pestis’s cell surface before host cell contact (22), and it is present in the outer membrane (13). To determine which cell fraction within Salmonella V-Ag localizes, Salmonella-(F1+V)-Ags cells cultured from LB broth were harvested and separated into the following three subcellular components: spheroplastic fraction, periplasmic fraction, and outer membrane (including cell walls) fraction by conventional methods. The cell culture supernatant was also collected to assess V-Ag secretion. Western blot analysis clearly showed that V-Ag primarily appears in the spheroplastic (56%) and outer membrane fractions (35.7%) with trace amounts present in the periplasmic fraction (8.3%) with no detectable V-Ag secreted into the surrounding medium by 12 h (Fig. 5F). By 24 h, increased amounts of V-Ag were detected in the periplasmic fraction. This distribution pattern suggests that a portion of the V-Ag expressed in Salmonella can localize to the outer membrane as seen in Y. pestis.

To discern the immunogenicity of Salmonella-(F1+V)-Ags vaccine, BALB/c mice were orally immunized with 2 × 10⁹ CFU Salmonella-(F1+V)-Ags, and they were boosted with 3 × 10⁹ CFU 6 weeks later. It was observed at 4 weeks that Salmonella-(F1+V)-Ags elicited elevated serum IgG anti-F1-Ag and anti-V-Ag Ab responses (Fig. 6, A and B) similar to those obtained with Salmonella-F1-Ag (Fig. 2A) and Salmonella-V-Ag(pV55) vaccines (Fig. 4A). However, the mucosal IgA Ab titers were significantly less than those obtained with the individual vaccines (Figs. 2A and 4A). By 8 wk (2 wk after the boost), there were no significant differences in serum IgG anti-F1-Ag Ab titers between Salmonella-(F1+V)-Ags and Salmonella-F1-Ag vaccines; mucosal IgA Ab titers were slightly reduced (p = 0.032) when compared with a similar time point for Salmonella-F1-Ag-vaccinated mice. By 8 wk, the serum IgG anti-V-Ag Ab titers were elevated in Salmonella-(F1+V)-Ags-vaccinated mice when compared with Salmonella-V-Ag(pV55) (p < 0.001); copro-IgA anti-V-Ag Ab titers did not differ.

Serum IgG subclass responses were also evaluated in Salmonella-(F1+V)-Ags-vaccinated mice. IgG1 and IgG2a anti-F1-Ag titers
results show that simultaneous expression of F1-Ag and V-Ag in Salmonella does not lessen the immunogenicity for either Y. pestis Ags, although the mucosal compartment appears to be affected more than the systemic immune compartment. These results also show that Salmonella can successfully mimic Fl- and V-Ags’ expression as with native Yersinia and further supports our contention that dual Ag expression is feasible.

To determine which supportive T cell cytokines were responsible for the elevated Ab titers, cultured lymphocytes from spleens derived from Salmonella-(F1+V)Ags-immunized BALB/c mice were assessed by cytokine ELISPOT (Fig. 6, E–H). Lymphocytes were restimulated for two days with 10 µg/ml of recombinant GST-V-Ag, Fl-Ag, or media. Elevated IFN-γ (Fig. 6E) and IL-4-producing cells (Fig. 6F) were particularly noted in response to both V and Fl-Ags. Significant increases in IL-10- (Fig. 6G) and IL-13-producing cells (Fig. 6H) were also detected, but were not as great as the number of IFN-γ- and IL-4-producing cells. These results indicate that a mixed Th cell response was induced.

Recombinant Salmonella vaccines are protective against bubonic plague

To test the efficacy of our Salmonella plague vaccines, BALB/c mice were orally immunized with Salmonella-F1-Ag, Salmonella-V-Ag(pV55), Salmonella-(F1+V)Ags, H647, or with sPBS, as described previously. Four weeks after the booster immunization, mice were challenged sc with 1 × 10^6 Y. pestis 195/P (1000 LD_{50}), and the mean survival rate and splenic and blood CFU in mice that developed terminal plague were determined. The Salmonella-(F1+V)Ags showed the greatest efficacy (87.5% survival), as did the Salmonella-F1-Ag (83.3% survival) group, which succumbed to bubonic plague 7 days after challenge (Table 7).

To ascertain whether partial protection was evident in any of vaccinated mice, those mice that developed terminal plague in general showed elevated Y. pestis CFU in their spleens and blood except for the one mouse in the Salmonella-(F1+V)Ags group, which succumbed to bubonic plague 7 days after challenge (Table 7).
PBS-dosed mice, and significance determined: Survival fractions obtained from vaccinated mice were compared with and H647-immunized and the sPBS-dosed groups showed no protection. V)Ags vaccine confers protection against pulmonary plague. Mice (F1/H11001 with /H11001 mouse of 8 succumbing to infection; (F1 V)Ags-immunized group showed the best protection with only one group was partially protective with three of seven mice succumbing to Madagascar strain (H11011 immunization, mice were challenged nasally with 1 the more lethal pneumonic plague. To test the efficacy of our live Salmonella Since the Recombinant Salmonella protective against pneumonic plague Since the Salmonella-(F1 + V)Ags vaccine was protective for bubonic plague, we then questioned whether it would protect against the more lethal pneumonic plague. To test the efficacy of our live Salmonella vaccines, BALB/c mice were orally immunized similarly as that previously described, and 105 days after the booster immunization, mice were challenged nasally with 1 × 10⁹ Y. pestis Madagascar strain (~100 LD₅₀) (Fig. 8). The Salmonella-(F1 + V)Ags showed the best efficacy (87.5% survival), which was identical to that obtained with the bubonic plague challenge. Surprisingly, the Salmonella-F1-Ag vaccine recipients showed poor efficacy (40% survival). In contrast, mice vaccinated with Salmonella-V-Ag(pV55) were partially protected (57.1% survival), which is similar to that observed with the bubonic plague challenge. None of the H647 nor the sPBS recipients were protected (Fig. 8). No culturable Y. pestis was obtained from organ homogenates of mice surviving 30 days after challenge (at termination of study). In summary, these results show that coexpression of both F1- and V-Ags confers the best protection against bubonic and pneumonic plague.

**Table II. Y. pestis 195/5 CFU in tissues obtained from mice with terminal bubonic plague**

<table>
<thead>
<tr>
<th>Mousea</th>
<th>TTDb (h)</th>
<th>Spleen (CFU/mg)</th>
<th>Blood (CFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salmonella-V-Ag(pV55)</td>
<td>68</td>
<td>2.2 × 10⁶</td>
<td>nd</td>
</tr>
<tr>
<td>Salmonella-V-Ag(pV55)</td>
<td>58</td>
<td>5.0 × 10⁶</td>
<td>2.0 × 10⁸</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>3.6 × 10⁶ ± 2.0 × 10⁶</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salmonella-F1-Ag</td>
<td>144</td>
<td>9.2 × 10⁵</td>
<td>nd</td>
</tr>
<tr>
<td>Salmonella-(F1 + V)Ags</td>
<td>164</td>
<td>2.3 × 10⁸***</td>
<td>nd</td>
</tr>
<tr>
<td>H647</td>
<td>90</td>
<td>3.0 × 10⁵</td>
<td>3.3 × 10⁷</td>
</tr>
<tr>
<td>H647</td>
<td>105</td>
<td>1.7 × 10⁵</td>
<td>1.2 × 10⁸</td>
</tr>
<tr>
<td>H647</td>
<td>42</td>
<td>2.7 × 10⁶</td>
<td>nd</td>
</tr>
<tr>
<td>H647</td>
<td>42</td>
<td>4.7 × 10⁵</td>
<td>nd</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>6.2 × 10⁶ ± 7.4 × 10⁶</td>
<td>7.6 × 10⁵ ± 6.1 × 10⁷</td>
<td></td>
</tr>
<tr>
<td>sPBS</td>
<td>78</td>
<td>6.4 × 10⁶</td>
<td>1.4 × 10⁹</td>
</tr>
<tr>
<td>sPBS</td>
<td>68</td>
<td>2.7 × 10⁶</td>
<td>nd</td>
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<tr>
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<td>8.6 × 10⁶</td>
<td>1.6 × 10⁹</td>
</tr>
<tr>
<td>sPBS</td>
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<td>9.4 × 10⁶</td>
<td>nd</td>
</tr>
<tr>
<td>sPBS</td>
<td>54</td>
<td>1.0 × 10⁷</td>
<td>2.2 × 10⁹</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>7.4 × 10⁶ ± 3.0 × 10⁶</td>
<td>1.7 × 10⁹ ± 4.2 × 10⁸</td>
<td></td>
</tr>
</tbody>
</table>

*a Mice from the immunization groups that succumbed to plague were evaluated for extent of Y. pestis colonization. 
*b TTD, Time to death; nd, Not detected.
** Refers to statistical differences in colonization when compared with the sPBS-dosed mice.

Discussion

Salmonella vaccine vectors are adept for delivering vaccines and mimicking natural infections of the gut (21). These recombinant Salmonella vaccines can target mucosal inductive tissues (PP) and ultimately disseminate and stimulate both mucosal and systemic arms of the immune system (29, 30). It has been shown that the attenuated strain S. Typhi can serve as a safe and effective oral vaccine to prevent typhoid fever and serve as a live vector to deliver heterologous Ags in humans (31–34). To enhance the immunogenicity of passenger Ag expression by live Salmonella vaccines (35), various types of promoters have been tested to enhance passenger Ag expression. Such examples include the use of stationary phase inducible promoters, spv and dps, or macrophage-inducible promoters to drive heterologous Ag expression in Salmonella vaccine strains to achieve greater immunity (26, 27), or the use of a mucosal Ag-uptake adjuvant as an enhancer to strengthen immunogenicity (36). In this study, we focused on two factors for enhancing immunity against plague. One was to adjust the V-Ag expression level in Salmonella via the use of a chimeric promoter, and the other was to express both vaccines, V- and F1-Ags, on a single plasmid to eliminate vaccinating with two different, and possibly, competing vaccines.

Heterologous Ag delivery by Salmonella used to be largely dependent upon antibiotic selectable genes (37, 38) or by alternative methods, including nonantibiotic-based vectors (39), balanced lethal stabilization systems (20, 40), or by chromosomal insertions (41, 42). Owing to the apparent drawbacks of stability and antibiotic gene leakage, the antibiotic-based vector delivery systems cannot be further developed for human use. Chromosomal-dependent delivery systems do have promise, especially if stability and

**FIGURE 8.** Oral immunization of BALB/c mice with Salmonella-(F1 + V)Ags vaccine confers protection against pulmonary plague. Mice were orally immunized with 2 × 10⁹ CFU and 4 wk later boosted with 3 × 10⁹ CFU of the following strains: Salmonella-(F1 + V)Ags (eight mice), Salmonella-F1-Ag (five mice), Salmonella-V-Ag(pV55) (seven mice), H647 (eight mice), and sPBS (eight mice). Mice were nasally challenged with ~100LD₅₀ Y. pestis Madagascar 105 days postboost. The Salmonella-(F1 + V)Ags-immunized group showed the best protection with only one mouse of 8 succumbing to infection; Salmonella-V-Ag(pV55)-immunized group was partially protective with three of seven mice succumbing to infection; Salmonella-F1-Ag-immunized group was less protective at 40%; and H647-immunized and the sPBS-dosed groups showed no protection. Survival fractions obtained from vaccinated mice were compared with PBS-dosed mice, and significance determined: *, p = 0.0014.
expression can be improved. The advantage of adapting nonantibiotic-based vectors or balanced lethal vectors for *Salmonella* delivery systems is the elimination of antibiotic selection and in vivo use of antibiotics to maintain vaccine stability while maintaining the ability to stimulate potent immune responses (20, 25).

Previous attempts to express V-Ag by expression in *Salmonella* required multiple (five) doses to elevate Ab responses despite being immunized with relatively high vaccine (1–5 × 10^10 CFU) doses (18). This frequent number of oral vaccinations may have been attributed to poor Ag stabilization since antibiotic selection for V-Ag expression was used. As a result, this V-Ag vaccine only conferred 30% protection in a bubonic plague challenge (18). Likewise, similar problems with plasmid stability were evident in earlier studies using attenuated *Salmonella* for expressing F1-Ag (43); however, later this group was able to express F1-Ag on a *Salmonella* cell surface to achieve more rigorous protection (44). More recently to circumvent the use of antibiotic selection, an operator-repressor titration system was used to achieve stable and high level expression of F1-Ag (45). Protection in mice against bubonic plague was observed when immunized with a very high vaccine (1–5 × 10^10 CFU) dose (45). In our study, to avoid antibiotic selection, we used the balanced-lethal asd^+ plasmid to ensure stable expression of the plague Ags. In addition, this asd^+ plasmid was further manipulated by fusing PretA and PphpO in tandem to elevate V-Ag expression (pV55), hypothesizing that increasing V-Ag expression would enhance immunity. Improvement in V-Ag expression was confirmed by Western blot analysis in which the 37-kDa band of *Salmonella*-V-Ag (pV55) was enhanced ~4-fold and ~2-fold greater than *Salmonella*-V-Ag (pV3) or *Salmonella*-V-Ag (pV4), respectively, suggesting that the chimeric promoter in pV55 improves V-Ag expression in a synergistic fashion. Considering PphpO is macrophage inducible, its in vivo regulation capacity to enhance V-Ag expression is believed to effect immunity, as evidenced in this study. When *Salmonella*-V-Ag(pV55) was orally administered to BALB/c mice and compared with *Salmonella*-V-Ag(pV3) and *Salmonella*-V-Ag(pV4) vaccines, the *Salmonella*-V-Ag (pV55) elicited significantly more S-IgA and serum IgG Ab responses. These results show that the chimeric promoter was more effective, possibly, through higher Ag expression which, in turn, elicited greater Ab responses. A significant attribute of *Salmonella*-V-Ag (pV55) is that fewer doses of vaccine are required, and possibly a single dose may be sufficient to elicit a sustained Ab response to V-Ag, unlike that previously described (18) in which multiple oral doses were required.

The isolation of F1-Ag-negative *Y. pestis* strains from different animal species (46–48), as well as from humans, (49) accounts for 15.8% of all atypical *Yersinia* strains (50). Thus, the reliance of F1-Ag as the sole vaccine is not feasible, and a plague vaccine must include either V-Ag (51) or other protective epitopes (52, 53) to protect against nonencapsulated *Y. pestis*. While there are limited reports regarding potential new vaccine targets (52, 53), V-Ag was protective against nonencapsulated *Y. pestis* (51). Thus, V-Ag with other vaccine targets can possibly be used to address infection with nonencapsulated *Y. pestis*.

In addition to having F1-Ag and V-Ag coexpressed or administered in their recombinant form, previous studies have shown that constructing a live vaccine that expresses both F1- and V-Ags as an F1/V fusion protein, achieves protection against *Y. pestis* bubonic challenge (19). However, plasmid stability is problematic, thus questioning the practicality of this vaccine. In contrast, the F1/V fusion protein’s application as a subunit vaccine was proven efficacious against bubonic and pneumonic plague (54). Contrary to the fusion strategy, in this study, we expressed both of the F1- and V-Ags in their native forms. Based upon our results from *Salmonella*-V-Ag (pV55), a new plasmid, pV55F, was constructed, which can express both F1- and V-Ags at 37°C by *Salmonella*. *Salmonella*-F1+/+V)AgS express V-Ag in the periplasm and outer membrane, while F1-Ag is exported to the *Salmonella* cell surface. Advantage of this strategy is that the F1- and V-Ags will be produced and presented in a fashion similar to *Y. pestis*, thus, avoiding immunization with abnormally presented (nonneutralizing) epitopes, which is often the case with inclusion body formation during Ag overexpression in bacterial vectors. The placement of the F1/V fusion protein in *Salmonella* in a compartment not normally containing this Ag may have destabilized the F1/V fusion protein disabling effective immunization. Thus, the strategy used in our study can be adapted for future delivery of multiple Ags or the creation of a multivalent vaccine. It was evident that the *Salmonella*-F1+/+V)AgS vaccine was efficacious against both bubonic and pneumonic plague. Serum IgG subclass analysis revealed that the immune IgG1 and IgG2a Ab levels to F1-Ag and V-Ag were elevated when using our *Salmonella* vector. It has been previously suggested that elevated IgG1 Abs to F1-Ag and V-Ag were indicative of protection (55). Whether this is the case remains to be determined, but clearly the *Salmonella*-F1+/+V)AgS vaccine did stimulate elevated IgG1 Ab titers. In support of these elevated Ab responses, elevations in both IFN-γ and IL-4 were obtained in Ag restimulation assays. To a lesser extent, IL-10 and IL-13 were induced.

Since *Y. pestis* remains a concern for bioterrorism, a highly effective and safe plague vaccine is urgently needed. In the past, live and killed whole-cell plague vaccines for humans were inefficient and required repeated boosting to maintain elevated Ab titers and were reactogenic (56, 57). Thus, alternative approaches to formalin inactivation are needed to develop efficacious vaccines for plague. One approach is the development of a live vaccine, as described here. The advantage of a mucosal vaccine is that immune Abs may be able to prevent growth of *Y. pestis* that is deposited in the nasal mucosa as the result of aerosol exposure. Further studies are warranted to determine the contribution of the mucosal immune system to protection against pneumonic and mucosal plague challenges.

Acknowledgments

We thank Clayton Jarrett for technical assistance with the bubonic challenge studies and Nancy Kommers for her assistance in preparing this manuscript.

Disclosures

The authors have no financial conflict of interest.

References
