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Effect of Expression Level on Immune Responses to Recombinant Oral *Salmonella enterica* serovar Typhimurium Vaccines

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Abstract

Live, attenuated *Salmonella* has been used to express heterologous antigens for development of oral vaccines. Often, expression must be regulated because of deleterious effects on the *Salmonella* vector. The effect of varying expression levels on immune response parameters has not been well defined. In this study we introduced mutations in the -10 region of the *trc* promoter in the expression plasmid to generate series of vaccine strains with different levels of expression of a model antigen, the hemagglutinin HagB from *Porphyromonas gingivalis*. There was no difference in growth rates of the *Salmonella* vaccine strains containing the wild-type, the mutant plasmids or the empty expression vector. The primary IgG response in serum in mice orally immunized with the wild type strain peaked 3-4 weeks earlier than the intermediate expression level strains, suggesting that high expression levels may favor an earlier response. While there was a trend for anti-HagB recall responses to correlate with higher expression level, the peak levels were not significantly different even for expression levels as low as 33% of wild type. A similar trend in terms of response level was seen with serum and salivary IgA. The subclass of the IgG response was predominately IgG2a regardless of expression level, consistent with a Th1 response. These data suggest that isotype distribution, immune response level and T helper cell profile is largely unaffected over a wide range of expression levels.

1. Introduction

Live, attenuated *Salmonella* has been shown to be an effective vector for oral delivery of recombinant vaccine antigens, resulting in mucosal and systemic responses [1-4]. Recombinant antigen expression avoids the problem of antigen purification and stability when administered via the oral route. Synthesis of vaccine antigen can occur *de novo* in inductive lymphoid tissues resulting in a rich immunological response with long-term memory [5]. As with any system involving expression of foreign proteins in bacteria, certain proteins can be toxic to the expression vector when expressed at high or even moderate levels. This can be particularly problematic with live recombinant vaccines. Vector fitness can affect growth rate, membrane integrity, and determine the ability to survive transit through the gut colonize and persist in inductive tissues. Modulation of expression level can be used to avoid toxicity but could have effects on the subsequent immune response parameters. In this study, we introduced defined mutations into the promoter of a plasmid expressing a nontoxic heterologous hemagglutinin

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gene of *Porphyromonas gingivalis*, a putative pathogen in human periodontal disease, in the cytoplasm of avirulent *Salmonella enterica* serovar Typhimurium and determined the effects of different levels of expression on the magnitude of the primary and recall responses in mucosal and systemic compartments. The effects on the IgG subclass distribution of the response in serum were also evaluated.

2. Materials and Methods

2.1 Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. Strains were cultured aerobically at 37°C on Luria-Bertani (LB) medium [6] or on agar plates, with the addition of DL- α , ϵ -diaminopimelic acid (50 μ g/ml) for the plasmid-free Δ *asd* strains. Cultures were maintained at -80°C as glycerol stocks.

2.2 Construction of HagB expression plasmids

The plasmid pCRB2922 expressing the *hagB* hemagglutinin of *P. gingivalis* under control of the *trc* promoter was constructed as previously described [7]. Briefly, the *hagB* gene was amplified by PCR from the plasmid pAX-*hagB* which contains the entire open reading frame and flanking regions of *hagB* cloned from *P. gingivalis* strain 381 [8]. A 3' *SalI* site introduced into the amplified *hagB* gene was ligated into *EcoRI-SalI* digested pYA292, an expression plasmid containing the gene coding for aspartate β -semialdehyde dehydrogenase (*asd*) from *S. enterica* serovar Typhimurium [9]. The *EcoRI* site was blunted by digestion with mung bean nuclease and ligated to the blunt 5' end of the *hagB* gene in frame with the *trc* promoter. The plasmid was initially transformed into *E. coli* χ 6097, a Δ *asd* cloning/expression strain. The *asd* gene expressed by the recombinant plasmid complements the deletion in the *E. coli* cloning and *Salmonella* vaccine strains and promotes stable maintenance of the plasmid without the need for antibiotics [9,10].

Mutations were introduced into -10 region (TATAAT) of the *trc* promoter to regulate expression of *hagB* as previously described [7]. Briefly, pCRB2922 was amplified using a primer pair, one of which contained random nucleotide substitutions in the -10 region (TANNNT). Clones of *E. coli* χ 6097 generated from the mutated library were screened for variable expression using colony immunoblotting with an IgG fraction of rabbit anti-HagB IgG raised against histidine-tagged recombinant HagB purified by nickel-nitriloacetic acid agarose (Qiagen Inc., Valencia, CA) [5]. The IgG fraction was obtained using immobilized Protein A chromatography (Sigma-Aldrich, St. Louis, MO). The promoter regions of candidate mutant plasmids were sequenced to determine the mutations and then a restriction fragment containing the promoter and *hagB* reading frame was cloned into a similarly restricted copy of pYA292 to minimize chances that any mutations occurred elsewhere in the plasmid. Plasmids from selected clones were electroporated into the vaccine strain *S. enterica* serovar Typhimurium χ 4072.

2.3 Growth curves

Growth curves were obtained using the Bioscreen C Microbiology Reader (Oy Growth Curves AB Ltd., Helsinki, Finland). Overnight LB static cultures of *Salmonella* vaccine strains containing the empty expression vector (pYA292) or mutated expression plasmids were diluted 1:40 in LB and grown with shaking to OD= 0.5. The cultures were diluted 1:100 in fresh LB, and the growth of all strains was monitored simultaneously at 30 min intervals at 600nm at 37°C with continuous shaking.

2.4 Quantitation of expression level

Cell lysates of vaccine strains were prepared and assayed for expression level as previously described [7]. Briefly, strains were grown in LB medium to an optical density of 0.8 at 600nm. The cells were collected by centrifugation of a 1.5ml portion at $13,000 \times g$ for 5 min at 4°C. The pellet was washed 2x using 7mM sodium phosphate buffer (pH 7.4) containing 272mM sucrose and 1mM MgCl₂. The pellet was resuspended in 1x SDS-PAGE sample buffer (0.0625M Tris-HCl, pH 6.8, containing 2% (w/v) sodium dodecyl sulfate (SDS) and 10% (v/v) glycerol) and boiled for 10 minutes to solubilize proteins.

To quantitate HagB, ELISA plates were coated with purified polyclonal rabbit anti-HagB IgG. After blocking with 0.1% bovine serum albumin, serial dilutions of purified HagB standard [5], or bacterial lysates were applied to the plates. Biotinylated anti-HagB IgG was applied and the plates were incubated overnight at 4°C. After washing, the plates were incubated for 30 min with avidin peroxidase (1 µg/ml), washed, and developed with 0.04% *o*-phenylenediamine dihydrochloride in 0.1 M phosphate buffer, pH 5.0, containing 0.012% H₂O₂. The absorbance at 490nm was recorded using a BioRad model 550 microplate reader (BioRad, Hercules, CA). Samples were compared to standard curves generated employing 4-parameter logistic regression using Microplate Manager III software (BioRad).

The total protein concentration of each lysate was determined using the Micro BCA™ Protein Assay Reagent Kit (Pierce, Rockford, IL). The specific HagB concentration in each lysate was normalized to total protein concentration to allow comparison of the expression level of each strain as ng HagB/µg total protein in lysate. The expression level of the wild-type promoter strain pCRB2922 (143.3 ng HagB/µg protein) was taken as 100%.

2.5 Animal immunization and sampling

Female BALB/c VAF/Plus mice, 6-8 wks of age (Charles River, Wilmington, MA) were housed in filter-top cages in the Infectious Disease Isolation Unit at the University of Florida Animal Resource Center and given sterilized food and water *ad libitum*. The bedding was changed to reduce coprophagy and the food supply was removed 4 hours prior to immunization to minimize the amount of material in the stomach. Vaccine strains were grown as a static cultures in LB broth overnight at 37°C, diluted 1/20 in fresh LB broth and grown at 37°C with aeration to an OD₆₀₀ of 0.8. Cultures were centrifuged and resuspended in LB broth to a density of 10¹⁰ CFU/ml based on calibrated curves of OD₆₀₀ vs cell number. Mice were given 0.1ml of 0.1M NaHCO₃ by gastric intubation 10 min prior to the vaccine to neutralize gastric acid. Groups of seven mice were immunized by gastric intubation with 10⁹ cells on days 1, 3, and 5 of week 0. The food supply was returned following immunization. Boosting was carried out in the same manner at week 14. Serum and saliva were collected and assayed as previously described [11,12].

2.6 Statistical methods

Differences in immune responses between groups were determined by one-way analysis of variance on log-transformed data using Tukey's post test for multiple comparisons. Analysis was performed using InStat software (GraphPad Software, San Diego, CA).

3. Results

3.1 Selection and analysis of expression level mutants

The library of promoter mutations in χ 6097 was screened by colony immunoblotting using anti-HagB. Clones with variable levels of expression could be detected by observing differences in staining intensity (Fig. 1). Quantitative expression levels were determined in the *Salmonella* vaccine strain using pCRB2922 (143.3 ng HagB/µg protein), which possesses the

wild-type *trc* promoter, was taken as 100%. Three reduced expression mutants, pCRB65 (75.8%), pCRB1232 (33.2%), and pCRB148 (1.7%), were chosen for immunization studies. There was no difference in growth rates of the *Salmonella* vaccine strains containing the wild-type, the mutant plasmids or the empty expression vector pYA292 (Fig. 2).

3.2 Immune responses in orally immunized mice

To examine the effects of the cytoplasmic expression level of HagB on the magnitude and kinetics of the immune response, mice were orally immunized at week 0, then boosted at week 14 with the wild-type and expression level mutant vaccine strains. Immune responses were assessed in serum and saliva. In both serum and secretions, there was a general correspondence between expression level and peak responses (Fig. 3). Primary serum IgG responses to the wild-type strain peaked at around 5 weeks while the peak primary response to pCRB65 (75.8%) and pCRB1232 (33.2%) appeared to be delayed by 3-4 weeks (Fig 3a). Peak recall responses occurred around week 19, with the wild-type strain exhibiting a more rapid rise and reaching the highest level. Differences between peak recall responses at week 19 were not statistically significant except for pCRB148 (1.7%), which was significantly lower than the other vaccine strains ($p < 0.001$), reaching around 800 ng/ml during weeks 17-21 (ca. 1% of wild-type).

Peak primary serum IgA responses occurred somewhat later (weeks 7-9) than IgG responses (Fig. 3b). This delay in the response is consistent with other cytoplasmic strains we have examined [12]. Peak recall serum IgA responses occurred between weeks 17-21, with the wild-type reaching the highest levels. Differences in peak recall responses were not statistically significant except for the pCRB148 (1.7%), which was significantly lower than the other vaccine strains ($p < 0.001$). For both serum IgG and IgA, there was little difference in response between the intermediate pCRB65 (75.8%) and pCRB1232 (33.2%) expression level mutants.

Primary salivary IgA responses followed kinetics similar to serum IgA (Fig. 3c). Peak responses were similar among the wild-type and two intermediate expression level strains.

3.3 Serum IgG subclass distribution

The subclass distribution of IgG anti-HagB antibodies was determined during the early primary response (week 3), peak primary (week 5), pre-boost (week 13), and peak recall responses (week 19). Regardless of expression level, IgG2a responses to HagB predominated during the course of the primary and recall response (Fig 4 a-c), which is characteristic of a Th1 type response [13, 14].

4. Discussion

The majority of pathogens initially colonize and/or invade via mucosal surfaces. As a consequence, much effort has been directed at designing vaccines to stimulate mucosal immunity [15,16]. Because of compartmentalization of the immune system, parenteral immunization routes are ineffective at inducing mucosal responses. Stimulating a mucosal response requires delivering the vaccine antigen to a mucosal inductive site such as the Peyer's patches in the gut via the oral route. Oral mucosal vaccine approaches must preserve the integrity of the antigen during transit through the gut, and deliver the antigen in such a way that enhances its immunogenicity. For purified component vaccines, a variety of methods have been used including physical encapsulation, and conjugation to mucosal adjuvant molecules [16]. Live, attenuated whole-cell vaccines, such as the oral cholera vaccines have been shown to be capable of inducing effective responses [15].

For recombinant antigens, live, attenuated viral or microbial vectors have been used for delivery. *Salmonella* has the advantage of having a tropism for the mucosal inductive tissues

of the Peyer's patches, allowing *in situ* expression of antigen while enhancing its immunogenicity. Attenuated *Salmonella* has been used to express over 50 different foreign antigens of various origins [1-4].

In the present study, expression of HagB at any level had no effect on the growth rate of the vaccine strains. However, the metabolic burden resulting from overexpression of heterologous gene products, or even low levels of expression of certain more toxic genes, can compromise the live vaccine vector [17-19]. Reductions in growth rate, interference with membrane stability or altering expression of important vector genes may reduce its ability to colonize in the gut and persist long enough in mucosal inductive tissues to induce an effective immune response.

We have used attenuating promoter mutations to allow successful expression HagB on the surface of *S. enterica* serovar Typhimurium using the Lpp-OmpA fusion system, which is toxic at moderate expression levels [7]. Using *in vivo*-inducible promoters can avoid toxicity during vaccine growth, although the actual expression level *in vivo* is difficult to predict, and the problems associated with toxicity would resurface following *in vivo* induction. It may be desirable to use promoter attenuation in combination with *in vivo*-inducible promoters for particularly toxic vaccine antigens.

How expression levels affect immune response parameters has not been well defined. In the present study, we examined the effect of different constitutive expression levels on immune responses using a model antigen that we have previously shown to be immunogenic and nontoxic at high expression levels [5,11,12,20]. The primary IgG response in serum to the wild type strain peaked 3-4 weeks earlier than the intermediate expression level strains (Fig 3a), suggesting that high expression levels may favor an earlier response. While there was a trend for anti-HagB recall responses to correlate with higher expression level, the peak levels were not significantly different even for expression levels as low as 33% of wild type. A similar trend in terms of response level was seen with serum IgA, however the peak primary response occurred later than the peak IgG response regardless of expression level. Likewise, primary mucosal IgA responses in saliva follow a similar pattern to serum IgA responses and peak later than the IgG response.

IgG subclass distribution is influenced by several factors including the cytokine environment during presentation, type of antigen presenting cell and costimulatory signals, as well as the nature and dose of antigen [21]. While antigen dose can affect T helper cell polarization, there is controversy over whether high or low doses of antigen favor Th1 or Th2 responses. Over the range of expression levels we studied, the predominant subclass of anti-HagB was IgG2a which is characteristic of a Th1 response [13,14]. The predominance of IgG2a antibodies agrees with our previous studies with HagB [12]. Most recombinant antigens expressed in oral, avirulent *Salmonella* vaccines induce a predominately Th1 response, as does the vector itself [12,22,23]. An exception is cholera toxin B subunit, which induces a Th2 response [24,25]. Some temporal changes in Th bias have been seen in during immune responses to oral *Salmonella* vaccines [26,27].

Normally, mucosal IgA production is strongly Th2-dependent. In particular IL-5, IL-6, and IL-10 are major cytokines that enhance IgA responses. While IL-4 is necessary for mucosal IgA responses to soluble proteins, it is not necessary for mucosal IgA responses to passenger antigens delivered by live *Salmonella* [23,28]. In the presence of a strong Th1 response induced by live, recombinant *Salmonella* vaccines, IL-6, IL-10 from alternative sources can compensate for the lack of the Th2 cytokines IL-4 and IL-5 [15,23,28].

The present study suggests that there is a wide latitude in expression level, and that the highest levels of expression are not necessarily required for an elevated immune response to heterologous recombinant antigens expressed in live attenuated *Salmonella* vectors.

Furthermore, expression level does not significantly affect isotype distribution and IgG subclass profile, an indication that the T helper cell profile is unaffected. With toxic heterologous recombinant antigens, promoter attenuation can be of value in increasing the *in vivo* fitness of the vaccine vector. Even with *in vivo*-inducible promoters, limiting maximum expression level by introducing promoter mutations could be of additional benefit with potentially toxic antigens.

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Fig. 1. Colony immunoblot of promoter mutation library of χ 6097/pCRB2922 developed with anti-HagB showing examples of reduced expression level mutants (*). Arrow indicates wild-type control, pCRB2922 (100%).

Fig. 2. Growth curves of *Salmonella* vaccine strains containing the wild-type pCRB2922 (100%), and promoter mutants pCRB65 (75.8%), pCRB1232 (33.2%), and pCRB148 (1.7%), and the empty expression vector pYA292.

Fig. 3.

Time course of anti-HagB responses (mean and standard error) in serum IgG (a), serum IgA (b) and salivary IgA (c) in mice immunized with wild-type pCRB2922 (100%), and promoter mutants pCRB65 (75.8%), pCRB1232 (33.2%), and pCRB148 (1.7%). Arrows indicate priming (week 0) and boosting (week 14). Differences between peak serum IgG and IgA recall responses at week 19 were not statistically significant except for pCRB148 (1.7%), which was significantly lower than the other vaccine strains (*, $p < 0.001$).

Fig. 4.

IgG subclass distributions (mean and standard error) of anti-HagB antibodies in serum from mice immunized with (a), wild-type pCRB2922 (100%), and promoter mutants (b), pCRB65 (75.8%), (c), pCRB1232 (33.2%). Distributions were measured during the early primary response (weeks 3 and 5), pre-boost (week 13) and post-boost (week 19).

TABLE 1

Bacterial strains and plasmids

Bacterial strains or plasmids	Relevant characteristics	Reference
Strains		
<i>E. coli</i> χ 6097	F' Δ [<i>lac-pro</i>] <i>tpsL</i> Δ <i>asdA4</i> [<i>zbf-2</i> ::Tn10] <i>thi</i> Φ 80d <i>lacZ</i> Δ M15	[10]
<i>S. enterica</i> serovar Typhimurium χ 4072	SR-11 derivative (pSISR100 ⁺ <i>gyrA</i> 1816 Δ <i>cya-1</i> Δ <i>crp-1</i> Δ <i>asdA1</i> Δ [<i>zbf-4</i> ::Tn10])	[10]
Plasmids		
pAX- <i>hagB</i>	<i>AccI</i> - <i>XbaI</i> subclone in pUC18 containing <i>hagB</i> ORF	[29]
pYA292	<i>asd⁺</i> expression plasmid	[9]
pCRB2922	<i>hagB</i> cytoplasmic, derivative of pYA292; wild type <i>trc</i> promoter (100% expression)	[7]
pCRB65	promoter mutant of pCRB2922; 75.8% expression	[7]
pCRB1232	promoter mutant of pCRB2922; 33.2% expression	[7]
pCRB148	promoter mutant of pCRB2922; 1.7% expression	[7]