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***Salmonella enterica* serovar Typhi Live Vector Vaccines Finally Come of Age**

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Abstract

Attenuated bacterial vaccine strains hold great promise as live vectors for presentation of foreign antigens from unrelated bacterial, viral, and parasitic pathogens to the immune system. While this approach has proven quite successful in experimental animal models for eliciting antigen-specific mucosal, humoral, and cellular responses, results have been disappointing for clinical trials carried out thus far. We hypothesize that the paucity of human responses to foreign antigens delivered by live vectors suggests that the strains and genetic approaches used to date have resulted in over-attenuated vaccine strains with severely reduced immunogenicity. However, remarkable advances have now been made in the genetics of foreign antigen expression, understanding mechanisms of live vector immunity, and refining immunization strategies. The time has now come for development of multivalent live vectors in which stable antigen expression is balanced with metabolic fitness to create highly immunogenic vaccines.

INTRODUCTION

A vaccinology strategy that has generated much interest is the adaptation of attenuated strains of *Salmonella enterica* serovar Typhi (*S. Typhi*) or *S. Typhimurium* to create live vectors that synthesize protective antigens from unrelated pathogens and deliver these heterologous antigens to the immune system, thereby eliciting protective immune responses^{1,2}. Advantages of *Salmonella* strains as live vector vaccines include: 1] they can be delivered orally, 2] they target M cells overlying gut-associated lymphoid tissue (inductive sites for immune responses), 3] they are readily internalized by dendritic cells and macrophages, and 4] they stimulate broad immune responses including serum antibodies, secretory IgA intestinal antibodies and a panoply of cell-mediated immune (CMI) responses, including cytotoxic lymphocytes (CTL).^{3–7} Using *S. Typhimurium* as the live vector and mice as the model, *Salmonella* live vector technology has been extraordinarily successful in stimulating serum, mucosal and CMI responses to many foreign antigens of other bacteria, viruses, protozoa and helminths. Moreover, challenge models have documented the ability of many of these *S. Typhimurium* vaccines to confer upon mice protection against challenge with the heterologous pathogen.^{8,9}

Based on the success of *S. Typhimurium* live vectors in mice, other investigators have attempted to use the highly human host restricted serovar *S. Typhi* as a live vector for humans and to achieve similar levels of safety and immunogenicity to foreign antigens. Whereas much progress has been made in molecular biologic aspects of adapting *S. Typhi* as a live vector, only a few constructs have advanced to Phase 1 clinical trials where they

elicited only modest immunogenicity compared to analogous studies with *S. Typhimurium* in mice. Nevertheless, each clinical trial with a *S. Typhi* live vector construct has taught one or more invaluable lessons and identified limitations to live vector technology that have guided further development and improvements. In this review, we highlight various advances in the transition of attenuated *Salmonella* strains from monovalent oral vaccines to multivalent live vectors. We first explore the hypothesis that less-attenuated vaccine strains may be required for use as live vectors because expression of potentially toxic foreign antigens may over-attenuate the strain and compromise both anti-vector and foreign antigen-specific immunity. We then highlight technological advances that allow sufficient levels of foreign antigen to be expressed to induce immune responses, while avoiding over-attenuation of the strain; we also address improvements in pre-clinical immunization regimens. This discussion serves as a backdrop for appreciating the limitations of the clinical trials carried out to date using attenuated *S. Typhi* live vector constructs. Finally, we emphasize the need for clinical trials to prove the hypothesis that consequent to incorporation of the various described innovations, *S. Typhi* live vectors have finally come of age.

SELECTION OF ATTENUATED *Salmonella* STRAINS TO SERVE AS LIVE VECTORS

Attenuated *S. Typhi* strain Ty21a, derived in the early 1970s by chemical mutagenesis, is licensed as a live oral typhoid vaccine but requires 3–4 doses.^{10,11} Recombinant DNA technology has created several attenuated *S. Typhi* strains that have been well-tolerated in clinical trials and have proficiently elicited anti-Typhi immune responses following oral administration of a single dose.^{12–22} Among the best known recombinant strains are Ty800,¹² CVD 908,^{13–15} CVD 908-*htrA*,^{16,17} CVD 909,^{18,19} and M01ZH09.^{20–22} These improved strains have also been proposed to serve as live vectors. However, data demonstrate that over-attenuated vaccine strains are insufficiently immunogenic. Therefore, the possibility exists that an optimally attenuated strain, ideal for use as a single-dose live oral typhoid vaccine, may not necessarily function as an ideal live vector due to the various metabolic stresses of expressing foreign antigens.

This point has been explored by Curtiss et al^{23,24} who compared the immunogenicity of classically attenuated *S. Typhimurium* strains, in which critical biochemical loci were deleted from the chromosome, versus strains in which attenuation was genetically engineered to be “conditional”, manifesting itself only after vaccine strains have colonized and penetrated into lymphoid tissues of the host. The key distinction between these two types of strains is that classically attenuated strains are fully attenuated at the time of vaccination and early colonization of the host, while delayed attenuation vaccine strains in theory are wild phenotype at the time of immunization and become attenuated only after reaching deep tissue immunological sites of induction. When used as live vectors to present a foreign protective antigen from *Streptococcus pneumoniae*, it was demonstrated in orally immunized mice that some conditionally attenuated strains colonized deeper tissues such as the liver and spleen up to 10-fold more efficiently than conventionally attenuated strains, and that conditionally attenuated strains elicited significantly higher humoral and cellular immunity to foreign antigen and vector antigens than conventional strains, as well as better protection against heterologous challenge with virulent *S. pneumoniae*. These results in a mouse model support the hypothesis that, for a given antigen, the degree of attenuation of the live vector vaccine strain itself significantly influences the immunogenicity of the foreign protein. However, the genetic engineering techniques chosen for expression and delivery of immunogenic levels of foreign protein must also be carefully considered.

THE SCIENCE OF STABLE FOREIGN ANTIGEN EXPRESSION

Chromosomal integration of antigen cassettes

Following the deletion of a given chromosomal locus to create an attenuated vaccine strain, that locus can be replaced later with a cassette encoding the desired foreign antigen. Chromosomal expression of foreign antigens offers maximum genetic stability, since chromosomal genes are rarely lost by spontaneous deletion. Chromosomal expression simplifies construction of multivalent vaccines,²⁵ in which multiple chromosomal loci can be chosen for expression of distinct antigens from the same or unrelated pathogens. However, since the copy number of genes encoding foreign antigens is usually one per bacterial cell, it is a challenge to ensure that sufficient levels of foreign antigen are expressed to be immunogenic.

Not surprisingly, early attempts at chromosomal expression of heterologous antigens focused on the use of constitutive promoters to drive continuous synthesis of foreign protein in hopes of inducing the desired humoral and CMI responses.^{26,27} However, Hohmann et al²⁸ demonstrated in mice that foreign antigens expressed constitutively from the chromosome of *S. Typhimurium* failed to elicit antigen-specific immune responses. In contrast, expression of the identical antigen using P_{pagC} (which is induced after phagocytosis of live vectors by murine macrophages) elicited strong serum IgG responses against the vectored antigen. It was hypothesized that the intracellular location and timing of heterologous antigen expression, rather than constitutive expression of large amounts of the protein, are critical to foreign antigen immunity.

Building upon the observations of Hohmann, Stratford et al²⁹ reasoned that the use of the more powerful P_{ssaG} *in vivo*-inducible promoter to drive chromosomal expression within the phagosome would stimulate more robust antigen-specific immune responses. Since P_{ssaG} is a tightly regulated SPI-2 promoter that is induced 400-fold following phagocytosis of the *Salmonella* by macrophages³⁰, it was hypothesized that a live vector could be constructed in which foreign antigen expression is minimal prior to vaccination but maximizes at critical sites and times of induction of immune responses. Therefore, an expression cassette was assembled encoding a P_{ssaG} -controlled *eltB* gene directing synthesis of the B subunit of heat-labile enterotoxin (LT) from enterotoxigenic *Escherichia coli* (ETEC). This cassette was integrated to replace the deleted *aroC* gene in *S. Typhi* vaccine candidate M01ZH09. Mice immunized i.n. with a single dose of live vector displayed potent antigen-specific IgG anti-LT responses.^{29,31}

Plasmid-based antigen expression

In addition to chromosomal integration of genes encoding foreign antigens, plasmids can also be used. A wide variety of plasmid-based expression technologies are available for achieving adequate production of antigens to generate the relevant immune response(s). However, the metabolic burden imposed by resident expression plasmids has the potential to render a vaccine strain over-attenuated and thus sub-immunogenic. Since spontaneous plasmid loss would remove any metabolic burden and allow plasmidless bacteria to quickly outgrow the population of plasmid-bearing bacteria, such a shift in the live vector population would reduce its capacity to stimulate immune responses. A critical challenge facing successful development of plasmid-based live vectors is to ensure stable maintenance of expression plasmids, while reducing any toxicity associated with synthesis of the foreign proteins they encode.

One strategy for engineering genetic stability into expression plasmids is to borrow from themes developed in nature. Paradigms of plasmid organization that promote stable plasmid replication and maintenance have been reviewed by Thomas^{32,33} to include: 1] a conjugative

transfer system for plasmid mobilization between bacteria; 2] at least one locus that confers a selective advantage to the host bacterium upon inheritance of the plasmid; 3] a self-regulating origin of replication; 4] an active partitioning mechanism to promote the non-random distribution of plasmids into bacterial daughter cells; 5] a post-segregational killing system to remove plasmidless daughter cells from a growing population of bacteria, and; 6] a multimer resolution mechanism for decatenating recombined plasmids that must segregate independently for proper inheritance.

However, regulatory agency considerations limit the choices of genetic systems that can be incorporated into plasmid backbones to accomplish stable expression, since the safety of the vaccine strains contemplated for human use must be assured. For example, live vectors intended for human use cannot carry self-transmissible plasmids, and the use of selectable genes encoding resistance to “clinically irrelevant” antibiotics is currently being strongly discouraged by regulatory agencies. Development of expression plasmids for use in *S. Typhi* (as well as other) live vectors has therefore taken advantage of small non-transmissible multicopy replicons that enable more DNA sequence to be devoted to expression of heterologous antigens, while minimizing the size and genetic instability of the replicons.

Improvements to plasmid backbones

To prevent plasmidless daughter cells from overtaking a growing population, plasmid retention systems have been developed based on the strategy of removing plasmidless bacteria from a growing population, thereby preventing loss of expression of the desired foreign antigen. Removal of plasmidless bacteria can be accomplished by several methods that are not mutually exclusive and can be used in combination: 1] plasmid partitioning systems that enhance the proper segregation and inheritance of expression plasmids into dividing cells; 2] post-segregational killing systems to remove bacteria in which proper inheritance of plasmids has not occurred, and; 3] “balanced-lethal” or conditionally lethal systems in which expression plasmids are engineered to encode a critical protein (often an enzyme) whose gene has been deleted from the live vector chromosome; plasmid loss would then lead to the eventual death of plasmidless progeny due to a cessation of normal metabolism.

A combination of both a plasmid partitioning function and a post-segregational killing system was reported to improve plasmid retention³⁴. The post-segregational killing system that was used encoded a self-contained toxin-antitoxin system in which the protective antitoxin is unstable and requires constant synthesis from resident expression plasmids; plasmid loss activates the toxin, again leading to cell lysis. To remove the random partitioning of multicopy plasmids during cell division, plasmid segregation functions were also introduced to ensure non-random inheritance of plasmids into all daughter cells. Quantitative *in vitro* analysis of plasmid retention clearly demonstrated that as toxin-antitoxin and partitioning maintenance functions were incrementally introduced, plasmid stability improved accordingly³⁴. Use of this plasmid maintenance system has recently progressed into pre-clinical trials in non-human primates, where expression plasmids combined this plasmid retention system with a novel antigen export system to test the immunogenicity of exported fusions of Protective Antigen from anthrax toxin (PA83). Monkeys primed mucosally (i.n.) with attenuated *S. Typhi* live vector CVD 908-*htrA* expressing exported PA83 fusions were boosted 3 months later with a single parenteral dose of Biothrax® vaccine (the complex licensed anthrax vaccine that contains PA83 among other antigens). Notably, within 7 days after administration of the single parenteral booster, robust toxin neutralizing antibody levels were detected in serum.³⁵

Another approach to improving the inheritance and retention of expression plasmids borrows from motifs observed in nature that reduce the multimerization of plasmids, thereby

increasing the actual number of individual replicons available for proper segregation^{36,37,38}. Several resolution systems have been described originating from self-transmissible factors isolated from a variety of enteric strains^{39,40}. Using the *crs-rsd* site-specific resolution system, originally identified in the virulence plasmid pSDL2 from *Salmonella* Dublin, Stephens et al⁴¹ observed that incorporation of this stability module into ColE1 replicons dramatically improved plasmid retention in *S. Typhi* vaccine strain CVD 908-*htrA*. Interestingly, the highest retention frequencies were observed only after additional transcription elements were incorporated into these expression plasmids to tightly regulate foreign antigen expression levels⁴¹. This system awaits further immunogenicity testing in pre-clinical models.

Retention of expression plasmids has also been accomplished using conditional lethal systems. Conditional lethal approaches also function as selection systems, thereby removing the need for recovery of incoming plasmids in live vectors using antibiotic selection. One such non-antibiotic balanced lethal system is based on the expression of the *asd* gene encoding aspartate β -semialdehyde dehydrogenase (Asd), an enzyme critical to synthesis of the cell wall and several amino acids⁴². Loss of plasmids encoding Asd is lethal for any bacterium incapable of synthesizing Asd from the chromosome, resulting in lysis of the bacterium due to an inability to correctly assemble the peptidoglycan layer of the cell wall. The *asd* system thus improves the apparent stability of expression plasmids by removing plasmid-cured bacteria from the population (i.e., a post-segregational killing system). The *asd* system has been successfully employed in attenuated *S. Typhimurium* live vector strains⁴³ expressing a variety of antigens including tetanus toxin fragment C⁴⁴, *E. coli* LT⁴⁵, synthetic hepatitis B viral peptides⁴⁶ and more recently *Yersinia pestis* F1 and LcrV antigens⁴⁷. Mice immunized mucosally with these recombinant strains elicited potent immune responses including serum IgG and secretory IgA.

A clever variation of the balanced lethal non-antibiotic strategy for plasmid selection and maintenance involves construction of a conditionally lethal transcriptional control circuit in which the *lacO-lacI* operator-repressor genes controlling the *E. coli* lactose operon are engineered to control the synthesis of a chromosomally encoded protein critical for bacterial survival. Introduction of multicopy expression plasmids carrying *lacO* into the carrier organism titrates LacI repressor away from chromosomal *lacO* to enable synthesis of the required protein and bacterial growth. Loss of such an expression plasmid would then assure that surplus LacI binds to chromosomal *lacO*, shutting down synthesis of the essential protein, and resulting in the death of plasmidless cells.⁴⁸ The repressor-titration technology was incorporated into a low copy number plasmid encoding the F1 antigen from *Y. pestis* and transferred to *S. Typhimurium*. This vaccine construct protected 5 out of 6 orally immunized mice from subcutaneous lethal *Y. pestis* challenge. The stabilized expression plasmid was retained *in vivo* for two weeks.⁴⁹

We have recently developed a novel conditional lethal plasmid selection system based on plasmids encoding the single stranded binding protein, which has been removed from live vector chromosomes. SSB, a non-catalytic 177 amino acid protein with a relative molecular weight of 19 kDa, binds as a tetramer with high affinity to single-stranded DNA (ssDNA) and plays an essential role as an accessory protein in DNA replication, recombination, and repair.^{50,51} Loss of expression plasmids encoding this essential non-enzymatic protein would therefore quickly lead to cell death due to failure of proper DNA metabolism. We engineered a set of isogenic expression plasmids in which PA83 fused to an antigen export system was expressed from SSB-stabilized medium copy (pSEC91-83S, ~ 15 copies per chromosomal equivalent) or low copy (pSEC10-83S, ~ 5 copies per chromosomal equivalent) plasmids (Figure 1). Plasmids were introduced into either CVD 908-*htrAssb* or the less attenuated parent CVD 908-*ssb*. As a control, we also included CVD 908-

htrA(pSEC91-83) in which conventional plasmids expressing resistance to kanamycin were used. We tested the immunogenicity of these constructs in BALB/c mice primed intranasally on days 0 and 14 with 5×10^9 cfu of live vector and boosted intramuscularly 28 days later (day 42) with 10 μ g purified PA83 plus alum. Preliminary results with pooled sera are shown in Table 1 (serum IgG). Surprisingly, humoral responses engendered using CVD 908-*htrAssb*(pSEC91-83S) carrying an SSB-stabilized medium copy expression plasmid were clearly lower than responses elicited by the conventional CVD 908-*htrA*(pSEC91-83) live vector carrying the kanamycin resistance medium copy pSEC91-83 plasmid (**group 2 versus group 7**). However, when the copy number for SSB-stabilized plasmids was reduced, serum responses with CVD 908-*htrAssb*(pSEC10-83S) rose 10-fold versus conventional CVD 908-*htrA*(pSEC91-83) on day 41, and remained elevated 2 weeks after boosting (**group 3 versus group 7**). These data clearly suggest that as the plasmid copy number is reduced, the immunogenicity of the foreign antigen improves. We hypothesize that this effect is due to the reduced metabolic burden associated with the lower copy number of pSEC10-83S. Indeed, when pSEC10-83S is carried by the less attenuated CVD 908*ssb* live vector, immunogenicity of the foreign antigen increases yet again and remains elevated 4 weeks post boost (**group 3 versus group 6**). Here we stress that CVD 908-*htrAssb* and CVD 908*ssb* are isogenic strains, differing only in the deletion of a single *htrA* chromosomal locus. However, identical expression plasmids elicit higher serum antibody responses in the less attenuated CVD 908*ssb* live vector than when carried by CVD 908-*htrAssb*. We hypothesize that any metabolic burden still associated with pSEC10-83S is compensated for in the less attenuated background of CVD 908*ssb*. These data strongly support the hypothesis that the metabolic burden imposed upon a live vector by multicopy expression plasmids can be overcome using less attenuated live vectors to achieve more robust immune responses.

Improvements to techniques of antigen expression

If one succeeds in engineering expression plasmids in which non-antibiotic selection systems coupled with inheritance functions improve plasmid retention, little is gained without consideration of the potential metabolic impact of foreign antigen expression. Techniques to minimize the potential toxicity of foreign antigen expression have focused on regulated expression of foreign antigens and export of antigens out of the cytoplasm of live vectors.

Regulated expression of multicopy foreign antigen cassettes reduces the overall metabolic burden imposed by multicopy expression plasmids, thereby improving the fitness and immunogenicity of a vaccine construct. Such controlled expression can be achieved either at the level of transcription, translation, or both. Chatfield et al⁸ demonstrated that constitutive expression in *S. Typhimurium*, using the powerful *tac* promoter [*P_{tac}*] to express tetanus toxin fragment C (frag C) from a multicopy plasmid, resulted in undetectable fragC-specific antibodies after oral immunization of mice. Isogenic expression using the re-engineered *nirB* promoter *P_{nir15}*, which responds to the environmental signal of low oxygen, elicited antitoxin antibodies that protected orally immunized mice against lethal tetanus toxin challenge. It was further shown that plasmids carrying the constitutive *P_{tac}*-fragC cassette were rapidly lost *in vivo* from bacteria colonizing deep tissues⁸. This concept of using “*in vivo* - induced” expression cassettes to enhance the stability of multicopy plasmids was soon expanded to include eukaryotic antigens from parasites such as *Schistosoma*⁵² and *Leishmania*⁵³. Bumann⁵⁴ demonstrated that regulated expression of foreign antigens strongly influences the immunogenicity of live vectors by affecting colonization levels within the host. When comparing constitutive expression of green fluorescent protein (GFP) fusions from *P_{tac}* versus regulated expression from the PhoQ/PhoP-controlled *P_{pagC}*

promoter, Bumann showed that while both constructs induced comparable cellular immune responses ~1,000-fold lower doses were needed if the antigen was expressed from P_{pagC} .

Optimization of *in vivo* expression levels does not necessarily ensure appropriate antigen-specific immune response.⁵⁵ Problems with inherent antigen toxicity within the cytoplasm of live vectors may diminish the colonizing ability of live vectors and lower the levels of antigen delivered to immunological inductive sites. In addition, proper folding may be required for conformationally specific epitopes to trigger protective serum antibody responses. In attempts to address potential toxicity and protein folding problems, various antigen export technologies have been developed for periplasmic, extracellular, and surface expression of foreign proteins.

Kang et al⁵⁶ used a secretion signal sequence from β -lactamase to dramatically improve the immunogenicity of the pneumococcal surface protein A (PspA) from *S. pneumoniae* delivered by attenuated *S. Typhimurium* strains carrying a multicopy Asd-stabilized expression plasmid. Interestingly, for these plasmid constructs, expression of Asd in this balanced lethal plasmid maintenance system was intentionally reduced by removing the wild type promoter of *asd*, functionally reducing synthesis of Asd to theoretically enhance retention of more copies of the expression plasmid⁵⁷. The β -lactamase signal sequence allowed PspA fusions to be secreted out of the original site of synthesis in the cytoplasm and into the periplasmic space, improving the humoral immunogenicity of this fusion protein 10,000-fold versus unfused cytoplasmic PspA in mice orally immunized with a single dose of live vector⁵⁶. The improved immunogenicity may have resulted either from better folding of immunogenic epitopes in the periplasmic space, reduced metabolic burden associated with over-expression of the foreign protein, or a combination of these factors. When this technology was merged with the strategy of using live vector strains with conditional attenuation, excellent secretion-dependent protection was achieved in mice orally immunized with a single dose of live vector and challenged 6 weeks later with 100 lethal dose units (LD₅₀) of *S. pneumoniae*⁵⁸.

Extracellular export of heterologous antigens into the extracellular milieu of *Salmonella* live vectors has also been reported by several groups to enhance the immune response to a foreign protein. Hess and colleagues⁵⁹ reported that cytoplasmic expression of the protective T-cell antigen listeriolysin O (LLO) within recombinant *Salmonella* vaccine strains did not confer protection in mice against lethal challenge with virulent *L. monocytogenes*. However, in-frame insertion of LLO within a truncated form of the *E. coli* hemolysin A (HlyA) allowed extracellular secretion of this fusion in the presence of the co-expressed *E. coli* HlyB/HlyD/TolC export apparatus, and resulted in protection against lethal challenge with *L. monocytogenes*. Similar results have been reported by other groups using type III secretion systems encoded either by *Salmonella* pathogenicity islands 1 (SPI-1)^{60,61} or SPI-2⁶² to elicit protection using secreted antigens from both prokaryotic and eukaryotic pathogens.

A novel antigen export system has also been described that is derived from a cryptic hemolysin encoded by *clyA* within the chromosome of *S. Typhi* vaccine strains CVD 908-*htrA*⁶³ and Ty21a⁶⁴. The molecular biology of cytolysin A (ClyA) from *S. Typhi* is well characterized^{64–67}, and it has been conclusively demonstrated that ClyA is not secreted by any of the known secretion pathways, but is instead exported via outer membrane vesicles⁶⁸. Such a mechanism for vesicle formation raises the intriguing possibility of engineering ClyA to export antigens out from live vectors that are otherwise potentially toxic when expressed cytoplasmically; these vesicles would also carry lipopolysaccharide (LPS) which might improve the immunogenicity of a foreign antigen.

This ClyA-mediated export technology has been successfully applied to the development of *S. Typhi*-based vaccines carrying antigens from prokaryotic and eukaryotic organisms^{35,69,70}. The usefulness of this system has been extensively demonstrated using genetic fusions of ClyA to the cell binding Protective Antigen (PA83) subunit of *Bacillus anthracis* anthrax toxin. Delivery of ClyA-PA83 protein fusions by the licensed typhoid vaccine strain Ty21a to mice⁷⁰ and by candidate vaccine strain CVD 908-*htrA* to mice and monkeys³⁵ by priming mucosal immunization allowed high titers of toxin neutralizing antibodies to be achieved when animals were boosted parenterally with the licensed Biothrax® anthrax vaccine. The biological relevance of antitoxin responses against ClyA-PA83 was proven in work carried out with *S. Typhimurium*; mice were protected against a lethal aerosol anthrax spore challenge when orally immunized with live vectors that expressed ClyA-PA83 but not when live vectors delivered PA83 fused to *E. coli* HlyA⁷¹. It is clear therefore that the regulated expression of foreign antigens in the appropriate bacterial compartment can have a profound impact on immunogenicity.

Antigen expression by eukaryotic target cells

An interesting alternate approach, which theoretically avoids toxicity issues with antigen production by the live vector, involves the use of DNA vaccines in which antigen expression is controlled by plasmid-based eukaryotic promoters and ribosome-binding sites. With this approach, the purpose of the live vector is to invade eukaryotic target cells and release the DNA vaccine upon lysis of the strain. The DNA vaccine is then taken up by the eukaryotic target cell and is transcribed and translated to produce the intended foreign antigen.

Salmonella delivering DNA vaccines have been shown to induce cellular and humoral immune responses against bacterial pathogens⁷² and their toxins⁷³, viruses^{74,75}, and against tumor antigens^{76,77}. Although this phenomenon was first reported over a decade ago by Sizemore et al.^{78,79}, the exact mechanisms involved in DNA delivery and immunological priming remain unknown. This lack of understanding has severely hampered efforts to improve the efficiency of the technique⁸⁰.

It is readily appreciated that the efficiency of DNA vaccine delivery will explicitly depend on retention of DNA vaccines within invading live vectors prior to their delivery into target cells. Although plasmid maintenance functions would be expected to improve DNA vaccine retention, this approach has yet to be tried. However, the impact of copy number on DNA vaccine stability within live vectors has been systematically examined by Gahan et al⁸¹, who reported that very high copy number pUC-based plasmids are unstable in attenuated *S. Typhimurium* strains (both *in vitro* and *in vivo*), and that lower copy DNA vaccines induce antigen-specific antibody responses after oral immunization of mice. When antigen-specific immune responses were compared for an *S. Typhimurium* live vector strain delivering tetanus toxin fragment C from a conventional medium copy prokaryotic expression plasmid versus the same strain carrying the identical plasmid backbone with a eukaryotic expression cassette (ie a DNA vaccine), toxin-specific serum antibody responses were significantly higher using prokaryotic plasmids versus DNA vaccines⁸¹.

The work of Gahan et al^{81,82} shows that the use of DNA vaccines cannot simply rely on plasmid genetics previously developed for prokaryotic expression systems, but rather may require development of novel approaches to achieve desired immunity. Support for this point comes from recent work by Pasetti et al⁸³ who developed a DNA vaccine against measles that contained the nonstructural protein gene sequences from a human Sindbis virus that would allow direct cytoplasmic amplification within infected eukaryotic cells of RNA encoding the measles hemagglutinin and fusion proteins. When Sindbis virus-derived DNA vaccines encoding the H antigen were introduced into *S. Typhi* and administered to cotton rats, neutralizing measles antibodies and protection against respiratory measles challenge were demonstrated⁸³.

OPTIMIZING IMMUNIZATION STRATEGIES

The type of immune response elicited by recombinant antigens expressed by live vectors depends both on the capacity of the bacteria to gain access to the appropriate immune cells (e.g. antigen presenting cells; APC) and the characteristics and efficacy of the ensuing antigen presentation. In this regard, the propensity of *Salmonella* to target M cells overlying gut-associated lymphoid tissue and gain access to dendritic cells (DC), macrophages and other cells is an inherent advantage to the use *Salmonella* as a live vector for delivery of foreign antigens to APC. The accessibility of sufficient amounts of antigen in the appropriate bacterial cell compartment(s) is likely to directly influence the nature and strength of the immune responses elicited. Therefore, depending on whether humoral or CMI to the foreign antigen is deemed desirable to provide protection against the pathogen from which the foreign antigen was derived, delivering antigen to the appropriate bacterial and APC cellular compartment(s) becomes critical. In fact, antigens expressed on the bacterial cell surface, or exported from the bacterial cell, have been shown to enhance both humoral⁵⁶ and CMI responses.⁵⁹ Moreover, advances in regulation of antigen expression and delivery technology have allowed the targeting of APC cellular compartments that favor stimulation of CMI. For example, hemolysin-mediated (via HlyA) antigen secretion into the phagosome was shown to enhance priming of CD4 and CD8 T cell responses.⁶⁰ Another strategy involves the use of Type III secretion systems (TTSS), which are virulence factors that Gram-negative bacteria use to translocate proteins into the cytoplasm of eukaryotic host cells. *Salmonella* pathogenicity island 1 (SPI1), a TTSS, which activates on contact with host cells, is expressed in growth conditions that are consistent with the intestinal environment and promotes phagocytosis of the bacteria. SPI2, another TTSS that is induced when the bacterium is phagocytosed, promotes intracellular growth and survival within APC and other cells. Thus, SPI2 proteins are more likely to be processed endogenously by an infected APC, leading to expression of antigenic epitopes restricted predominantly by MHC class I (and perhaps even class II) molecules. Indeed, a few of these SPI2 proteins have been already used as carrier molecules based on this hypothesis. A chimeric antigen-SspH2 protein induced both antigen specific CD4 and CD8 T cells, while antigen-SifA induced CD8 specific T cell responses.⁸⁴ Hussein et al.⁶² used the SPI2 effector protein SseF to efficiently deliver *Listeria monocytogenes* antigen and observed protection against wild type *Listeria monocytogenes* in vaccinated animals. Studies with *S. Typhimurium* live vectors by Russmann et al. also showed that SPI-1 and SPI-2-mediated antigen delivery into the cytoplasm of APC enhances CMI.^{60,85,86} SPI-1-dependent translocation of *Listeria* peptides into the APC cytosol led to efficient MHC I-restricted antigen presentation demonstrated by IFN- γ production and cytotoxic responses by peptide-specific CD8 T cells, which conferred protection against lethal challenge with wild type *Listeria*.⁶⁰ Similarly, SPI-2-mediated antigen delivery resulted in efficient priming of central and effector memory CD8 T cells.⁸⁵ Because of the efficiency in priming T cells, this live vector-based strategy has been explored for treatment of tumors.⁸⁷ Future studies in primates and eventually in humans will be required to prove the validity of these approaches.

Immune responses elicited by oral vaccination with attenuated *S. Typhi* in humans: support for the use of *S. Typhi* as live vector vaccines

Recent advances in the identification, characterization and measurement of memory T and B cell subsets, the hallmark of adaptive immunity, have the potential to accelerate vaccine development by enabling more accurate predictions of the effectiveness of vaccine candidates based on the type of memory induced and the longevity of these responses. In this regard, we and others^{4,7,19,88–96} have started to evaluate the relevance of these measurements in T and B cells, including the various memory T cell subpopulations. Over the past 15 years, we have studied the immune responses elicited in orally immunized

subjects given different attenuated *S. Typhi* live vaccines, with an emphasis on measuring CMI. We showed that immunization with attenuated *S. Typhi* strains, including Ty21a, CVD 906, CVD 908, CVD 908-*htrA* and CVD 909, resulted in the appearance in peripheral blood of sensitized lymphocytes that exhibit significantly increased proliferative responses and Th1-type cytokine production patterns (i.e., IFN- γ , TNF- α , etc., in the absence of interleukin 4 [IL-4] and IL-5) to *S. Typhi* antigens.^{7,19,88–94} Moreover, CD3⁺ CD4⁺ CD8[–] CD56[–] T helper cells were identified as the predominant IFN- γ -secreting effector cell population responding to soluble *S. Typhi* antigens.⁹¹ The observed Th1-type responses are likely important in resistance to *S. Typhi* by a variety of mechanisms, including augmentation of macrophage bactericidal activity and enhancement of antigen presentation. In addition, we observed that immunization with attenuated *S. Typhi* vaccine strains elicited CD8⁺ specific cytotoxic T cells (CTL) that were able not only to kill *S. Typhi*-infected targets, but also secrete IFN- γ .^{5,91,94,97}

Regarding humoral responses, oral immunization with attenuated *S. Typhi* elicits gut-derived circulating IgA antibody secreting cells (ASC) that are detected among peripheral blood mononuclear cells (PBMC) approximately 7–10 days after ingestion of live oral typhoid vaccines. These measurements have been used to estimate the degree of priming of the local intestinal immune system.^{12,19,92,93} These cells are believed to eventually home to the lamina propria of the intestinal mucosa and other mucosal sites where they will synthesize and release SIgA antibody.

Advances in understanding of lymphocyte homing have enabled us to predict with reasonable accuracy the homing potential of antigen specific lymphocytes in circulation by studying the expression of homing molecules. In fact, selective homing of effector/memory cells to the small intestine is believed to be driven, to a large extent, by the expression of integrin $\alpha_4\beta_7$ and CCR9.^{98–101} It is difficult to obtain tissues from the local microenvironments of vaccinated subjects (e.g., gut in the case of organisms that enter the host via the gastrointestinal tract). Therefore, the ability to determine whether specific B and T lymphocytes in circulation have the potential to home to the appropriate tissues is important in helping to predict the effectiveness of immunity to *Salmonella* and to the unrelated organisms whose antigens are expressed when *Salmonella* is used as a live vector. A study of Ty21a vaccinees suggested that virtually all ASC in blood elicited by oral immunization were integrin $\alpha_4\beta_7^{\text{hi}}$.¹⁰² To take advantage of these recent advances, we developed a sophisticated multichromatic flow cytometric approach (up to 13 colors, 16 parameter/cell) to study concomitantly the phenotypic and functional characteristics of effector populations (e.g., T effector/memory [T_{EM}], T central/memory [T_{CM}], etc.) including expression of homing molecules, intracellular cytokines, etc. These responses are likely representative of those that might be expected in the gut microenvironment, since evidence suggests that circulating active T cells and ASC expressing gut homing molecules are in transit to the gut associated lymphoid tissue (GALT).⁹⁹ Using *S. Typhi*-specific CD8⁺ T cell clones derived from Ty21a vaccinees up to 40 months after immunization, we demonstrated for the first time that *S. Typhi*-specific cells bearing a T_{EM} phenotype (i.e., CCR7[–] CD27[–] CD45RO⁺ CD62L^{low}) and co-expressing high levels of integrin $\alpha_4\beta_7$ and intermediate levels of CCR9 (suggesting that these specific clones have the potential to home to the GALT) remain in circulation for long periods of time.¹⁰³ We have also recently concluded extensive studies to evaluate T_M subsets (and their homing potential), up to 90 days after oral immunization with CVD 909.¹⁰⁴ These studies not only confirmed the remarkable immunogenicity of CVD 909, but also showed that oral immunization with attenuated *S. Typhi* strains elicits diverse *S. Typhi*-specific IFN- γ -secreting CD4⁺ and CD8⁺ T_{CM} and T_{EM} subsets that express, or not, integrin $\alpha_4\beta_7$, i.e., they are able to migrate to the gut, as well as secondary lymphoid tissues. Moreover, we provided the first demonstration in a bacterial system in humans of the induction of specific CD3⁺ CD8⁺

T_{EMRA} (CD62L⁻ CD45RO⁺) subsets, widely regarded as the most active effector cells in viral systems.¹⁰⁴

This panoply of effector CMI and antibody responses, as well as the homing characteristics of the antigen specific lymphocytes elicited in humans, makes *Salmonella* an attractive vector to carry foreign antigens. *S. Typhi* targets appropriate immunological afferent sites, elicits strong and varied immunity to the foreign antigens by providing bystander effects and other immune enhancing mechanisms, and endows specific lymphocytes with the potential to home to the appropriate effector sites.

Heterologous prime-boost strategy

One emerging vaccination strategy that has proven to be generically effective at enhancing immune responses, particularly against poorly immunogenic antigens encoded by live vectors, is the “heterologous prime-boost” approach.¹⁰⁵ The heterologous prime-boost strategy involves sequential administration of a target antigen, in different vaccine formulations, administered by the same or different routes. This strategy can induce cellular and humoral immune responses that are superior to those achieved using a homologous prime-boost regimen with a given vaccine formulation. A variety of complex mechanisms is undoubtedly involved in controlling the magnitude and breadth of responses induced by prime-boost immunization. However, one central hypothesis holds that administration of the target antigen using two distinct delivery vehicles allows for expansion of antigen-specific memory T and B cells, while minimizing anti-vector immunity that might reduce responses elicited to the foreign antigens upon booster immunization.^{105,106} Upon re-exposure to antigen, primed antigen-specific memory cells expand rapidly, mounting enhanced and broadened anamnestic responses. Moreover, administration of vaccines via the mucosal (live-vector vaccine prime) and parenteral (boost) routes has the theoretical potential to elicit effector immune responses present at high levels in both local (e.g., mucosal microenvironment) and systemic sites.

Initially, this approach was shown to improve immune responses against purified *Plasmodium* and HIV antigens.^{107–111} More recently this technique has been demonstrated to enhance the immunogenicity of live vector-expressed prokaryotic and eukaryotic heterologous antigens. *Salmonella* have been successfully used as mucosally delivered priming agents, followed by subsequent parenteral boosting with the target antigen given as a purified protein in the presence of adjuvant or encoded by a DNA vaccine.

Londono et al.¹¹² first described the use of an *S. Typhi*-based live vector in a heterologous prime-boost regimen. In this study, attenuated vaccine strain CVD 908-*htrA* and its earlier parent strain CVD 908 were engineered to express *Helicobacter pylori* urease from plasmid-borne genes transcriptionally controlled by the stress-regulated promoter P_{*htrA*}. In mice primed i.n. with live vectors, followed by two subcutaneous booster doses of purified urease plus alum, urease-specific IgG responses elicited by the heterologous prime-boost regimen were at least 10-fold higher than in mice receiving purified urease or live vectors alone. A balanced Th1/Th2 response was also observed. Most importantly, partial protection was observed after prime-boost immunization upon intragastric challenge with a mouse-adapted virulent strain of *H. pylori*, whereas mice vaccinated with protein and live-vector alone remained unprotected.¹¹²

Vindurampulle et al.¹¹³ also investigated a heterologous mucosal prime/parenteral boost strategy, using CVD 908-*htrA* expressing tetanus toxin fragment C. Mice primed i.n. with CVD 908-*htrA* expressing frag C and boosted intramuscularly (i.m.) with tetanus toxoid mounted enhanced and more rapid antitoxin responses in comparison to mice primed and boosted with parenteral tetanus toxoid alone.

Live vectors can also successfully prime the immune system to further respond to a DNA vaccine boost. The plasmid-based ClyA antigen export strategy was used to improve the immunogenicity of a problematic eukaryotic antigen derived from *Plasmodium falciparum*.⁶⁹ Mice were primed i.n. with CVD 908-*htrA* exporting ClyA fused to a truncated version of the *P. falciparum* circumsporozoite protein (CSP) expressed by the sporozoite stage of the *P. falciparum* parasite. After boosting intradermally via jet injection with a DNA vaccine encoding a eukaryotic codon-optimized version of CSP, mice developed both CSP-specific IFN- γ -secreting cells and IgG capable of recognizing the native CSP protein in the membrane of *P. falciparum* sporozoites. Attempts to express unfused CSP within the cytoplasm of the *S. Typhi* live vector were unsuccessful.⁶⁹ This report exemplifies a successful combination of refinements in expression technology, use of different vaccine formulations, and improved immunization strategies to induce potent immune responses against an otherwise poorly immunogenic antigen.

Salmonella live vector vaccines used in heterologous prime-boost strategies have overcome the immature immune system of young hosts and have effectively primed the neonatal immune system for potent anamnestic responses to a parenteral antigen boost given later in life. Capozzo et al.¹¹⁴ demonstrated that two 10⁹ cfu doses of CVD 908-*htrA* expressing frag C of tetanus toxin administered to neonatal mice at 7 and 22 days after birth could elicit serum tetanus antitoxin responses in mice, even when they were offspring of tetanus toxoid immunized mothers and the infants had maternal antibodies to tetanus toxin. Expanding on this observation, Ramirez et al.¹¹⁵ primed newborn mice intranasally with a single dose of *S. Typhi* expressing *Y. pestis* F1 antigen and detected mucosal antibodies and IFN- γ secreting cells one week after immunization. The mice developed a potent and rapid anamnestic response to a subsequently administered parenteral boost with F1-alum; the response surpassed those of newborns primed and boosted only with F1-alum or *S. Typhi* delivering F1. Neonatal priming with F1-expressing live vector, as opposed to priming with F1-alum, resulted in a more balanced IgG2a/IgG1 profile, enhanced avidity maturation and stimulation of B memory cells, and strong Th1-type cell mediated immunity. This is a promising approach to immunizing a population unable to respond to conventional vaccines early in life.

HUMAN CLINICAL TRIALS

To our knowledge, there have been only eight published clinical trials of *S. Typhi* live vector constructs (Table 2); collectively, a review of these data is instructive. The first trial established proof-of-principle that *S. Typhi* live vectors can elicit serum, mucosal IgA and CMI responses in humans;⁸⁸ however, only a minority of vaccines responded. The constructs tested were made from six separate live vector strains with different methods of attenuation (one chemical and the others recombinant deletions). Four trials tested *S. Typhi* vectors expressing antigens known to be weak (CSP and urease) and the immune responses were meager for CSP and rare for urease. Fragment C and LT B are known strong antigens and trials with these constructs gave some positive results. The two constructs that incorporated chromosomal integration of foreign antigen genes also gave some positive results.^{88,116} In contrast, the various plasmid stabilization systems overall gave disappointing results. All but two constructs expressed the antigen from constitutive promoters. Notably, each of the inducible promoters regulated expression of a strong antigen, frag C of tetanus toxin under P_{nir15} and LT B under P_{ssaG}. However, only the P_{ssaG}-LT B was immunogenic. In contrast frag C under constitutive control did elicit seroconversion in some subjects. Finally, all but the LT B construct involve cytoplasmic expression of the foreign antigen.

THE FUTURE OF LIVE VECTOR VACCINES

Our summation of results of the clinical trials with *S. Typhi* live vectors leads us to conclude that, heretofore, no trial has tested a robust live vector strain that expresses an antigen of predictable immunogenicity (based on other vaccine or natural infection experience) from a stabilized gene (chromosomal or state-of-the-art plasmid maintenance system), under a suitable promoter and (critically!) with either surface expression or export of the antigen out of the bacteria. Thus, to date, no clinical trial has been performed with an optimum construct. The exciting pre-clinical advances in recent years of *S. Typhi* vector vaccinology presented in this review beg for such a trial to be undertaken in the near future.

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Figure 1.

Genetic maps of isogenic expression plasmids encoding full-length Protective Antigen (PA83) from anthrax toxin genetically fused to the carboxyl-terminus of cytolysin A (ClyA). Unique restriction sites are shown in bold. Abbreviations: *P_{ompC}*, modified osmotically controlled *ompC* promoter from *E. coli*; *clyA*, encodes cytolysin A from *S. Typhi*; *pa83*, encodes full-length anthrax toxin Protective Antigen from *Bacillus anthracis*, codon-optimized for expression in *S. Typhi*; *clyA::pa83*, encodes *pa83* fused to the carboxyl terminus of ClyA; *ori15A*, origin of replication from p15A, providing an expected copy number of ~15 per chromosomal equivalent; *ori101*, origin of replication from pSC101, providing an expected copy number of ~5 per chromosomal equivalent; T1, transcriptional terminator from the *rrnB* rRNA operon of *E. coli*; *par*, passive partitioning function from pSC101; *ssb*, encodes single stranded binding protein; *parM* and *parR*, two loci comprising the *parA* active partitioning system from the multiple antibiotic resistance R plasmid pR1.

Table 1

Anti-PA83 IgG responses in pooled serum from mice primed intranasally with PA83-expressing *S.Typhi* live vectors and subsequently boosted parenterally with purified PA83.

Group	Strain	Plasmid Copy number	Foreign Antigen	SSB?	Day 41 (pre-boost)	Day 49 (1 wk post boost)	Day 55 (2 wks post boost)	Day 70 (4 wks post boost)
1	<i>htrAssb</i> (pSEC91dS)	~15	(-) control	YES	25 [*]	1,105	5,690	41,824
2	<i>htrAssb</i> (pSEC91-83S)	~15	ClyA-PA83	YES	25	2,944	80,710	598,700
3	<i>htrAssb</i> (pSEC10-83S)	~ 5	ClyA-PA83	YES	4,436	314,166	475,045	209,389
4	908 <i>ssb</i> (pSEC91dS)	~15	(-) control	YES	25	25	8,484	206,628
5	908 <i>ssb</i> (pSEC91-83S)	~15	ClyA-PA83	YES	2,631	296,222	630,795	1,180,372
6	908 <i>ssb</i> (pSEC10-83S)	~ 5	ClyA-PA83	YES	18,431	856,485	1,219,323	504,111
7	<i>htrA</i> (pSEC91-83)	~15	ClyA-PA83	NO	443	108,283	108,283	755,215

* Reciprocal titer

Table 2

Summary of salient features of clinical trials with *S. Typhi* live vectors expressing foreign antigens

Live vector vaccine	Foreign antigen	Immunization schedule, route and dose (cfu per dose)	Gene stabilization	Promoter	Location in bacterial cell of expressed antigen	No. subjects	Immune responses to the foreign antigen			Ref.
							Serum Antibodies	Mucosal antibodies or IgA antibody-secreting cells	CMI responses	
CVD 908so(Δ arcC1019::p <i>tac</i> -csp)	<i>Plasmodium falciparum</i> circumsporozoite protein (CSP)	2 oral doses (5×10 ⁷ cfu), 8 days apart	Chromosomal integration into Δ arcC	Constitutive (P <i>tac</i>)	Cytoplasm	10	2 of 10 developed serum antibodies to sporozoite or CSP antigens	4 of 7 tested had 4-fold rise in jejunal SIgA antibodies to CSP	CTL to targets expressing CSP, 1 of 10	88
Δ cva Δ csp-cdtA Δ sd <i>S. Typhi</i> χ 4632pYA3167) expressing hepatitis B virus core pre-S protein	Hepatitis B virus core pre-S protein	3 doses (3×10 ⁸ , 3×10 ⁹ and 1×10 ⁹ cfu, respectively on days 1, 10 and 42) given either orally or rectally	Plasmid with <i>asd</i> /balanced lethal stabilization system	Constitutive	Cytoplasm	7 oral 6 rectal	0 of 7 oral vaccinees 1 of 6 rectal vaccinees developed a rise in serum IgG anti-pre-S1 antibodies	0 of 7 oral 0 of 6 rectal	No data	117
Δ cva Δ csp-cdtA Δ sd <i>S. Typhi</i> χ 4632pYA3167) expressing hepatitis B virus core pre-S protein	Hepatitis B virus core pre-S protein	1 oral dose (3×10 ⁷ or 7×10 ⁸ cfu)	Plasmid with <i>asd</i> /balanced lethal stabilization system	Constitutive	Cytoplasm	10 (5 at each dosage level)	No significant rises in serum antibody to the foreign antigen	No significant rises in mucosal antibody to the foreign antigen	No data	118
Δ phoPphoQ Δ purB <i>S. Typhi</i> strain Ty1033 expressing <i>Helicobacter pylori</i> urease A & B	<i>Helicobacter pylori</i> urease A & B subunits	1 oral dose (10 ¹⁰ cfu); 1 subject received 2 doses 3 months apart	PurB balanced lethal plasmid maintenance system	Constitutive (P <i>cztB</i>)	Cytoplasm	8 (7 got 1 dose & 1 got 2 doses); 3 were boosted orally with 60 mg of recombinant H. pylori urease plus 2.5 μ g of <i>E. coli</i> LT as adjuvant	No significant rises in serum antibody to the foreign antigen	No significant rises in mucosal antibody to the foreign antigen	No data	119
CVD 908- <i>htrApTET1pp</i> or CVD 908- <i>htrApTET1prt15</i>)	Fragment C of tetanus toxin	Study #1: 17 subjects with baseline levels of serum tetanus antitoxin were given a single oral dose of 2×10 ⁷ or 3×10 ⁸ cfu of CVD 908- <i>htrApTET1pp</i> or 9×10 ⁷ cfu of CVD 908- <i>htrApTET1prt15</i> ; Study #2: 3 seronegative subjects (tetanus antitoxin < 0.01 IU/ml) ingested a single dose of 5×10 ⁹ cfu (N=2) or of 8×10 ⁹ cfu (N=1) of CVD 908- <i>htrApTET1pp</i>	Unstabilized plasmids	Constitutive (P <i>1pp</i>) or anaerobically induced (P <i>prt15</i>)	Cytoplasm	17 seropositive subjects (most with moderate or high levels of serum tetanus antitoxin); 3 tetanus seronegative subjects	2 of 17 subjects with tetanus antibodies at baseline mounted four-fold or > rises in serum tetanus antitoxin (both had low baseline serum antitoxin titers and received the 3×10 ⁸ cfu dose of CVD 908- <i>htrApTET1pp</i>); 0 of 2 seronegative subjects had rises following ingestion of 5×10 ⁹ cfu of CVD 908- <i>htrApTET1pp</i> ; The 1 seronegative subject who got 8×10 ⁹ cfu of CVD 908- <i>htrApTET1pp</i> mounted a 43-fold rise in serum tetanus antitoxin	Mucosal antibodies to the foreign antigen were not measured	19 of 20 subjects exhibited proliferative responses to tetanus toxoid	120
<i>thyA</i> Ty21a(pDB1)	<i>Helicobacter pylori</i> urease	3 oral doses (6×10 ⁹ , 7×10 ⁹ and 9×10 ⁹ cfu, respectively on days 0, 2 and 4)	ThyA balanced lethal plasmid stabilization system	Constitutive	Cytoplasm	9	No significant rises in serum antibody to the foreign antigen	No foreign antigen-specific IgA ASCs	1 subject had a proliferative and an interferon gamma response	121
<i>thyA</i> Ty21a(pDB1)	<i>Helicobacter pylori</i> urease	9 subjects ingested 3 doses of Ty21a (10 ⁹ cfu, every other day interval) followed 2 months later by Ty21a(pDB1) (6×10 ⁹ , 7×10 ⁹ and 9×10 ⁹ cfu, respectively on days 0.2 and 4); 4 subjects who had received three doses of Ty21a(pDB1) 12 months earlier were re-immunized with the same 3-dose regimen; 3 subjects received only 3 doses of Ty21a(pDB1) by the same regimen	ThyA balanced lethal plasmid stabilization system	Constitutive	Cytoplasm	9 got Ty21a followed by Ty21a(pDB1); 4 got Ty21a(pDB1) booster 12 months after initial oral vaccination with Ty21a(pDB1); 3 got only Ty21a(pDB1)	No rises in serum antibody to urease	Mucosal antibodies were not measured	7 subjects had proliferative responses to urease antigen; 2 subjects had significant interferon-gamma responses	122
Δ arcC Δ ssaV attenuated <i>S. Typhi</i> strain M01Z109 expressing <i>E. coli</i> LT B subunit	<i>E. coli</i> LT B subunit	12 subjects ingested 2 doses (10 ⁸ cfu) of the live vector 56 days apart; 24 subjects ingested 2 doses (10 ⁹ cfu) of the live vector 56 days apart	Chromosomal integration in Δ arcC	Inducible SP12 (P <i>ssaG</i>)	Periplasm	Total of 36 (12 low dose, 24 high dose)	Rises in serum IgG anti-LT followed in 42% of subjects following the first dose of either 10 ⁸ (5/12) or 10 ⁹ cfu (10/24); After 2 doses, 22/36 had rises in anti-LT (61%)	Mucosal antibodies were not measured; 11% of subjects IgA ASCs were induced on days 7, 11 & 14 after each dose of vaccinee)	No data	116