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Review

Animal models paving the way for clinical trials of attenuated *Salmonella enterica* serovar Typhi live oral vaccines and live vectors

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Abstract

Attenuated *Salmonella enterica* serovar Typhi (*S.* Typhi) strains can serve as safe and effective oral vaccines to prevent typhoid fever and as live vectors to deliver foreign antigens to the immune system, either by the bacteria expressing antigens through prokaryotic expression plasmids or by delivering foreign genes carried on eukaryotic expression systems (DNA vaccines). The practical utility of such live vector vaccines relies on achieving a proper balance between minimizing the vaccine's reactogenicity and maximizing its immunogenicity. To advance to clinical trials, vaccine candidates need to be pre-clinically evaluated in relevant animal models that attempt to predict what their safety and immunogenicity profile will be when administered to humans. Since *S.* Typhi is a human-restricted pathogen, a major obstacle that has impeded the progress of vaccine development has been the shortcomings of the animal models available to assess vaccine candidates. In this review, we summarize the usefulness of animal models in the assessment of the degree of attenuation and immunogenicity of novel attenuated *S.* Typhi strains as vaccine candidates for the prevention of typhoid fever and as live vectors in humans.

Keywords: *Salmonella*; Live vector vaccines; Animal models

1. Introduction

It has been unequivocally established that attenuated *Salmonella enterica* serovar Typhi (*S.* Typhi) strains can serve as safe and effective oral vaccines to prevent typhoid fever [1–4]. It has also been proposed that attenuated *S.* Typhi strains can function as live vectors to deliver foreign antigens to the immune system, either by the bacteria expressing antigens through prokaryotic expression plasmids or by delivering foreign genes carried on eukaryotic expression systems (DNA vaccines) that are activated in mammalian antigen-presenting cells (APCs) [5–7]. The practical utility of such live vector vaccines relies on achieving a proper balance between minimizing the vaccine's reactogenicity and maximizing its immunogenicity. The background of the live vector strain, the type of mutation selected to achieve attenuation, the appropriate expression of the foreign antigen and the efficiency of DNA delivery, are among the factors that appear to be crucial in determining the extent and quality of the immune response elicited [8].

To advance to clinical trials, vaccine candidates need to be pre-clinically tested in relevant animal models that attempt to predict what their safety and immunogenicity will be when administered to humans. Since *S.* Typhi is a human-restricted pathogen, a major obstacle that has impeded the progress of vaccine development has been the shortcomings of the pre-clinical animal models available to assess vaccine candidates. In this review, we summarize the role of animal models in the assessment of the degree of attenuation and immunogenicity of a new generation of attenuated *S.* Typhi strains that have the potential to be used as live oral typhoid vaccines and as live vectors in humans.

2. Typhoid fever: pathogenesis and immunity

S. Typhi is estimated to cause each year approximately 16 million cases of typhoid fever and 600,000 deaths [9]. Much of our knowledge of the pathogenesis of human typhoid infection comes from observations made in clinico-pathologic studies, mainly carried out in the first half of the 20th century, and in the course of experimental challenge studies in healthy adult volunteers carried out in the 1950s and 1960s [10] to estimate the efficacy of licensed typhoid vaccines and of candidate vaccines under development (reviewed in [11,12]). In addition, two animal models have contributed significantly to our understanding of the pathogenesis of

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human typhoid infection: oral challenge of chimpanzees with virulent *S.* Typhi [13,14] and oral or systemic challenge of mice with *S. enterica* serovar Typhimurium (*S.* Typhimurium) or serovar Enteritidis (*S.* Enteritidis) [15,16]. We will first briefly summarize how these two animal models have helped to unveil the steps involved in the pathogenesis of human typhoid fever and discuss their assistance in the development of *S.* Typhi vaccine candidates.

2.1. Insights from the chimpanzee model

Studies performed at the Walter Reed Army Institute of Research [13,14] showed that chimpanzees infected with *S.* Typhi by the orogastric route develop a clinical illness that resembles human typhoid fever. Wild type *S.* Typhi strain Ty2 given in doses of 1×10^{11} colony forming units (CFU) produced a mild clinical illness in which most of the animals developed bacteremia and exhibited fever for up to 10 days, and excreted bacteria in their stools [13]. Differences between clinical typhoid infection in chimpanzees and humans include the shorter incubation period followed by a milder and briefer clinical course in the animal model [13]. Following invasion of the intestinal lining, typhoid bacilli were subsequently detected in aggregate intestinal lymphoid follicles and draining mesenteric lymph nodes [14]. From these sites bacilli spilled into the thoracic lymph and then into general circulation. *S.* Typhi was found in the liver and spleen, as well as in the bile and gallbladder; bile containing bacteria resulted in re-infection of the intestinal mucosa [13]. *S.* Typhi carriage in the gallbladder was also documented in rabbits following intravenous inoculation [17–19]. Pathological examination of infected chimpanzees revealed granulomatous lesions in intestinal and mesenteric lymphoid tissues, spleen and liver, just like those seen in human typhoid infection. The chimpanzees mounted serum antibody responses to *S.* Typhi LPS and flagella [13].

2.2. Insights from the mouse typhoid model

S. Typhimurium causes a lethal disease in susceptible rodents that in a number of ways resembles human typhoid infection. *S.* Enteritidis produces a similar systemic infection. Also known as the “mouse typhoid model”, *S.* Typhimurium and *S.* Enteritidis infection in mice has been adopted and extensively used as a model to study pathogenesis and immunity of typhoid fever (reviewed in [20–25]). The information obtained in this model has greatly advanced our understanding of *Salmonella*–host interactions, innate resistance and acquired immunity [20,26–29]. It has also helped to identify and determine the role of numerous virulence mechanisms such as the sophisticated type III secretion systems (TTSS) encoded by *Salmonella* pathogenicity island (SPI)-1 and SPI-2, among others [28–31]. Furthermore, it has assisted in the design of molecular strategies to obtain attenuated mutants derivatives, and the evaluation of immunogenic properties of recombinant vaccine strains. The molecular pathogenesis of *Salmonella* infection in mice and the characteristics of the immune responses induced have been extensively reviewed elsewhere [20–33]. We will briefly summarize the more salient features of the mouse typhoid model to lay the ground for a detailed discussion of its advantages and limitations to assist *S.* Typhi vaccine development.

After oral infection, *S.* Typhimurium colonizes the small intestine and enters the Peyer’s patch (PP) through the M cells [34] by means of bacteria–host cell interaction that involves the TTSS [20,28–31,33]. *Salmonella* may disseminate beyond the intestinal epithelial barrier after being taken by migrating CD18-expressing phagocytes [35]. Others have suggested that dendritic cells (DC) that breach the gut intestinal epithelial barrier and sample intestinal bacteria might also contribute to the transport of non-invasive *Salmonella* from the intestinal lumen, in an alternative route to M cell invasion [36,37]. Once across the intestinal epithelium, the bacteria disseminate via the lymphatics into the bloodstream, seeding spleen, liver and bone marrow. Bacteria in the bloodstream are removed by phagocytes in the reticuloendothelial system organs [20]. Subsequent to the initial spread, *S.* Typhimurium replicates in PP of the terminal ileum as well as in mesenteric lymph nodes, liver and spleen [15], likely within macrophages [38,39]. These first stages, which are normally completed within a few hours, are followed by several days during which the bacteria replicate and re-enter the systemic circulation in the form of a secondary bacteremia [15].

Both innate and acquired immunity contribute to the responses against *S.* Typhimurium infection in mice (reviewed in [8,21,23,25]). During the initial stages of infection, bacterial components such as LPS, lipoproteins and flagella—agonists of Toll receptors—as well as specific bacterial proteins delivered by *Salmonella* through the SPI-1 and SPI-2 TTSS [28,33] induce a massive pro-inflammatory response and recruit activated macrophages and neutrophils to eliminate the bacteria. An array of pro-inflammatory and immunoregulatory cytokines has also been described in mice infected with *S. enterica* serovar Dublin (*S.* Dublin) [40]. Although innate mechanisms constitute the primary line of defense restricting the initial bacterial multiplication, they fail to achieve sterile immunity. The generation of specific T- and B-cell responses results in the effective clearance of the bacteria and protection from subsequent infection.

It has been established that *S.* Typhimurium is taken up by macrophages and DCs from bone marrow, spleen, mesenteric lymph nodes and PP, and both cell types appear to play a critical role in antigen presentation [37,41–44]. Both $CD4^+$ and $CD8^+$ T cells have been found to play an important role in protection [23,25] and a number of studies have shown the induction of Th1-type responses, characterized by the production of IFN-γ, IL-12 and TNF-α [45–47].

The role of antibodies and B-cell responses in the protective immunity to murine typhoid infection has been controversial [48]. Studies provide evidence that B cells play an

important—though not exclusive—role in the protection against systemic infection with virulent *S.* Typhimurium [49–51]. Moreover, B cells appear to strengthen T-cell responses and this "cross-talk" between humoral- and cell-mediated immunity is believed to be critical in host defense against systemic infection [8,25,52]. *Salmonella* challenged mice elicit antibody responses to a large number of bacterial antigens including LPS, flagella, fimbriae, porins, and bacterial proteins (i.e. outer membrane proteins (OMPs), lipoproteins and heat-shock proteins). T-cell responses against bacterial proteins and flagella have also been described [8]. Their contribution in protection, however, is not completely understood. The protective role of *Salmonella* virulence (Vi) antigen has been demonstrated using the mouse typhoid model. A recombinant *S.* Typhimurium Vi4072 which had the ability to produce Vi capsular polysaccharide induced serum and secretory antibody responses to Vi antigen and delayed type hypersensitivity following footpad injection. Immunization with Vi4072 at 5×10^7 CFU afforded complete protection against intraperitoneal (i.p.) challenge with virulent Ty2, whereas same doses of Vi negative strain failed to protect [53]. *Salmonella* OMPs [54] and porins [55] also appear to contribute to protection against typhoid infection, as documented in the mouse model.

One caveat of the typhoid murine model is that resistance or susceptibility is directly linked to the innate host response and largely dependent on the host-genetic background [56]. For example, mouse strains that express the immunity to typhimurium-resistant (*Ity*^{r}) allele are relatively resistant to *Salmonella* infection whereas strains that express the *Ity*^{s} (sensitive) allele are susceptible [57]. A gene product encoded within the *Ity*/*Bcg*/*Lsh* locus, a pH-dependent transporter expressed in the phagosomal membrane known as natural-resistance-associated macrophage protein 1 (Nramp-1), mediates such resistance by enhancing bactericidal activity, cytokine production and antigen presentation [58] in resident peritoneal and splenic macropohages, where it is exclusively expressed [59,60]. Other genes known to influence experimental infection include *Lps*^{d}, *xid*, *LBP*, CD14 and the Toll-like receptor (*Tlr*) 4, which has gained considerable attention recently [56,61,62]. TLR4 mediates LPS recognition and signaling, therefore regulating innate responses to bacterial LPS [62]. Endotoxin-tolerant mice with mutations in *Tlr4* are hyporesponsive to LPS and, as a consequence, extremely susceptible to *Salmonella* infection [63,64]. Both the *Nramp1* and *Tlr4* genes are critical during early control of bacteria replication. Mayor histocompatibility complex (MHC) class II antigens also appear to be involved in the clearance of *Salmonella* in mice [25,65]. These findings raise the question of which mouse strain better resembles human infection. Although most research was performed in susceptible mice, some investigators contend that resistant mice might be closer to humans [66].

Albeit *S.* Typhimurium and *S.* Enteritidis have been the serovars most extensively used in the murine typhoid model, other serovars such as *S.* Dublin have also been utilized to study pathogenesis and immunity in infected mice [40,67,68].

2.3. An understanding of the pathogenesis and immunity of human typhoid infection based on a composite of data from human and animal model infections

Information gleaned from natural disease and experimental infections in humans, and from animal models allows a composite model of human typhoid infection to be described (reviewed in [11,12,69]). Following ingestion of contaminated food or water vehicles containing typhoid bacilli, the M cells overlying the PP of the small intestine, as well as specialized phagocytic cells, provide the portal of entry for the bacteria. In successive steps the bacilli reach draining lymph nodes, enter the lymph and then the blood circulation, and are filtered by fixed macrophages in the organs of the reticuloendothelial system including the liver, spleen and bone marrow [10,13,14]. Bacilli are believed to replicate within intracellular compartments of these macrophages. During this primary bacteremia, typhoid bacilli reach the gallbladder and the bile, which leads to a re-seeding of the intestinal mucosa as bile is discharged into the intestine, as documented in patients with acute typhoid fever [70,71]. The end of the incubation period, which usually varies from 8 to 14 days (in part depending on the dose ingested), is followed by the onset of fever (which increases in step-wise fashion), headache and abdominal discomfort, accompanied by a low level bacteremia. Individuals with abnormal gallbladder mucosa from cholelithiasis or other underlying gallbladder disease may become chronic biliary carriers of *S.* Typhi following acute infection [12]. In some individuals, typhoid infection remains sub-clinical. It is reasonable to speculate that host-genetic mechanisms play a key role in controlling immunity or immunopathology related to typhoid infection. Supporting this hypothesis, a recent study demonstrated a significant association between susceptibility to typhoid fever and genes of the class II and class III MHC loci on human chromosome 6—alleles of HLA-DR and HLA-DQ—and the gene encoding TNF-α [72]. An association between MHC class II antigens and susceptibility had also been described in the murine typhoid model [65]. However, in contrast to the murine model, susceptibility to typhoid fever in humans does not appear to be related to Nramp-1 [73].

Specific immune responses to *S.* Typhi, e.g. serum IgG and IgA against bacterial LPS and flagellar (H) antigens [12,74], gut secretory IgA [75], and cell-mediated immunity [12,76], have been demonstrated in patients with typhoid fever. Chronic carriers display high titers of serum anti-Vi antibodies [77]. In contrast, only 20% of patients with acute typhoid fever manifest significant rises in serum Vi antibodies [77]. The relative contributions of cellular and humoral immunity in giving rise to infection-derived immunity remains a matter of controversy, as correlates of protection have not been clearly defined.

3. Attenuated *Salmonella* strains as live oral vaccines and live vectors

3.1. The need for a typhoid vaccine and the potential advantages of S. Typhi as a live vector

In the developing world, typhoid fever remains a disconcerting public health problem because of the emergence of strains resistant to previously useful antibiotics. As a result, the global incidence of severe and fatal disease is increasing, thereby intensifying interest in new typhoid vaccines as possible control measures [11]. Three populations are at particularly high risk of developing typhoid fever and would benefit from immunoprophylaxis with a safe, effective, inexpensive, and practical vaccine. These include children in endemic areas, travelers and military personnel from industrialized countries who visit endemic areas in developing countries and clinical microbiological technicians [12].

The history of typhoid vaccination has been extensively reviewed elsewhere [78,79]. Inactivated whole-cell vaccines were effective but unacceptably reactogenic for public vaccination. As of now two types of vaccines lead the way for the prevention of typhoid fever: purified Vi polysaccharide vaccine [80] and attenuated *S.* Typhi strain Ty21a live oral vaccine [2]. Tested extensively in the field, Vi polysaccharide vaccines are safe and immunogenic. Protection is mediated by high levels of serum Vi antibodies [80,81]. A new generation of Vi polysaccharide–protein conjugate vaccines is being tested [82]. In contrast, IFN-γ production and cytotoxic activity by sensitized T cells, as well as antibodies to *S.* Typhi LPS and flagella, are likely among the protective effector immune responses induced by live attenuated *S.* Typhi [83–86]. Ty21a has been evaluated in several clinical trials and found to be well tolerated although only modestly immunogenic, requiring three to four doses to confer protection [1–4].

The demonstration that oral typhoid vaccine strain Ty21a is safe and efficacious in preventing typhoid fever, spurred research to develop other attenuated strains with defined genetic mutations that would be as safe and well tolerated as Ty21a but considerably more immunogenic, such that a single oral dose would elicit protective immunity [78,79]. This has been considerably boosted by the recent progress in the understanding of the genetics of *Salmonella* virulence as well as recombinant DNA technology. Live vaccines are particularly attractive because they can be administered orally, avoiding the risk of parenteral injection; they are able to induce cell-mediated immunity in addition to antibody responses and since they are delivered through mucosal surfaces they have the potential to induce mucosal responses at multiple local and distant sites as well as systemic responses. Attenuated *Salmonella* strains can also be used for expression of heterologous antigens/proteins that can be delivered to the immune system. Furthermore, *Salmonella* can transfer plasmids encoding foreign antigens under control of eukaryotic promoters (DNA vaccines) to APCs, resulting in targeted delivery of DNA to these cells. The possibility of using *Salmonella* as a live vector to express heterologous antigens or deliver them in genetic form, in a multivalent vaccine that could protect against several diverse pathogens, is an attractive alternative in vaccine delivery [5,7,21]. The sections below will attempt to describe the process of identifying novel attenuated strains as potential vaccine candidates and how animal models can assist the pre-clinical evaluation of their safety and immunogenicity paving the way to clinical trials.

3.2. Identifying attenuated Salmonella strains

A better understanding of the pathogenesis of human and mouse typhoid infections has allowed a search for “rational” attenuating mutations to achieve the right balance between attenuation and immunogenicity. The mouse typhoid model has played an invaluable role in identifying target genes involved in house-keeping, biosynthesis of structural components and metabolites, pathogenesis and bacterial resistance to host-defense mechanisms into which defined, sometimes multiple, attenuating and irreversible mutations were introduced. Target genes for the development of *Salmonella* vaccine strains include galactose epimerase *galE*, aromatic amino acid and purine biosynthesis pathway *aro* and *pur*, heat-shock protein *htrA*, regulatory components *cya*/*crp* and *phoP*/*phoQ*, outer membrane proteins *ompC* and *ompF*, DNA recombination and repair pathway *recA* and *recBC*, stationary-phase survival *surA*, SPI-1 and SPI-2 TTSS components *ssa*, *sse*, *spt*, *sop*, *sip*, and DNA adenine methylase *dam*, among others (reviewed in [8,24,87,88]). The immunogenicity of these mutants was typically assessed in the mouse typhoid model. Although several other animal models of infection with *Salmonella* have been described such as *S.* Gallinarum, *S.* Pullorum [89] and *S.* Enteritidis in chickens [90], *S.* Dublin and *S.* Typhimurium in cattle [22,24] and *S.* Choleraesuis [91] and *S.* Typhi in pigs [92], their usefulness to assist in the development of typhoid vaccine candidates for human use remains undefined.

Among the attenuating mutations first identified in the typhoid model that were later applied to *S.* Typhi to develop typhoid fever vaccine candidates for humans, are those introduced into genes required for biosynthesis of bacterial components (*galE*, LPS), nutrients (*purD*, *aroA*), global regulatory systems (*cya*/*crp*), heat-shock proteins (*htrA*), and two-component regulatory system (*phoP*/*phoQ*) (reviewed in [7,79,87,88]). Thus, new live attenuated typhoid strains carrying mutations in *aroC* and *aroD* [93], *aroC*, *aroD* and *htrA* [94], *aroA*, *aroC* and *htrA* [95], *cya* and *crp* [96] or *phoP*/*phoQ*, *aroA* [97] were generated. These mutations considerably reduced the virulence of *S.* Typhimurium in mice while retaining immunogenicity. However, when introduced into homologous *S.* Typhi genes, they sometimes failed to produce adequately attenuated strains for humans, as we will discuss in detail later. In part, the difficulty in assessing attenuation of *S.* Typhimurium strains in mice and homologous

S. Typhi in humans derive from the measurement of different outcomes, i.e. lethality in the murine *S.* Typhimurium model versus fever and other adverse reactions after oral delivery of *S.* Typhi strains to humans. Although it is important to consider these issues when extrapolating data to humans, the strength of the mouse typhoid model clearly surpasses its limitations [21,24].

4. Animal models to assess the degree of attenuation and protective efficacy of candidate live *S.* Typhi vaccines

Once attenuated strains of *S.* Typhi have been engineered, they must be evaluated in pre-clinical animal models, particularly to assess their putative safety and protective efficacy, prior to entering clinical trials. Although chimpanzees will manifest clinical illness following ingestion of wild type *S.* Typhi, this is not a practical pre-clinical model. Not only are these higher primates in scarce supply and extremely expensive, but the fact that a high inoculum is needed to cause clinical infection with wild type parent strains makes this model insensitive to detect attenuation. One animal model that has proven to be practical for assessing the safety (adequate attenuation) and protective efficacy of *S.* Typhi vaccine candidates involves i.p. injection of mice with *S.* Typhi adsorbed to hog gastric mucin.

4.1. Intraperitoneal inoculation of mice with S. *Typhi and hog gastric mucin to evaluate attenuation and efficacy of typhoid vaccines*

Overall, mice are resistant to *S.* Typhi infection, particularly if administered orally [15]. Mice injected i.p. or intravenously (i.v.) with *S.* Typhi at doses of $\sim 10^6$ CFU show a short-lived, usually nonlethal infection [98,99], while massive i.p. doses (10^8–10^9 CFU) result in an overwhelming peritonitis leading to death within 24 h post-challenge. This acute infection pattern—also called toxin syndrome—bears little resemblance to typhoid infection in humans [100]. O'Brien examined the basis for this natural resistance of mice to *S.* Typhi and proposed that it may be due to the inability of the bacterium to multiply rather than to rapid killing by resident macrophages. These investigators implicated iron as at least one nutrient in insufficient supply to the microbe [101]. Latter studies have linked host specificity with the ability of the bacteria to survive intracellularly [102] and suggested that bacterial virulence also influences survival and intracellular multiplication [103]. The virulence of *S.* Typhi strains administered parenterally to mice has also been associated to their Vi content [81].

In spite of this resistance, when mice are infected i.p. with moderate doses ($>10^3$ CFU) of *S.* Typhi suspended in mucin, the bacteria become pathogenic, being able to infect and survive within peritoneal phagocytic cells [104]. Sein et al. [105] reported a murine LD_{50} of 10^5 for *S.* Typhi suspended in 5% mucin and showed that mice survived approximately 48 h after injection. These animals contained a high number of bacteria within vesicles in peritoneal cells. Death in this model is believed to result from the toxic effects of endotoxin associated with the rapidly expanding peritoneal *S.* Typhi load [99,104].

The hog gastric mucin model has been used to evaluate the degree of attenuation of the new generation of *S.* Typhi vaccine strains including Δ*aroA* Δ*aroC* BRD mutants [106], Δ*aroC* Δ*aroD* CVD 906 and CVD 908 [107], Δ*aroC* Δ*aroD* Δ*htrA* CVD 908-*htrA*, and Δ*guaBA* CVD 915 [108]. Mice that have been inoculated i.p. with parent virulent *S.* Typhi strain or attenuated derivative strains mixed with hog gastric mucin showed increasing LD_{50} values which corresponded with higher levels of attenuation [106–109].

Challenge of immunized mice with wild type *S.* Typhi resuspended in mucin has also been used to predict the efficacy of killed whole-cells typhoid vaccines [110]. In this assay, serial dilutions of the vaccines are injected into mice i.p. Seven days after immunization, mice are challenged by the same route with wild type *S.* Typhi strain in hog gastric mucin. The end point is survival, and ED_{50} is reported. It is believed that previously vaccinated animals are able to control the extracellular growth of the challenge population within the peritoneal cavity by increasing the rate of phagocytosis of *S.* Typhi in the presence of specific opsonins. Most of the intracellular bacilli are then removed from the peritoneal cavity and are rapidly inactivated in the liver and spleen. The hog gastric mucin model has been applied to evaluate the efficacy of parenteral vaccines that contain Vi antigen and a positive correlation was observed between in vivo potency in mice and vaccine efficacy in the field [81]. In recent studies, it has been extended to assess the efficacy of a mucosally (intranasally) administered attenuated *S.* Typhi strain, CVD 909, that constitutively expresses Vi antigen. In this model, CVD 909 exhibited a higher level of efficacy than CVD 908-*htrA* (the parent attenuated strain from which it was derived in which the expression of Vi is highly regulated) when vaccinated and control mice were challenged i.p. with virulent *S.* Typhi suspended in hog gastric mucin [111].

5. Results of Phases 1 and 2 clinical trials with engineered attenuated *S.* Typhi strains

The results of clinical trials with *S.* Typhi strains harboring specific attenuating mutations have sometimes been discordant with those from the mouse model in which *S.* Typhimurium harboring the homologous mutations were tested. For example, *S.* Typhimurium *galE* strains displayed the required level of attenuation in mice and were also protective against lethal challenge with wild type *S.* Typhimurium [112]. Following a similar approach, Hone et al. [109] constructed a *S.* Typhi Δ*galE* mutant by introducing a precise deletion in the *galE* gene on a Vi^- derivative of

S. Typhi wild type Ty2. Compared with the analogous *S.* Typhimurium mutant, this strain was expected to be even further attenuated due to the absence of the Vi antigen. Although this strain, like Ty21a, was found to be highly attenuated in mice, it remained virulent for humans [109,113]. Two out of four volunteers who ingested 7×10^8 viable organisms became ill and developed a typhoid like illness with fever and bacteremia [109]. Similarly, a Δ*cya* Δ*crp* mutant of *S.* Typhimurium was avirulent in mice [114], whereas a *S.* Typhi Δ*cya* Δ*crp* mutant from Ty2 (χ3927) was unacceptably reactogenic in humans causing severe illness in 1 out of 12 volunteers and asymptomatic vaccine bacteremia in two additional volunteers [96]. Hone et al. also constructed *S.* Typhi strain CVD 906, a recombinant derivative from *S.* Typhi field isolate ISP1820 with deletion mutations in *aroC* and *aroD* that encode enzymes in the aromatic amino acid biosynthesis pathway, rendering the strain nutritionally dependent on substrates that are not available in sufficient quantity in human tissues [107]. In a Phase 1 trial, this strain proved to be highly immunogenic but caused fever and other adverse reactions in a proportion of subjects, as well as vaccinemia—in which vaccine organisms were recovered from blood cultures [115]. Notably, another attenuated *S.* Typhi strain, CVD 908, in which the identical *aroC* and *aroD* mutations were introduced into another wild type parent (strain Ty2) [107], was clinically well tolerated and immunogenic in Phase 1 clinical trials [93,96,116]. In addition to eliciting strong serum antibody responses and mucosal antibody secreting cells (ASC) [93,96], CVD 908 triggered cell-mediated responses by circulating peripheral blood mononuclear cells (PBMC) to *S.* Typhi antigens, including T cells proliferation, IFN-γ production and $CD8^+$ cytotoxic T lymphocytes [12,83,84]. This strain, however, also caused silent self-limited vaccinemias, in subjects who ingested the highest doses (5×10^7 to 5×10^8 CFU) between days 4 and 8 after vaccination [93]. In mice, however, both CVD 906 and CVD 908 were attenuated and highly immunogenic [107]. A further derivative of CVD 908, with an additional mutation in the gene encoding the stress shock protein *htrA*, CVD 908-*htrA*, has been developed [94]. This strain has been administered to more than 100 young adults in Phases 1 and 2 trails proving to be as well tolerated as CVD 908, and likewise immunogenic, but without causing vaccinemia [94,117]. Phase 2 trials in pediatric subjects in typhoid endemic areas and Phase 3 efficacy trials are forthcoming.

Other approaches of attenuation of *S.* Typhi have also been pursued. *S.* Typhi Ty800, a derivative of Ty2 which harbors a deletion mutation in a regulatory system that allows survival in the phagosome (*phoP*/*phoQ*), was evaluated in a dose/response Phase 1 trial in young adults and was found to be well tolerated and immunogenic, eliciting IgG anti-LPS antibodies and IgA anti-LPS secreting cells [97]. Finally, *S.* Typhi strain χ4073, a triple deletion mutant in a global regulatory system *cya*, *crp* and in *cdt* (which is involved in the dissemination of *Salmonella* from gut associated lymphoid tissue to deep organs), was found to be well tolerated in healthy adults, although somewhat less immunogenic than CVD 908-*htrA* and Ty800 [118].

The promising CVD 908-*htrA*, Ty800 and χ4073 vaccine strains, although they elicit anti-LPS and H antibodies, rarely elicited Vi responses in humans [97,117]. Expression of Vi is highly regulated in relation to certain environmental signals such as osmolarity [119] and Vi may be expressed only when the typhoid bacilli are in the host's extracellular compartment in order to protect the bacteria from complement-mediated antibody-dependent bactericidal killing [81,111]. In an attempt to elicit Vi antibodies along with the immune responses generated by live organisms, CVD 909, a further derivative of CVD 908-*htrA*, was engineered to constitutively express Vi antigen [111]. This strain induced Vi antibodies and conferred greater protection compared with CVD 908-*htrA*, in mice immunized intranasally. Phase 1 clinical trials with CVD 909 are in progress.

Hindle et al. [120] assessed the safety and immunogenicity of two novel *S.* Typhi and *S.* Typhimurium vaccine strains harboring defined mutations in *aroC* and SPI-2 TTSS *ssaV* in volunteers immunized with a single oral dose of 10^7, 10^8 or 10^9 CFU. *S.* Typhi ZH9 vaccine was well tolerated and immunogenic; six out of nine volunteers responded with *S.* Typhi LPS-specific IgA ASCs, and five out of nine responded with either IgA or IgG against LPS or flagella [120]. The percentage of responders and the magnitude of the response to this vaccine, however, was found to be lower than that obtained over a similar dose range for CVD 908-*htrA* [94,120].

Another recently developed attenuated *S.* Typhi strain, CVD 915, which has a deletion mutation in the *guaBA* locus that interrupts the synthesis of guanine nucleotides [108], has proven highly immunogenic as a mucosal live vector in pre-clinical studies [108,121] and is regarded as a promising vaccine candidate to enter Phase 1 clinical trials. A derivative of this strain that also expresses Vi constitutively, CVD 916, has also been constructed and is currently undergoing pre-clinical evaluation.

6. Animal models to assess the immunogenicity of attenuated *Salmonella* live vector vaccines expressing foreign antigens

In addition to their application as live oral typhoid vaccines, attenuated *S.* Typhi have remarkable potential for use as live vectors to carry "foreign" or "guest" antigens as they can stimulate a wide array of humoral and cellular immune responses (reviewed in [5,8,88,122–124]). This approach might elicit protection against typhoid fever and other infections simultaneously. The improved knowledge on the physiology and molecular biology of *Salmonella* as live vector, the possibility of selecting different mutants, and highly sophisticated gene expression systems, as well as the co-administration of immunomodulators such as cytokines

and adhesion molecules, make it an appealing system to trigger specific responses tailored to the particular needs [125]. The ability to express multiple guest antigens in attenuated *Salmonella* also provides the opportunity to produce a polyvalent vaccine to protect against several diverse pathogens.

A major benefit of using *Salmonella* as a live vector is the possibility of using mucosal routes of immunization. In some experimental systems, mucosal delivery has proven more efficacious than parenteral vaccination for generating both systemic and local protective immunity [126]. In addition, the mucosal route of administration has several potential advantages compared with parenteral vaccination. For pathogens that use this route of entry, it is more likely to confer protection against infection as well as disease. It is usually associated with lower reactogenicity and logistics for vaccine administration are easier. Since it is perceived as a less-invasive way of administering a vaccine, mucosal immunization generally has higher public acceptance and increased compliance [127]. By using a live vector to deliver specific protective recombinant antigens, other products of the pathogen that might be reactogenic—and not critical for protection—are avoided. Such vaccines would be expected to have relatively low manufacture costs compared to many other types of vaccines [127]. These potential advantages make the *Salmonella* live vector strategy particularly attractive for mass immunization programs [127].

6.1. Salmonella *live vector strains in mice*

The mouse model using oral immunization with attenuated *S.* Typhimurium has been extremely useful in identifying the key factors that influence the immunogenicity of *Salmonella* as live vectors, including the choice of the vector strain, the nature of the foreign antigen, gene copy number, stability of the foreign antigen gene, promoter controlling foreign antigen expression, and site of foreign antigen expression [8].

A number of antigens from other bacteria, viruses and parasites have been cloned and expressed in *S.* Typhimurium, and the ability of these constructs to stimulate specific immunity has also been investigated using the mouse typhoid model (reviewed in [8,32,87,128–131]). The presence of a wide array of systemic and mucosal responses against the live vector and foreign antigens has been demonstrated in different studies including *S.* Typhimurium expressing tetanus toxin Frag C [132–136], *Yersinia pestis* F1 capsular antigens [137], influenza A nucleoprotein [138], enterotoxigenic *E. coli* fimbriae [139], *M. tuberculosis* and *M. bovis* secreted T cells antigen ESAT-6 [140], *Leishmania major* gp63 [141–143], *Plasmodium* spp. sporozoite and merozoite surface proteins [144,145], *H. pylori* urease [146,147], *Streptococcus* spp. antigens [148,149], Hepatitis B nucleocapsid (HBc) [150], herpes simplex virus (HSV), glycoprotein D (gD) [151,152], measles virus B and T epitopes [153], and lymphocytic choriomeningitis virus epitopes [154], among others. Potent immune responses have also been demonstrated in mice immunized with *S. dublin* expressing viral antigens [155].

Although most of these studies used oral, i.p. or i.v. immunization, alternative mucosal routes such as intranasal, vaginal and rectal have also been employed successfully to assess immunogenicity of vaccine candidates [149,156–158], providing useful information on protective responses at distant mucosal sites.

The protective efficacy of attenuated *S.* Typhimurium strains against murine typhoid has been measured in this model using i.p. [159], i.v. and oral challenge [160] with virulent *S.* Typhimurium strains. Protective responses against other pathogens induced by *Salmonella* live vectors were also demonstrated using bacterial [156,161], virus [154,162] and parasitic challenge [163]. Many of these studies have been reviewed elsewhere [88,128–130].

Some of the results of immunogenicity obtained in this model, however, have been contradictory and difficult to interpret due to the strong influence of the host's genetic background in the responses stimulated by the live carrier itself or the expressed foreign antigen. For example, immunization of *Ity*^{r} mice with *S.* Typhimurium expressing *Leishmania* gp63 antigen in *Ity*^{r} mice induces Type-1 T-cell responses that lead to enhanced resolution of *Leishmania* challenge infection; in contrast, immunization of *Ity*^{s} mice induces Type-2 responses and exacerbated lesion growth after challenge [142]. *Ity* alleles have also been found to influence antigen processing and presentation [164] as well as IFN-γ production [47,165–167]. The Nramp-1 protein encoded in the Ity locus has been related to increased surface expression of MHC class II molecules and inflammatory cytokines by macrophages stimulated with LPS [58]. Fayolle et al. studying the responses induced by *S.* Typhimurium expressing *E. coli* maltose-binding protein (MalE) demonstrated decreased antibody responses to MalE in *Ity*^{r} mice compared with other phenotypes. They also showed that H-2 major histocompatibility complex genes can significantly affect the antibody responses to the foreign antigen [168]. The diversity of H-2 haplotypes influences the outcome of $CD4^+$ Th1 responses to MalE expressed in *Salmonella* [169]. These observations should be considered in attempting to explain results of *S.* Typhi live vector studies in humans.

Although there is a consensus in the literature that recombinant *S.* Typhimurium has been extremely effective at inducing protective immunity against foreign antigens in mice, the few studies conducted in humans so far with similar recombinant *S.* Typhi strains have been only moderately successful, as discussed in detail later (Section 7). It would appear that responses to foreign antigens are weaker than those expected, based on pre-clinical studies in various animal models using similar constructs. For example, the Δ*cya* Δ*crp* mutant of Ty2 expressing Hepatitis B core and pre-S antigens was unable to elicit virus-specific responses in volunteers [118], despite the promising results observed in mice orally immunized with a similarly attenuated *S.* Typhimurium strain carrying the same plasmid [170].

The responses to Frag C delivered by Δ*aroC* Δ*aroD* attenuated *S.* Typhi CVD 908-*htrA* in humans were also modest [171], compared with the immunity elicited by Δ*aroA* Δ*aroC* attenuated *S.* Typhimurium strains in the murine model [136,172]. Similarly, a *phoP*/*phoQ*-deleted *S.* Typhimurium expressing *H. pylori* urease delivered nasally triggered strong serological and mucosal antibody responses to urease antigens in mice [173]. However, when a similar Δ*phoP*/*phoQ* mutant *S.* Typhi strain expressing *H. pylori* urease from a multicopy plasmid (Ty1033) was given to eight subjects, it failed to induce detectable immune responses to the foreign antigen [173]. Interestingly, a Phase 1 clinical trial using the attenuated *phoP*/*phoQ*-deleted *S.* Typhimurium as live vector expressing *H. pylori* ureases A and B [174] showed the induction of urease-specific antibodies in three out of six subjects, although this recombinant vaccine was not well tolerated.

The murine typhoid model has also been used to investigate different innovative approaches to enhance the effectiveness of live vectors (reviewed in [8,124,175]), including the use of two-phase variation systems for the expression of antigens that are toxic to *Salmonella* [176,177], evaluation of in vivo inducible promoters [178,179], in vivo measurement of antigen expression related to bacterial colonization [180], the possibility of enabling antigen expression in different compartments within the bacteria or the infected host cells [138,154,181,182], antigen secretion [140,145,183,184], and co-expression of immunomodulatory molecules such as cytokines [143,185–188].

The problem of pre-existing immunity, using *Salmonella* as live vectors, has also been addressed in the typhoid murine model. Some studies have shown that pre-existing immunity against *Salmonella* lowers recall serum responses [189,190] and interferes with the response against the foreign antigen [191]. Kohler et al. [189] found reductions of serum IgG and mucosal IgA in a time-dependent fashion, although overall, pre-immunization with a live vector did not appear to affect recall responses to the foreign antigen. In contrast, early studies from Bao and Clements [192] found that prior exposure of mice to the *Salmonella* potentiates subsequent serum and mucosal responses. Similarly, Whittle and Verma [155] showed that antibody responses to a viral B cells epitope expressed by *Salmonella* were enhanced in mice that had been primed with the carrier strain alone. In a more recent study, *S.* Typhimurium expressing the glucan-binding domain (GLU) of the enzyme glucosyltransferase, a virulent factor of *S. mutans*, was effective in priming the host for a secondary response to either soluble GLU peptide or *Salmonella* expressing GLU, and pre-existing immunity did not inhibit memory responses [149]. Our group has performed a large number of studies evaluating serum antibodies against bacterial and foreign antigens induced by attenuated *S.* Typhimurium and *S.* Typhi live vector strains in mice immunized intranasally using a two-dose (28 days apart) immunization schedule. In our hands, the booster doses consistently produced significant increases in antibody titers, indicating that pre-existing immunity did not abrogate the responses to a second dose [121,136,145,172,193].

6.2. Mouse model of intranasal immunization with S. Typhi live vectors

6.2.1. Development and applications of the model

In an effort to establish an animal model to assess immunogenicity of *S.* Typhi vaccines candidates at a preclinical level, Galen et al. studied the possibility of eliciting immune responses against bacterial and foreign antigens by delivering live recombinant *S.* Typhi vaccine strains into mice through the nasal route. They showed for the first time that *S.* Typhi strain CVD 908 carrying plasmids encoding tetanus toxin Fragment C alone, i.e. pTET*nir15* and pTET*lpp*, or fused to the eukaryotic cell receptor-binding domain of diphtheria toxin, i.e. pOG215, delivered to mice intranasally elicited serum IgG anti-tetanus toxin responses that protected against an i.p. challenge with 100 LD_{50} of tetanus toxin. In contrast, CVD 908 carrying pOG215 delivered orogastrically induced little, if any, serological responses against bacterial or foreign antigens [172]. Using a similar approach, Barry et al. [194] investigated the immune responses elicited by *S.* Typhi strain CVD 908 expressing pertussis toxin subunit S1 fused to Fragment C and showed that mice immunized intranasally with the live recombinant CVD 908 (pCFV-2) developed serum pertussis neutralizing antibodies. Wu et al. [195] showed the induction of antibodies against *Plasmodium falciparum* merozoite surface protein (MSP-1) in mice immunized with CVD 908 expressing MSP-1 fused to Fragment C.

We have compared side by side the systemic antibody and cell-mediated immune responses elicited by *S.* Typhi strain CVD 908-*htrA* and *S.* Typhimurium SL3261 alone or expressing Frag C, as well as the in vivo distribution of the vaccine organisms following intranasal and orogastric delivery [136]. The intranasal route of immunization proved remarkably more efficient at inducing serologic and cell-mediated immune responses against bacterial antigens and Frag C than the orogastric route for both *Salmonella* serovars. In this study, CVD 908-*htrA* carrying pTET*nir15* administered orally failed to induce serum responses; a low level of cellular proliferation was observed. Interestingly, results from this study also showed that the serologic Frag C responses elicited by *S.* Typhi CVD 908-*htrA* (TET*nir15*) given intranasally were significantly higher than those induced by *S.* Typhimurium SL3261 (TET*nir15*) delivered orally [136].

Other groups have also shown higher efficiency of the intranasal route as compared to orogastric administration in inducing systemic as well as mucosal responses to cloned antigens [157,158,196]. It has been suggested that the lower efficiency of the orogastric route for live bacteria may be due to the difficulty in achieving uniform infections as stomach acid and proteolytic environment, undigested food, and commensal bacteria in the gastrointestinal tract might interfere with vaccine uptake [16,32]. It is also conceivable that

the orogastric route might be less advantaged than the nasal route to prime the immune system.

In recent years, the murine model of intranasal immunization has been used to assess immunogenicity of a variety of new attenuated *S.* Typhi live vector vaccine strains. *S.* Typhi vaccine candidate CVD 909, which expresses Vi antigen constitutively, has been evaluated for induction of antibodies to Vi as well as *S.* Typhi LPS, in comparison with the parent strain CVD 908-*htrA*, in mice immunized intranasally [111]. CVD 909 was found to be more immunogenic than CVD 908-*htrA* in eliciting Vi antibodies, whereas LPS responses were virtually identical for both strains. This model was also used to assess immunogenicity of attenuated Δ*guaBA S.* Typhi strain CVD 915, both as typhoid vaccine, in comparison with strains Ty21a, CVD 908 and CVD 908-*htrA*, and as a live vector expressing Frag C as foreign antigen [108]. In related studies, we also characterized the T-cell responses against *Salmonella* antigens in this model. Immunization of mice with different attenuated *S.* Typhi vaccine strains elicited the appearance of *S.* Typhi-specific $CD8^+$, MHC class I-restricted CTL effector populations in cervical lymph nodes (CLN) and spleens [197].

The intranasal model has proven useful in comparing promoters to enhance in vivo expression of foreign antigens. Thus, Orr et al. evaluated the immunogenicity of several CVD 908-*htrA* recombinant strains carrying plasmids encoding Frag C under the transcriptional control of anaerobically induced promoters (i.e. *dmsA* from *E. coli* and two derivatives *dmsa2* and *dmsa3*). These promoters allowed Frag C to be expressed at low (pTET*dmsA*), moderate (pTET*dmsA2*) or high levels (pTET*dmsA3*) [198]. Results showed that CVD 908-*htrA* (pTET*dmsA2*) elicited moderate anti-Frag C antibody titers whereas CVD 908-*htrA* (pTET*dmsA3*) induced a remarkable response, even higher than that elicited by CVD 908-*htrA* (pTET*nir15*), which protected vaccinated mice against tetanus toxin challenge.

Lee et al. [199] used the intranasal model to investigate the immunogenicity of a recombinant Ty21a strain engineered to express viral antigens on the cell surface or intracellularly, and demonstrated that the former induced higher levels of serum antibodies. Ruiz-Pérez et al. [182] assessed in mice immunized intranasally the immunogenicity of a recombinant *S.* Typhi CVD 908 vaccine strain engineered to display the immunodominant B cells epitope of *P. falciparum* circumsporozoite protein (CSP) on the bacterial surface, and demonstrated the induction of $(NANP)_4$ antibodies that were able to recognize naive CSP. Recently, Londoño-Arcila et al. [200] evaluated the use *S.* Typhi strain CVD 908-*htrA* expressing *Helicobacter pylori* urease under control of the in vivo inducible *htrA* promoter in a prime-boost strategy. Mice primed by intranasal route with *S.* Typhi-expressing urease and boosted parenterally with urease plus alum were protected against *H. pylori* challenge, whereas neither delivery of urease-expressing *S.* Typhi alone nor immunization with urease and alum conferred protection [200].

In summary, the observation that *S.* Typhi administered intranasally to mice elicits an array of immune responses similar to those observed in volunteers given attenuated strains of *S.* Typhi orally [83,84,86,94,171] supports the use of this reliable small animal model to pre-clinically evaluate the immunogenicity and the efficacy of live vector vaccine candidates.

6.2.2. Characterization of the murine intranasal model

Pickett et al. [193] investigated the kinetics of *S.* Typhi distribution in the mouse intranasal model and showed that following intranasal delivery *S.* Typhi vaccine organisms can be recovered from nasal associated lymphoid tissue (NALT), lungs and PP as early as 2 min after inoculation and up to 3 days later. Interestingly, by 72 h after immunization, vaccine organisms were no longer recoverable from the lungs yet could still be isolated, albeit in small numbers, from the NALT, which has been suggested as the only site of multiplication. In these studies, bacteria could not be cultured from mesenteric and cervical lymph nodes, spleen, liver, bone marrow and blood samples, indicating that *S.* Typhi infection under these conditions does not become systemic. The lack of bacterial colonization in liver and spleen in mice immunized with *S.* Typhi intranasally was confirmed by others [200]. In contrast, mice immunized i.v. exhibited high number of organisms in these organs, indicating a wider spread of bacteria. Pickett and colleagues studied the distribution of vaccine organisms in murine tissues following orogastric immunization in an attempt to explain the higher immunogenicity of the intranasal versus the orogastric routes. Mice immunized orally or intranasal showed bacteria colonization in the same tissues and similar numbers of organisms were recovered from PP and lungs. However, after oral immunization, significantly lower numbers of bacteria were recovered from the NALT and by 10 h organisms had been virtually cleared from the lungs and NALT, while remaining very low in the PP, indicating a significantly lower persistence compared with intranasal delivery [136,193]. Lack of persistence in murine tissues likely accounts, in part, for the poor responses induced in orogastrically vaccinated mice [201].

In studies undertaken to explore the cellular events associated with priming of immunity in this model we found that intranasal delivery of *S.* Typhi is accompanied by a remarkable recruitment of Mac-1^+ ($CD11b^+$) cells (a marker of macrophages, monocytes and DC) and $CD8^+$ T cells to the NALT [202]. Of note, macrophages showed the highest percentage of in vivo *Salmonella* antigen expression in cells from NALT, PP, peripheral blood and lung of immunized mice, measured by multicolor flow cytometry using specific antibodies, within 12–18 h after inoculation [202]. Moreover, *S.* Typhi infected NALT cells obtained from these immunized mice induced in vitro proliferative responses of $CD3^+$ splenocytes previously sensitized for *S.* Typhi antigens, indicating priming of immune responses in the nasal tissue. Furthermore, cells from the nasal tissue collected from mice immunized with two doses, 28 days apart of CVD

908-*htrA* and restimulated in vitro with bacterial antigens showed proliferative responses and cytokine production, i.e. IFN-γ and IL-12 (Pasetti and Sztein, unpublished data). Taken together, these findings suggest that the nasal lymphoid tissue appears to be an inductive as well as an effector site of immune responses in this model. B cells and isotype switching and differentiation has also been shown to occur in the NALT [203]. It has been suggested that lymphoid cell populations present in the murine nasal tissue appear to be in a more naive state than those of the PP, which may allow them to respond more effectively when novel antigens are encountered [203]. Moreover, intranasal immunization in rodents has also been shown to be an effective route for the induction of mucosal immune responses expressed at remote sites. The remarkable efficiency of intranasal delivery for a variety of vaccine systems, including live bacteria has been reviewed [204–206].

7. Clinical trials of attenuated *S.* Typhi live vector vaccines expressing foreign antigens

7.1. Ty21a-based live vector vaccines

The concept of making a hybrid *S.* Typhi strain expressing foreign antigens was introduced by Formal et al. [207], who expressed the O polysaccharide of *Shigella sonnei* in Ty21a. Serum antibody responses in rabbits and protection of mice against systemic challenge with both *S. sonnei* and *S.* Typhi was demonstrated [207]. This construct, however, was unable to confer consistent protection in volunteers challenged with wild type *S. sonnei*. Although a first study with three doses of this *S.* Typhi-*Shigella* recombinant vaccine achieved 50–70% protective efficacy against *Shigella*-induced diarrhea [208], these results could not be confirmed in volunteers that received subsequent lots of vaccine [209].

Vibrio cholerae Inaba LPS was also expressed in Ty21a and assessed for immunogenicity and efficacy in clinical trials. Once again, responses against the foreign antigen were modest with only 25% of volunteers protected from challenge [210,211]. Bumann et al. [212] expressed *Helicobacter pylori* urease in Ty21a and the resulting strain, Ty21a (pDB1), was tested in nine volunteers while three others received the parent strain Ty21a. Ten out of twelve volunteers developed ASC specific to *Salmonella* antigens but only two volunteers seroconverted. Five volunteers also showed cell-mediated immunity against the live vector by either T cells proliferation or IFN-γ production. Three of the volunteers that had received Ty21a (pDB1) showed weak but significant T-cell responses to *H. pylori* urease, while none had detectable humoral responses [212].

7.2. Live vector vaccines based on other attenuated S. *Typhi strains*

As discussed above, progress has been made using a new generation of attenuated *S.* Typhi strains as live vector vaccines in humans. Hepatitis B virus (HBV) pre-S envelope proteins S1 and S2, fused to HBV core protein (HBc-pre-S), have been expressed in *S.* Typhi χ4632 and tested in clinical trials [118,213]. None of the volunteers who ingested a single dose of 3×10^7 or 7×10^8 CFU of HBc-pre-S-expressing *S.* Typhi seroconverted to the foreign antigen [118]. In a separate study, one out of six patients immunized rectally with the same strain elicited responses to pre-S1 and *S.* Typhi LPS. Notably, that subject manifested diarrhea [213]. CVD 908 has been employed successfully for the delivery of the CSP from *P. falciparum* in humans [116]. In this study, the CSP gene was integrated into the mutated *aroC* locus within the bacterial chromosome to achieve constitutive CSP expression. Two out of ten volunteers who received two oral doses of the recombinant strain mounted antibody responses to the CSP, while a third vaccine developed CSP-specific $CD8^+$ CTL response [116]. CVD 908-*htrA* expressing tetanus toxin Fragment C was tested in a Phase 1 trial [171]. Twenty-one healthy adult volunteers received a single dose of CVD 908-*htrA* carrying a Frag C-encoding plasmid, either pTET*nir15* (in which the Frag C gene transcription is controlled by an anaerobically-activated promoter) or pTET*lpp* (Frag C gene controlled by a powerful constitutive promoter), in dosage levels from 1.6×10^7 to 8.2×10^9 CFU. Vaccine strains were well tolerated and three of nine volunteers who received the highest doses of CVD 908-*htrA* (pTET*lpp*) construct developed rises in serum antitoxin antibodies [171]. *S.* Typhi Ty800 (a Δ*phoP*/*phoQ* mutant), was modified to express constitutively *H. pylori* ureases A and B (strain Ty1033) [173]. Despite the fact that all volunteers had strong mucosal immune responses to vaccination against the vector, as measured by increases in IgA ASC against bacteria antigens, and most of them had increased antibody titers against *S.* Typhi flagella and LPS, none had a detectable response to *H. pylori* urease following immunization. Responses were not recorded even following a booster with recombinant *H. pylori* urease A/B and *E. coli* heat labile toxin adjuvant 15 days after immunization with Ty1033 [173].

Clearly, the approach of using attenuated *S.* Typhi strains, as well as other live bacteria, to deliver heterologous antigens to the host's immune system needs to be improved before it can be successfully used in humans. To this end, the live vector needs to be appropriately attenuated, while still maintaining the ability to infect and trigger the immune system [214], and the foreign antigens have to be appropriately expressed to induce protective responses [8]. There are a number of factors believed to be critical in the development of live vector vaccines that still need thorough investigation. Among them are: (1) the selection of adequate plasmids and promoters to reduce metabolic burden and to ensure bacterial fitness for a productive infection and immunity [179]; (2) the need for recombinant stabilization systems to allow appropriate antigen expression [215]; (3) the use of adequate strategies to control the destination of the foreign antigen, whether it be in the cytoplasm or extracellular secretion according to the immune responses desired [184,216]; and (4)

the intrinsic difficulties in assuring that expressed epitopes will have the appropriate conformation [217]. These issues have gained considerable attention lately and have been reviewed elsewhere [7,8,87,88,124,127,175,216,218].

The concern of whether *Salmonella* live vectors can function in the face of pre-existing anti-*Salmonella* immunity has also been raised [7,124,189–192]. It has been suggested that this problem could be circumvented by using different live vectors or, alternatively, spacing the administration of the vector vaccine [127]. In a recent clinical trial with *S.* Typhi, Ty21a expressing ureases A and B from *H. pylori*, prior immunity to the carrier appeared to enhance the presentation of the foreign antigen [212].

8. Animal models to assess the immunogenicity of attenuated *Salmonella* live vector vaccines carrying eukaryotic expression systems encoding foreign antigens (i.e. DNA vaccines)

Recent studies support the use of attenuated bacteria not only as conventional live vectors expressing foreign antigens but also as a delivery system for DNA vaccines (reviewed in [6,88,125,175,219–221]). In the latter approach, plasmids encoding foreign antigens under the control of eukaryotic promoters are "directly" delivered by the live vector to the host APCs where they are expressed using the cellular machinery. This approach combines the advantages of mucosal delivery of a live carrier with targeted DNA vaccination. Moreover, it allows the co-delivery of antigens with cytokines or costimulatory molecules under eukaryotic transcriptional control, thereby exploiting their full potential for modulating immune responses [88,125]. *Salmonella* can infect antigen-presenting cells, i.e. macrophages, dendritic cells and B cells, resulting in targeted delivery of DNA vaccines directly to these cells. Further, since the foreign antigen is synthesized within the host cell, it should have an advantage for MHC class I presentation and induction of CTL responses compared with antigens expressed by the bacteria.

8.1. Salmonella *live vectors carrying DNA vaccines*

S. Typhimurium has been used as carrier for DNA vaccines in the mouse model (reviewed in [7,175,220,221]). An increasing number of groups have documented efficient in vivo transfer of functional DNA, transgene expression and immunogenicity of DNA-encoded antigens [222–227]. Delivery of eukaryotic expression vectors by *S.* Typhimurium strains has proven highly successful at inducing cellular and humoral immune responses against antigens from bacterial pathogens such as *L. monocytogenes* [222], *Chlamydia trachomatis* [228], viruses, including HIV [229], measles [230], Hepatitis B (HBsAg) [231], HSV [227] as well as tumor antigens [232]. Oral vaccination of mice with attenuated *S.* Choleraesuis carrying a DNA vaccine encoding the gD gene of pseudorabies virus (PrV) conferred protective immunity against PrV [233].

8.2. S. *Typhi live vectors carrying DNA vaccines*

Fenelly et al. first reported the ability of attenuated *S.* Typhi to serve as DNA delivery vehicle. Using attenuated vaccine strain Ty21a harboring a eukaryotic expression plasmid encoding measles virus antigens this group demonstrated the induction of cytotoxic responses against the virus nucleoprotein in mice inoculated i.p. [230]. Subsequently, Pasetti et al. [121] extended these observations by investigating the ability of attenuated *S.* Typhi to deliver DNA vaccines mucosally using the mouse intranasal immunization model. Plasmid pcDNA3*tetC* encoding Frag C under transcriptional control of the eukaryotic cytomegalovirus promoter was introduced into *S.* Typhi strain CVD 915, and the recombinant strain CVD 915 (pcDNA*tetC*) was evaluated for immunogenicity in comparison with CVD 915 alone or carrying a prokaryotic expression plasmid pTET*nir15*. Control groups included the same DNA vaccine, pcDNA*tetC*, and recombinant Frag C protein given intramuscularly. High titers of IgG1, IgG2a and IgG2b against Frag C were induced by CVD 915 (pcDNA*tetC*). Total serum IgG responses were significantly higher than those elicited by pcDNA*tetC* given i.m. or by the live vector carrying pTET*nir15*. Cell-mediated immune responses against bacterial antigens and Frag C, including proliferative responses in CLN and spleen as well as IFN-γ production were also induced by CVD 915 (pcDNA*tetC*) [121]. Woo et al. [234] used *S.* Typhi strain Ty21a to deliver DNA encoding HBsAg by the orogastric route in BALB/c mice and showed the induction of specific HBsAg antibodies when ampicillin was concomitantly administered.

Despite the fact that *Salmonella* remains in the phagosomal compartment, instead of escaping into the cytoplasm as *Listeria* or *Shigella*, efficient transgene expression can ensue. It has been suggested that the bacteria-carried plasmid reaches the cytoplasm of the host cell through leakage from the atypical vacuolar compartment in which the bacteria resides [125]. Interestingly, a modified *Salmonella* strain that can escape the phagosomal compartment by utilizing the *E. coli* hemolysin secretion system, adapted to deliver a eukaryotic antigen expression vector into the macrophage cytosol, exhibited an enhanced ability to transfect mammalian cells following lysis of the phagosomal membrane [235]. DNA delivery by *Salmonella* with phagosomal escape properties was able to present DNA-encoded antigens into MHC class I and stimulate $CD8^+$ T cells in vitro [236]. *Salmonella* harboring an inducible lysis system was also able to release DNA plasmid within APCs [237]. It is also likely that a yet unidentified specific phagosome-to-cytosol transport process—such as the one previously described for protein antigens in DC [238] could mediate plasmid translocation across the phagosomal membrane. Another hypothesis proposes that *Salmonella*-induced apoptotic macrophages might

be engulfed by APCs facilitating antigen presentation by cross-priming [239].

Despite the mounting evidence for the ability of *S.* Typhimurium to successfully deliver DNA vaccines in mice, it is unclear whether this approach will be effective in humans. Important safety issues, such as the possibility of integration of DNA vaccines carried by attenuated live vectors into the host cell genome which might promote oncogenesis [220,221] and the induction of anti-DNA antibodies or immune responses against transfected cells [240] need to be carefully addressed in pre-clinical studies before this vaccine strategy is evaluated in volunteers. A more precise understanding of the mechanisms involved and further evaluation of ways to optimize DNA vaccine delivery by *S.* Typhi through pre-clinical studies, will be required before this approach can be evaluated in humans.

9. Bridging the gap: from animal models to clinical trials

A new generation of attenuated *S.* Typhi strains is in various stages of development, including several that have been found to induce strong antibody and cell-mediated responses. From the experience accumulated during the clinical development of these *S.* Typhi strains, it has been learned that one must identify strains that successfully achieve a delicate balance that minimizes reactogenicity and maximizes immunogenicity. The use of attenuated *S.* Typhi strains to deliver foreign antigens or DNA vaccines has also emerged as a promising vaccine strategy. In this regard, multiple factors have been identified that influence the ability of these live vectors to stimulate the desired immune responses. These include characteristics of the live vector itself, specifics of the antigen expression system and the intrinsic properties of the immunogen, all of which affect the breadth, magnitude and quality of the immune responses elicited.

Because *S.* Typhi is a highly human-restricted pathogen, no animal model has proven to be completely satisfactory in predicting the potential of vaccine candidates for human use. Experience has shown that *S.* Typhimurium models are very useful in: (1) directing the construction of new attenuated strains of *S.* Typhi that can incorporate homologous genetic mutations and (2) identifying the critical parameters that affect expression and immunogenicity in vivo of live vector constructs. In contrast, once *S.* Typhi live vector constructs are available, it is the mouse model involving intranasal immunization that permits the pre-clinical assessment of the immunological potential of the vaccines. The safety profile of the *S.* Typhi constructs can be estimated by i.p. inoculation of several dosage levels of the construct suspended in hog gastric mucin so that an LD_{50} can be calculated. If the wild type *S.* Typhi parent strain and a known attenuated *S.* Typhi strain such as Ty21a are included in the experiment as controls, comparisons of the LD_{50} results can estimate the relative safety of the construct for humans. Obviously, great care should be taken in extrapolation of results to humans. Under any circumstances, carefully designed clinical trials in humans must serve as the final arbiter of the suitability of candidate *S.* Typhi vaccines. Progress and sophistication in the use of old and new animal models can greatly expedite the progression to clinical trials.

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