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Live attenuated vaccines for invasive *Salmonella* infections

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ABSTRACT

Salmonella enterica serovar Typhi produces significant morbidity and mortality worldwide despite the fact that there are licensed *S.* Typhi vaccines available. This is primarily due to the fact that these vaccines are not used in the countries that most need them. There is growing recognition that an effective invasive *Salmonella* vaccine formulation must also prevent infection due to other *Salmonella* serovars. We anticipate that a multivalent vaccine that targets the following serovars will be needed to control invasive *Salmonella* infections worldwide: *S.* Typhi, *S.* Paratyphi A, *S.* Paratyphi B (currently uncommon but may become dominant again), *S.* Typhimurium, *S.* Enteritidis and *S.* Choleraesuis (as well as other Group C *Salmonella*). Live attenuated vaccines are an attractive vaccine formulation for use in developing as well as developed countries. Here, we describe the methods of attenuation that have been used to date to create live attenuated *Salmonella* vaccines and provide an update on the progress that has been made on these vaccines.

1. Introduction

The first vaccines against typhoid fever consisting of heat-inactivated typhoid bacilli preserved in phenol administered parenterally, were developed in the late 19th century [1]. Experiences with implementation of typhoid vaccines in the British and US military in the early 20th century and subsequent large-scale controlled field trials sponsored by the World Health Organization documented that the inactivated whole cell vaccines were efficacious but were highly reactogenic [1]. Whole-cell vaccines against *Salmonella enterica* serovars Paratyphi A and B were also developed in the early 20th century and used by the U.S. military as a trivalent "TAB" vaccine against enteric fever [2]. However, these whole-cell vaccines lost favor due to their propensity to produce high fever, severe headache and malaise and gave way to the development of better tolerated *Salmonella* vaccines using other approaches such as parenteral polysaccharide and polysaccharide-protein conjugate vaccines and live attenuated oral vaccines. There are currently three types of licensed *Salmonella* vaccines: the live attenuated vaccine Ty21a marketed as Vivotif® (PaxVax Corporation); unconjugated Vi polysaccharide vaccine commercialized as Typhim Vi® (Sanofi Pasteur), Typherix® (GSK) and Typbar Vi® (Bharat Biotech), amongst others; and Vi polysaccharide conjugated to tetanus toxoid (Typbar TCV®, Bharat Biotech and Peda Typh™, Biomed).

Currently, licensed vaccines exist against no *Salmonella* serovars other than *S.* Typhi (although *S.* Typhi vaccine strain Ty21a confers moderate cross protection against *S.* Paratyphi B as well as *S.* Typhi) [3]. There is growing recognition that other invasive *Salmonella* serovars also cause a notable disease burden [4]. *S.* Paratyphi A is emerging as a pathogen in Asia [5]; the non-typhoidal *Salmonella* serovars *S.* Typhimurium and *S.* Enteritidis cause invasive disease throughout sub-Saharan Africa [6], and *Salmonella* Group C serovars such as *S.* Choleraesuis are associated with invasive disease in certain countries such as Taiwan [7]. As such, a multivalent vaccine that targets the following serovars is needed to control invasive *Salmonella* infections worldwide: *S.* Typhi, *S.* Paratyphi A, *S.* Paratyphi B (currently uncommon), *S.* Typhimurium, *S.* Enteritidis and *S.* Choleraesuis (as well as other Group C *Salmonella*).

At the Center for Vaccine Development, University of Maryland School of Medicine, we have developed and evaluated a variety of *Salmonella* live attenuated vaccines. There are several advantages of live oral attenuated vaccines over other vaccine formulations: (1) they can induce local immune responses at mucosal surfaces; (2) they are economical to produce; (3) they induce *Salmonella*-specific B and T cell immunity; (4) they are practical to administer to a large

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population; and (5) they do not generate hazardous waste (*e.g.*, needles and syringes) that needs to be discarded appropriately [8,9]. However, there are several limitations to live attenuated vaccines. First, one needs to balance immunity and reactogenicity, particularly if the vaccine is to be used as a live vaccine vector [10]. The vaccine may also need to be formulated differently for infants. For example, Ty21a at times has been available in both a sachet formulation for use in young children as well as enteric-coated capsules for use in older children and adults [11–13]. Finally, safety of live attenuated vaccines needs to be determined in immunocompromised subjects and also the very young prior to widespread use.

Here, we describe the methods of attenuation that have been used to date to create live attenuated *Salmonella* vaccines and provide an update on the progress that has been made on these vaccines.

2. Methods of attenuation

The first method used to mutate bacteria to create live attenuated vaccines was chemical mutagenesis. However, with the advent of molecular biology, live attenuated vaccines are now constructed by making focused site-directed mutations using genetic engineering.

2.1. Chemical mutagenesis

Here, bacteria are exposed to a mutagen and spontaneous mutants are selected and passaged. The licensed typhoid vaccine Ty21a was constructed in the early 1970s using chemical mutagenesis [14]. Spontaneous *galE* mutants were selected and shown to lack UDP-galactose-4-upimerase activity. In the absence of galactose, these mutants produce rough LPS whereas when galactose is supplied exogenously, smooth LPS is produced. Chemical mutagenesis is a simple procedure and highly effective if the mutation is not lethal to the bacteria. However, one disadvantage of this method is that additional mutations may occur in several locations in the genome and as such the mutations are not fully defined. For example, Ty21a has more than two dozen mutations in addition to *galE*, the sought mutation [15]. Interestingly, the *galE* mutation alone is not responsible for the attenuation of Ty21a [16]. Instead, attenuation is most likely due to a combination of the *galE* mutation and one or more of the other mutations.

2.2. Genetically engineered mutagenesis

With the introduction of recombinant DNA technology, bacteriologists were able to genetically engineer defined mutations in bacteria. This meant that researchers were able to accurately characterize the mutations in attenuated vaccine strains. Mutations can be introduced into the *Salmonella* genome using homologous recombination such that the final live attenuated vaccine is free of antibiotic resistance genes [17,18]. Presently, regulatory agencies such as the U.S. Food and Drug Administration require a live attenuated vaccine strain to possess two independently attenuating mutations. Interestingly, the choice of background strain also plays a role in generation of effective live attenuated vaccine strains. In some backgrounds, certain mutations were fully attenuating whereas in other strains, the effect on virulence was not as profound [19,20].

Below, we describe some the most commonly mutated genes in live attenuated *Salmonella* vaccines which have been evaluated in human volunteer studies.

2.2.1. Aromatic acid biosynthesis pathway

The first live attenuated *Salmonella* vaccines contained mutations in aromatic acid biosynthesis pathway genes [21]. Deletion of genes involved in aromatic amino acid synthesis (*e.g.*, *aroA*, *aroC* and *aroD*) produces bacteria that are auxotrophic for para-aminobenzoic acid (PABA) and 2,3-dihydrobenzoate. When administered to mice, *Salmonella aro* mutants are unable to scavenge enough PABA and dihydrobenzoate to replicate [21]. Multiple pre-clinical studies have shown that *Salmonella aro* mutants elicit robust immune responses which can protect animals against lethal challenge [22–25].

2.2.2. htrA

HtrA (also known as DegP) is a serine protease that is induced by heat shock in *E. coli* and other *Enterobacteriaeceae* [26]. This protein degrades misfolded proteins in the bacterial periplasm. *S.* Typhimurium Δ*htrA* mutants show decreased survival within macrophages, decreased virulence in mice and are protective [27–31].

2.2.3. ssaV

The *ssaV* gene has been used as an attenuating mutation in *S.* Typhi and *S.* Typhimurium vaccines [32]. This gene is encoded on *Salmonella* Pathogenicity Island 2 (SPI-2) a Type 3 Secretion System (TTSS) which is required for virulence of *S.* Typhimurium in mice [33]. SPI-2 mutants show decreased survival within macrophages [34–36]. This pathogenicity island translocates *Salmonella* effector proteins across the bacterial inner and outer membranes to the host cell cytoplasm. SsaV forms part of the TTSS needle apparatus. *Salmonella ssaV* mutants are unable to secrete SPI-2 effector proteins [37].

2.2.4. PhoP-PhoQ virulence regulon

The PhoP/PhoQ regulon is a two component regulatory system which controls the transcription of multiple genes [38–40]. PhoP is a cytoplasmic transcriptional regulator and PhoQ is a membrane associated sensor kinase. This operon contributes to survival within macrophages and resistance to antimicrobial peptides [38,41]. *S.* Typhimurium *phoP* mutants are avirulent and can induce a protective immune response in mice [42,43].

2.2.5. Adenylate cyclase and cyclic AMP receptor protein

Cyclic AMP (cAMP) and cAMP receptor protein (CRP) are required for multiple essential cellular processes including transport of metabolites [44]. The *cya* gene is required for adenylate cyclase synthesis and *crp* encodes cAMP receptor protein. *S.* Typhimurium *cya* and *crp* mutants are attenuated in mice and protective in various animal models [45–48].

2.2.6. clpPX

At the CVD, we have deleted *clpPX* in several live attenuated *Salmonella* vaccines [49]. This is an attenuating mutation in *S.* Typhimurium and other *Salmonella* serovars and also has an added benefit. The *clpPX* genes encode a protease that degrades the master flagella regulator FlhD/FlhC [50,51]. The FlhD/FlhC complex is a transcriptional activator of the flagella synthesis pathway. When ClpPX is absent, FlhD/FlhC accumulates and large amounts of flagellin are produced. We have used this phenotype to our advantage to enable economical purification of flagellin from recombinant *Salmonella* strains for use as a carrier protein in conjugate vaccines [49].

2.2.7. Other genes

Many other mutations have been shown to produce effective live attenuated *Salmonella* vaccines in preclinical studies. For example, *S.* Typhimurium DNA adenine methylase (Dam) mutants are avirulent and can protect mice against lethal challenge [52–54]. Dam methylates adenine in GATC sequences and controls the expression of multiple *Salmonella* virulence genes

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