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Recombinant live *Salmonella* spp. for human vaccination against heterologous pathogens

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Abstract

Live attenuated *Salmonella* spp. are promising candidates as oral vaccine delivery systems for heterologous antigens. Clinical trials have demonstrated that this approach is feasible for human vaccinations but further optimisation is necessary to obtain a better efficacy. Here, we discuss how existing clinical and pre-clinical data can be used to guide such optimisation efforts.

Keywords: Attenuated live carrier; Clinical trial; *Salmonella* vaccine

1. Introduction

Vaccines offer efficient, cost-effective measures against widespread infectious diseases. Among the various delivery systems, orally administered live attenuated *Salmonella* spp. that express heterologous antigens are promising candidates as they are safe and highly immunogenic and can elicit long-lasting protective systemic and mucosal immune responses against the heterologous pathogen [1–7]. As an example, it was recently shown that the *Salmonella*-based approach is highly efficacious in an animal infection model of the important human pathogen *Helicobacter pylori* [8,9].

Despite the great success of *Salmonella*-based heterologous vaccination in various animal models, it remains a challenge to adopt this promising approach to human applications. In this review, we summarise the results obtained in human heterologous vaccination trials and discuss recent developments which can help to develop efficacious human vaccines based on live recombinant *Salmonella*.

2. Small animal vaccination model

All human trials with *Salmonella* as carriers conducted so far have used attenuated strains of the human-adapted serovar *Salmonella typhi*, the causative agent of typhoid fever. In humans, this serovar is systemically infective, and attenuated vaccines of *S. typhi* can elicit both mucosal and systemic immune responses.

Mice are the most widely used pre-clinical model for vaccination studies. *S. typhi* does not cause a systemic infection in mice under physiological conditions but mice can be infected with the mouse-adapted serovar *Salmonella typhimurium* which causes a systemic, typhoid-like syndrome that mimics the human disease caused by *S. typhi*.

A major concern is the safety of live attenuated carriers of heterologous antigens. To generate safe carrier strains, defined genetic lesions are introduced into specific genes of a virulent wild-type isolate, rendering it highly attenuated. The close resemblance of the mouse *S. typhimurium* model to the human *S. typhi* situation makes it possible to first define appropriate attenuations in the mouse *S. typhimurium* model [10–14] and then to introduce analogous mutations into *S. typhi* strains for human trials (see below). Correlation of attenuations in both systems has been successfully demonstrated for several specific mutations including the genes *aroA*, *aroC*, *aroD*, *htrA*, *cya*, *crp*, *cdt*, and *phoPQ*.

Live vaccines should be safe also in immuno-compro-

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mised vaccinees. Various attenuated *Salmonella* strains have been tested in transgenic mice with defined defects in their immune system to assure that their attenuations are safe even if the immune response directed against the vaccine strain is impaired [15–20]. The relevance of these observations for human vaccination is underscored by recent findings that patients with genetic IL-12p40 [21] or IL-12 receptor deficiencies [22,23] exhibit strongly increased susceptibility to disseminated infection with non-typhoid *Salmonella*.

Based on the detailed mouse data and the human trials using prototype heterologous *Salmonella* vaccines, it is now possible to discuss various aspects concerning the construction of a second generation of improved *Salmonella* carriers for human use. Important parameters for this strain construction are the choice of the *Salmonella* serovar, the particular genetic background of the carrier, the type of attenuating lesions and the expression system for the foreign antigen.

3. *Salmonella* serovar

In various animal models, both host-adapted and non-host-adapted serovars have been successfully used. In humans, however, only derivatives of the human-adapted serovar *S. typhi* have been tested so far although there is no principle reason not to try other *Salmonella* serovars. In particular for pathogens that are restricted to mucosal surfaces like *H. pylori*, the mucosal immune response elicited in humans by enteritic *Salmonella* serovars (e.g. *enteritidis*, *typhimurium*) could be sufficient to confer protection. Such enteric serovars are especially attractive as their wild-types cause much less severe disease in humans. As a consequence, less severe attenuations might be sufficient to prevent unwanted side effects which enhances the chance to retain immunogenicity (see below). In a recent primate example using an enteric serovar, rhesus macaques have successfully been vaccinated using attenuated *S. typhimurium* expressing an SIV antigen [26].

4. Genetic background

The genetic background of the vaccine carrier strain can significantly influence the immune response. For example, in the mouse model, two *S. typhimurium* strains that carry identical attenuations but have been derived from different isolates elicit significantly different serum IgG and mucosal IgA antibody responses against a heterologous antigen [24]. In humans, identically constructed *S. typhi aroC aroD* deletion strains that have been derived from different isolates induce significantly different immune responses [16] which confirm and extend the mouse data.

Interestingly, all human trials for heterologous vaccination have used carrier strains that had been derived from a single *S. typhi* isolate (Ty2) that has been cultured in the laboratory since its isolation in 1916.

5. Attenuations

A prerequisite for the use of live recombinant *Salmonella* for vaccination is a sufficient attenuation to prevent unwanted side effects like bacteremia, diarrhoea or fever. On the other hand, over-attenuation can lead to poor immunogenicity of the vaccine, indicating that a delicate balance between attenuation and over-attenuation must be maintained.

Mouse data indicate that various independent genetic defects can yield adequately attenuated *Salmonella* strains. Among strains that are attenuated to similar levels, the specific type of attenuation can strongly influence the immune response [27–29], adding further complexity to the choice of the attenuating mutations. Human live vaccine strains should carry at least two independently attenuating mutations to minimise the hypothetical risk of reversion to virulence by DNA transfer from wild-type organisms in the field. Deletion is the preferred type of attenuating mutation as compared to point mutations that can spontaneously revert to wild-type. Except for the *S. typhi* Ty800 strain which carries an attenuating mutation at a single chromosomal locus (*phoPQ*) [30], all strains that have been tested in humans so far fulfil both criteria.

6. Strains that have been used in human vaccine trials

Table 1 summarises comparable results from phase I clinical trials with various heterologous *S. typhi* vaccines. For a comparison, clinical results obtained for *S. typhi* strains that do not express heterologous antigens are shown in Table 2.

6.1. Ty21a

The chemically induced *S. typhi* mutant Ty21a is characterised by mutations in the galactose biodegradation pathway including *galE* [31], and an inability to synthesise the Vi capsular antigen. However, these defects do not account for the safety of Ty21a, since a genetically defined site-directed *galE* mutant which is also Vi negative retains its virulence in human volunteers [32]. Therefore, the mutations that cause the attenuation of Ty21a remain undefined. Ty21a has been given as an anti-typhoid vaccine to more than 327 000 subjects world-wide in several controlled efficacy trials (recently reviewed in [33]). The strain is very well tolerated even at doses of 10^{11} orally applied living organisms [34], and pooled results from five clinical toxicity studies involving some 700 subjects of all age groups showed very few adverse reactions in vaccinees (2% fever and vomiting, and some 5% mild diarrhoea)

Table 1

Vaccine strain (background)	Mutation	Heterologous antigen	Vaccination regimen	Immune responses to heterologous antigen				% Vaccine efficacy (protected vaccinees)	Reference
				% Ig response[a]	% ASC (IgA)	ASC/10^6 PBMs	$CD8^+$ CTL		
5076-1C (Ty21a)	Several	*Shigella sonnei* O antigen (+120-MDa plasmid)	Oral, $\sim 10^9$ (3×)	21–35	100	10	n.d.	53–71	[41–43,68]
EX645 (Ty21a)	Several	*V. cholerae* O antigen	Oral, $\sim 10^{10}$ (3×)	7–43	7	n.d.	n.d.	25	[38,40]
χ4632 [pYA3167] (Ty2)	*cya*, *crp–cdt*, *asd*/*asd*$^+$	Hepatitis B core pre-S fusion on *asd*$^+$ plasmid	Oral, 10^7–10^9	0	0	0	n.d.	n.d.	[46]
χ4632 [pYA3167] (Ty2)	*cya*, *crp–cdt*, *asd*/*asd*$^+$	Hepatitis B core pre-S fusion on *asd*$^+$ plasmid	Oral, rectal, 10^8–10^9 (3×)	17	n.d.	n.d.	n.d.	n.d.	[48]
CVD908 Ω(Δ*aroC*::*tac*P-r*csp*) (Ty2)	*aroC*, *aroD*	*P. falciparum* circumsporozoite protein $CSP_{21\ 398}$, inserted into *aroC*, *tac* promoter	5×10^7 (2×)	20	n.d.	n.d.	10	n.d.	[53]

n.d.: not determined.
[a]Summary of all serological parameters including serum IgA and IgG and local IgA as well as bactericidal antibodies.

[33]. Depending on the vaccine formulation and the country where the study was performed, Ty21a exhibited between 33% and 96% protective efficacy, and protection lasted for at least 4 years [33]. However, the vaccine has a low immunogenicity and requires 3–4 oral doses to achieve optimal protection [35,36].

In addition to its world-wide use as an anti-typhoid vaccine, *S. typhi* Ty21a has also been used as a carrier for heterologous antigens [37–40]. Lipopolysaccharides (LPS) from *Shigella* were introduced into Ty21a via transfer of a 120-MDa *Shigella* plasmid which likely expressed additional *Shigella* protein antigens. Administration of three doses of this *S. typhi–Shigella* bivalent vaccine resulted in 50–70% protection against *Shigella*-induced bloody diarrhoea in human volunteer challenge studies [41]. Protection correlated with the presence of *Shigella* LPS-specific serum IgA and IgG levels prior to challenge [41], while no correlation was observed between protection and the frequency of *Shigella*-specific antibody-secreting cells (ASC), which were detected in all vaccinees [42]. Unfortunately, several subsequently prepared lots of the vaccine including a large scale lyophilised preparation were entirely ineffective [41,43], perhaps owing to frequent rearrangements of the LPS-encoding *Shigella* plasmid in *Salmonella* [44].

To construct a bivalent typhoid–cholera vaccine, *Vibrio cholerae* LPS biosynthesis genes were expressed in *S. typhi* Ty21a from a *thyA*-stabilised plasmid (see below). Small

Table 2
Comparison of clinical data for *S. typhi* human vaccines

Strain	Mutation	Oral dose[a]	Reactogenicity	Bacteremia	With diarrhoea (%)[b]	With rise in serum IgG (%)[c]	With ASC (IgA) in PBM (%)	ASC (IgA)/10^6 PBM	Reference
Ty21a	Multiple	10^{10} (4×)	None	?	None	63	60–80	n.d.	[54]
χ3927	*cya*, *crp*	5×10^5	Some	17	n.d.	33	50	n.d.	[25]
χ4073	*cya*, *crp–cdt*	5×10^8	None	0	20[d]	80	80	15	[46]
χ4632 [pYA3167]	*cya*, *crp–cdt*, *asd*/*asd*$^+$	3×10^8	Some	n.d.	Several	43	n.d.	n.d.	[48]
CVD906	*aroC*, *aroD*	5×10^7	Some	56	0	89	100	1750	[25]
CVD906-*htrA*	*aroC*, *aroD*, *htrA*	5×10^8	None	0	13	75	100	80	[52]
CVD908	*aroC*, *aroD*	5×10^8	Some	100	0	100	100	1060	[25,52]
CVD908-*htrA*	*aroC*, *aroD*, *htrA*	5×10^8	None	0	13	100	100	120	[52]
Ty800	*phoPQ*	5×10^8	None	0	0	100	100	830	[30]
Ty445	*phoPQ*, *aroA*	10^{10} (2×)	None	0	10	14	n.d.	n.d.	[54]

n.d.: not determined.
[a]Single oral dose unless otherwise noted.
[b]Trials involved between three and 14 vaccinees.
[c]*S. typhi*-specific serum IgG against somatic and/or flagellar antigen.
[d]At a dose of 5×10^7.

scale clinical trials demonstrated sero-conversion against *V. cholerae* LPS in 7–43% of human vaccinees by at least one of several parameters, and the vaccine protected 25% of volunteers from challenge with virulent *V. cholerae*, with the additional positive effect that diarrhoea in non-protected subjects was much milder than in unvaccinated controls [38,40].

6.2. χ4073 and χ4632

S. typhi Ty21a has been followed by a second generation of *S. typhi* vaccine strains that carry defined genetic lesions. In *S. typhi* χ3927 Δ*cya* Δ*crp*, deletions in the regulatory *cya* (adenylate cyclase) and *crp* (cyclic AMP receptor protein) genes affect the expression of a large variety of genes including carbon, phosphate and nitrogen metabolism, pH regulation, iron uptake, fimbria and flagella biosynthesis, and cause reduced growth rates and cell sizes in vitro [45]. This strain retains some reactogenicity (eliciting of adverse side effects) in human vaccinees [25], and has therefore been further attenuated by an additional deletion extending from the *crp* gene to the *cdt* gene, the latter being required for the colonisation of deep tissues in mice [12]. The resulting strain χ4073 was well tolerated in doses up to 5×10^8 orally applied colony forming units (cfu) in humans [46]. For a stable and high expression of heterologous antigens using a balanced–lethal system (see below), an additional mutation in *asdA1* has been introduced to obtain *S. typhi* χ4632 [47].

Hepatitis B virus (HBV) pre-S envelope proteins S1 and S2, fused to the HBV core protein (HBc-pre-S), have been expressed as heterologous protein antigens in *S. typhi* χ4632 [46,47]. HBc-pre-S is constitutively and stably expressed in vitro at $>1\%$ of total cellular protein from a pBR-based, *asd*-stabilised plasmid (see below). No sero-response to this heterologous antigen was elicited in humans after a single oral dose in a study at the Center for Vaccine Development in Baltimore, MD, USA [46]. A similar study using the same vaccine was performed at the Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland, with the exception that the vaccine was applied in three doses, spaced by several weeks, and was given orally or rectally to female volunteers only [48]. Out of six rectally immunised volunteers, only one sero-converted against the pre-S1 antigen. This individual was the only one that sero-converted against *S. typhi* LPS and in addition suffered from severe and prolonged diarrhoea, suggesting a possible link between severity of reaction to the vaccine and its immunogenicity. None of seven orally immunised subjects sero-converted against pre-S1 [48].

6.3. CVD908 and CVD908-htrA

The strain *S. typhi* CVD908 carries two independent deletions (*aroC* and *aroD*) in the common aromatic biosynthesis pathway [49]. The mutations cause an obligate requirement for *para*-aminobenzoic acid (PABA), which is present in only limiting amounts in host tissues relevant to infection. PABA is an intermediate in the synthesis of folate, which in turn functions as a cofactor in the majority of one-carbon transfer reactions, including the formulation of the translational initiator fMet-tRNA^{fMet} [50]. Thus, the ultimate inability to initiate protein biosynthesis after depletion of an intracellular pool of folate has been speculated to account for both the initial replication of various *aro* mutants in host tissues as well as their subsequent growth arrest [51]. In severely immunodeficient mice, however, *aroA* mutants have recently been shown to be virulent [15,16], indicating that *aro* mutant strains can replicate in host tissues. Possibly, replication is not fast enough to prevent the elimination by a functional host immune system whereas even slow bacterial growth could not be controlled in severely immunodeficient hosts. An insufficient attenuation by *aro* mutations was also observed in human trials in which the strain *S. typhi* CVD908 Δ*aroC* Δ*aroD* was found to cause unacceptably high levels of silent bacteremia in healthy volunteers. To further attenuate this strain, an additional mutation was introduced in the *htrA* gene that encodes a periplasmic serine protease [13] to obtain CVD908-*htrA*, which is save and immunogenic in humans [52].

A truncated version of the *Plasmodium falciparum* protein CSP has been constitutively expressed in *S. typhi* CVD908 by chromosomal integration of the *csp* gene into its already mutated *aroC* locus. Two oral doses of 5×10^7 cfu resulted in sero-conversion in two out of 10 vaccinees, while a third vaccinee developed antigen-specific $CD8^+$ cytolytic activity [53]. To our knowledge, no data for heterologous vaccination using the improved carrier strain CVD908-*htrA* have yet been reported.

6.4. Ty800 and Ty800-aroA

The *S. typhi* strain Ty800 carries a deletion in the *phoPQ* two component global virulence gene regulatory system. This strain is safe and highly immunogenic in humans as indicated by high frequencies of antigen-specific ASC [30]. An additional deletion in *aroA* leads to an obligate requirement for PABA (see above) which over-attenuates the resulting strain Ty800-*aroA*, rendering it only poorly immunogenic even at multiple high doses [54].

To our knowledge, no data for the strain *S. typhi* Ty800 as a carrier for foreign antigens in human trials have yet been reported.

7. Antigen expression

The expression level of foreign antigen can strongly influence the immune response that is induced by live recombinant *Salmonella* spp. (e.g. [24]). The desired high heterologous expression for a sufficient amount of time

is often hampered by the rapid in vivo loss of plasmids encoding foreign antigens [55].

Integration of the foreign gene into the *Salmonella* chromosome has been successfully used to obtain recombinant strains that very stably express heterologous antigens. In the case of *S. typhi* CVD908, chromosomal insertion of the heterologous gene *csp* leads to expression levels high enough to elicit some human immune responses against this foreign antigen (see Table 1) [53].

Because of the low copy number, the amount of heterologous antigen that can be expressed from the genes integrated into the *Salmonella* chromosome is usually smaller as compared to plasmid-based expression. An alternative for increasing the stability of expression at higher copy numbers is to use a balanced–lethal system in which a lethal chromosomal mutation is complemented by a plasmid that carries both a functional copy of the same gene and an expression cassette for the foreign antigen. In one such system, a mutation in the chromosomal aspartate β-semialdehyde dehydrogenase (*asd*) gene leads to an obligate requirement for diaminopimelic acid, which can be complemented by a plasmid carrying a functional copy of the *Salmonella asd* gene [56,57]. In another balanced–lethal system, thymidine requirement caused by a mutation in the chromosomal thymidylate synthetase (*thyA*) gene is complemented by a plasmid carrying a functional *thyA* gene [58]. Both systems allow for a stable, high expression of foreign antigens in recombinant *Salmonella* strains and have been used with some success in human vaccine trials [38,40,46,48]. In addition, both systems provide the additional advantage that no antibiotic resistance marker is required for the selection of transformed bacteria. This is an important aspect as antibiotic resistance markers are not acceptable for constructs to be used in human vaccination trials.

8. Conclusions

The results obtained for the various *S. typhi* prototype carriers and expression systems demonstrate the general feasibility of human vaccination with live *Salmonella* expressing foreign antigens. The results summarised in Table 2 demonstrate the general correlation between the pathogenic potential (reactogenicity and the ability to cause bacteremia) and the immunogenicity of the vaccine. Thus, the highly attenuated *S. typhi* Ty21a is poorly immunogenic. For the strains *S. typhi* χ3927, CVD906, CVD908 and Ty800, the introduction of additional attenuating mutations reduced both the reactogenicity and immunogenicity. The ability to induce mild diarrhoea appears to be a frequent problem with the *S. typhi*-based live vaccine strains.

Despite some success in human trials, the immune responses induced by live *Salmonella* expressing foreign antigens are weaker than those observed for analogous constructs in various animal models. It should be noted that with the exception of one trial (*S. typhi* CVD908 expressing CSP), only humoral responses but not the important cell-mediated immune responses against the heterologous antigen were monitored. Depending on the pathogen and the appropriate correlate of protection, measuring just antibody responses can considerable underestimate the efficacy of a vaccine as has been demonstrated, for example, for rhesus macaques receiving *Salmonella* expressing an antigen from SIV [26]. Nevertheless, further improvement of the immunogenicity of *Salmonella*-based vaccines for human use seems to be necessary. Based on the human trials and the extensive experience gained in the mouse system, a number of options for such an improvement exist.

As all human heterologous vaccination studies have used strains derived from a single isolate of only one serovar, the use of other *Salmonella* serovars and/or isolates might be an obvious option to get better carrier strains. Unfortunately, it seems to be difficult to predict the immunogenicity of a new isolate based on pre-clinical data. Hence, empirical testing in human trials would be necessary to obtain better carrier strains.

Only a limited set of attenuating mutations have been tested in the human trials. In the mouse model, several new mutations have been identified that yield safe strains with superior immunogenicity as compared to the commonly used *aro* strains. More such mutations are likely to be found in the future. Some of these mutations could be interesting candidates to be tested in carriers for human use.

Additional innovative approaches that were developed using animal models and have yet not been tested in human trials include the use of the two-phase variation system for the expression of antigens that are toxic to *Salmonella* [59,60], presentation of the antigen in different compartments of the bacteria or the infected host cell [61,62,68], co-expression of immuno-modulatory molecules [63–66]), and the use of in vivo inducible promoters to achieve localised high level expression of the foreign antigen [55,67]. These new approaches illustrate the great potential for improvement of live attenuated *Salmonella* spp. as vaccine delivery systems for efficacious and cost-effective human vaccination.

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