Novel strains of *Salmonella typhimurium* as potential vectors for gene delivery

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**Abstract**

DNA vaccines are known to induce long-term antigen specific cellular responses. We tested two new strains of *Salmonella typhimurium*, one carrying a mutation in a SPI-2 gene and the *aroC*-gene and another carrying mutations in the *sifA-* and *aroC-*genes, as potential DNA vaccine delivery vehicles. We compared them with the SL7207 strain and found that the new strains were more invasive, and that they were efficient mediators of gene transfer in vitro using EGFP as reporter gene. We tested the ability of the new strains to survive within the spleen, liver and mesenteric lymph nodes and evaluated their safety in C57/BL/6J mice.

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1. Introduction

DNA vaccines are known to induce long-term antigen specific cellular responses and show promise as anti-cancer agents. Unfortunately, conventional methods of vaccination with DNA often require prohibitively large amounts of DNA to be administered in order to induce appropriate immune responses. This problem is compounded in larger mammals such as cattle and man, and there is a clear need to develop more efficient and cost effective delivery systems to circumvent this problem. Live attenuated bacterial vectors such as *Salmonella enterica typhimurium* (*S. typhimurium*) have shown considerable promise as efficient delivery vehicles for DNA. The potential advantage of such bacterial vectors is their ability to efficiently transfer foreign DNA under eukaryotic promoter control to key inductive cells of the immune system, and to simultaneously up-regulate the expression of appropriate co-stimulatory molecules such as B7.1 and B7.2. The transferred DNA is then subsequently expressed by host cells leading to B- and T-cell stimulation and induction of a protective immune response (reviewed in [1]). In addition to their immuno-stimulatory and delivery capabilities, bacterial vectors have additional advantages over alternative approaches including their relatively low cost of manufacture and ease of administration. These characteristic make *Salmonella* an ideal vehicle for delivering cancer antigens in the development of novel anti-cancer immunotherapies.

Modern molecular biology techniques and increasing knowledge of *Salmonella* pathogenesis has provided numerous new targets for attenuation, this has lead to the generation of a variety of different mutant strains of *S. typhimurium*. Many of these strains have been used as safe and effective vehicles for delivery of foreign DNA...
The bacterial strains used in this study were CD12 (TML sifA-aroC-) and WT05 (TML ssaV-aroC-) obtained from Microscience Ltd, Winnersh Triangle, UK, and SL7207-aroA544::Tn10, kindly provided by Prof. B. Stocker, Stanford University, USA. Bacteria were grown in Luria–Bertani (LB) medium (Sigma) supplemented with a mixture of 4-amino benzoic acid (BDH), 2,3-dihydroxy benzoic acid (Sigma), L-phenylalanine, L-tryptophan and L-tyrosine (Sigma) (Aromix). Bacterial growth curves were obtained using optical density measurements (with BioPhotometer, Eppendorf AG Hamburg) and by plating serial dilutions of bacterial culture on Luria–Bertani agar (Sigma). The generation time for each strain was subsequently calculated and compared.

2.2. Isolation of murine macrophages

To obtain primary murine macrophages 1 ml of 3% (vol/vol) Brewer’s thioglycollate medium (Sigma) was injected into the peritoneal cavity of C57/BL/6J mice to produce inflammatory macrophages, which were harvested 7 days after this procedure. Isolated macrophages were allowed to adhere for 2 h at 37 °C, the non-adherent cells were then removed by two washes with antibiotic-free medium.

2.3. Isolation of murine dendritic cells

Spleens from naïve C57/BL/6J mice were perfused with collagenase type IV with addition of DNase I (50 µg ml⁻¹). Spleens were cut into small pieces and digested in 5 ml of collagenase/DNase for 45 min at 37 °C. Single spleen cell suspensions were prepared and incubated with colloidal superparamagnetic CD11c MicroBeads (Milteni Biotec). Following magnetic separation using autoMACS (Milteni Biotec) CD11c+ cells were immediately sorted out in a flow cytometer.

2.4. In vitro infection studies

The bacteria were grown in LB broth supplemented with Aromix at 37 °C with moderate shaking. Human U937 monocyte cell line (ECACC 85011440) and mouse monocyte/macrophage cell line J774.2 (ECACC 85011428) were used for all in vitro infection assays. Cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies), 2 mM glutamine, 100 units ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin (Sigma). Immediately prior to infection, cells were washed with saline and suspended in complete medium without antibiotic or serum. The bacteria were added at a ratio 50:1 for 30 min. Following the infection the medium was supplemented with gentamicin (100 µg ml⁻¹) (Gibco BRL) for 60 min, and then replaced with fresh medium containing 10% serum, 2 mM glutamine and gentamicin (16 µg ml⁻¹). The cells were subsequently lysed at different time intervals in 1% Triton X-100 (vol/vol with saline) for 20 min following three washes with saline.
2.5. In vivo infection studies

Female C57/BL/6J mice (8–10 weeks old) were used for all infection studies and were inoculated orally with 1 x 10^9 CFU in 200 μl of saline. To prepare the inocula bacteria were grown overnight at 37 °C in 3 ml of LB broth in a 10 ml falcon tube with shaking (300 rpm) and were then used to inoculate fresh medium (1:100). Fresh inocula were grown under the same conditions for 2–3 h until an optical density at 600 nm of 0.3–0.6 was reached. Bacterial cultures were then diluted in physiological saline and the CFU were enumerated by plating a dilution series of the inoculum. To determine the bacterial load in the spleen, liver and mesenteric lymph nodes (MLN) the organs were removed and placed in 1–3 ml of saline and homogenised. Eukaryotic cells were then harvested by centrifugation at 1500g and resuspended in 0.01% sodium deoxycholate at room temperature for 15 min to lyse the eukaryotic cells. Viable bacterial CFU were determined by plating aliquots of a dilution series of the lysate onto LB agar (Sigma).

2.6. Transformation of S. typhimurium with EGFP plasmid vector

Strains CD12 and WT05 were electroporated with pIRES2-EGFP plasmid (Clontech) according to the following protocol. A single colony of bacteria was inoculated into fresh medium and grown overnight at 37 °C to mid log phase, harvested bacteria were then chilled for 15 min on ice and washed twice with ice-cold water. Freshly prepared bacteria were then admixed with plasmid (0.1–0.5 μg) in a 0.2-cm cuvette and electroporated with a Bio-Rad Gene Pulser at 2.5 kV, 25 μF and 200 Ω. The bacteria were then added to 1 ml of S.O.C. Medium (GIBCO) and incubated with moderate shaking for 90 min at 37 °C. The transformed culture was then plated onto LB agar plates with kanamycin (50 μg ml⁻¹). Resistant colonies harbouring the DNA plasmid were cultured and stored in glycerol. Plasmid stability within both strains of S. typhimurium was established by growing bacteria on agar plates with and without kanamycin (as per antibiotic resistance gene in pIRES2-EGFP plasmid).

2.7. Assessment of gene transfer from S. typhimurium to primary macrophages

Gene transfer from S. typhimurium to murine peritoneal macrophages was determined using EGFP plasmid as described above. An attenuated strain of S. typhimurium harbouring EGFP plasmid was added to macrophages at a ratio 50:1 and incubated for 30 min at 37 °C. Cells were then incubated with medium containing gentamicin (100 μg ml⁻¹) for 1 h. After 1 h medium was changed to complete medium with 10% serum, 2 mM glutamine and gentamicin (16 μg ml⁻¹). After another 4 h incubation, 10 μg ml⁻¹ of tetracycline was added to block intracellular bacterial multiplication and thereafter incubation was continued for another 48 h. The medium was then removed and the cells were washed and evaluated by fluorocytometry on the Fluorescent Antibody Cell Sorter (FACS) and under fluorescent microscope.

3. Results

3.1. Infection and intracellular survival of CD12 and WT05 strains of S. typhimurium within murine macrophage cell line J774.2 and human monocyte cell line U937

To determine behaviour and viability of CD12 and WT05 strains of S. typhimurium we looked at the growth characteristics of the three strains in LB medium. The generation time for the WT05 strain was established at 41.3 min, strain CD12 – 86.4 min and strain SL7207 – 110 min. We subsequently infected U937 cells and compared their intracellular survival. The CD12 strain was more invasive (Fig. 1(a)). Similar results were seen with the murine macrophage cell line J774.2, with the CD12 strain proving more invasive at the early stage of infection (Fig. 1(b)). Intracellular survival times within different cell lines were comparable.

The infection patterns of CD12 and WT05 strains were compared to SL7207 strain carrying a single aroA mutation. SL7207 has traditionally been used in many gene transfer experiments [2,5] with some success. Our results indicate that the new strain CD12 is consistently more invasive (p-value 0.018–0.089) with larger numbers of bacteria detectable within cells after 2 h, which may enhance the strains ability to mediate gene transfer to host cells (Fig. 2(a) and (b)).
3.2. Infection of murine dendritic cells with CD12 and WT05 strains of *S. typhimurium*

To evaluate the new strains and compare them with SL7207 we infected murine dendritic cells. The infection rates resembled those observed using cell lines (Fig. 3), with both CD12 and WT05 proving more invasive than SL7207. The cells were only viable for 48 h due to known *Salmonella* toxicity to primary cells [11] (Fig. 2).

3.3. Assessment of gene transfer using CD12 and WT05 strains in primary mouse macrophages

We have shown using EGFP as a reporter gene that CD12 and WT05 mediated gene transfer is efficient in J774.2 cell line, with up to 98% of cells expressing detectable levels of reporter gene product as determined by FACS staining (Fig. 3(a)). Darji and co-workers have shown that gene transfer was also high in primary mouse macrophage cells, with up to 30% of infected cells expressing detectable levels of the reporter protein [2]. Therefore we also evaluated CD12 and WT05 mediated gene transfer efficiency in these cells. Following infection of primary macrophages with CD12 and WT05 strains carrying the EGFP reporter gene, we have shown that the efficiency of gene transfer was good despite poor plasmid stability (range 11.6–13.8%). Around 64% of viable macrophages infected with WT05 expressed GFP, for strains CD12 and SL7207 expression was lower with 32% and 28%, respectively (see Figs. 3(b) and 4).

Plasmid integrity was tested by preparation of the plasmid from *salmonella* and restriction analysis.

3.4. Isolation of live bacteria from liver, spleen and MLN following oral inoculation

To determine the in vivo growth kinetics of CD12 and WT05 we inoculated C57/BL/6J mice orally and isolated live bacteria from liver, spleen and MLN on day 2...
and 5. Oral administration of up to $10^8$ CFU to mice did not cause any noticeable adverse events. The numbers of bacteria recovered after oral inoculation were compared with the SL7207 strain. The numbers of CD12 and WT05 bacteria recovered from spleen, liver and MLN decreased steadily over time in a predictable fashion (see Fig. 5(a)–(c)). The SL7207 strain bacteria also decreased in number by day 5.

4. Discussion

The identification of numerous tumour-specific and tumour-associated antigens have already led to specific immunotherapy strategies for a range of human cancers. As in the context of viral antigens in infectious disease models, anti-cancer DNA vaccines comprise the nucleotide sequence of one or more of tumour-associated T cell epitopes cloned into a plasmid vector and constitutively expressed in the host tissues via a viral promoter [12]. In this way, marked and long-lasting cellular (both CD4+ and CD8+ T cell) and humoral anti-tumour immunity has been demonstrated, particularly in murine models of melanoma, suggesting great promise for human application [13]. *Salmonella* mediated delivery of DNA offers many advantages over more traditional methods of administration such as injection via the im or id routes. Firstly, the ability of these vectors to target key immunologically relevant cells should allow more efficient delivery of the DNA to the host. Secondly, *Salmonella* stimulates the expression of appropriate co-stimulatory molecules within APCs to maximise T-cell activation. Thirdly, bacterial/DNA vector systems are relatively cheap to manufacture and can be administered orally, allowing the bacteria to transfer DNA directly to the abundant APCs in the gut mucosa [2,3,14]. As in the case of im and id delivery of DNA, the precise mechanism of gene transfer and expression after oral administration is not clear, but the *Salmonella/plasmid* may be scavenged by DCs in the mucosa, expressed within these cells by the time they track to regional lymph nodes to prime naive T cells.

In this study, two new strains of *S. typhimurium* were assessed for invasiveness and gene transfer. Mutants with deletions in the *sifA* gene such as CD12 are unable to maintain the integrity of their SVC [8,9,15] and may consequently prove to be more efficient at transferring DNA to the host than alternative strains. The WT05

![Fig. 4. In vitro gene transfer assessment following infection of primary mouse macrophages with *S. typhimurium* strain WT05 (a), CD12 (b) carrying EGFP plasmid as seen under fluorescent microscope. Figure (c) represents a positive control cells – J774.2 murine macrophage cell line transfected with EGFP plasmid. Figure (d) shows negative control cells for the above experiment (macrophages without EGFP plasmid) (magnification 40×). The image has been enhanced for clearer picture.](https://academic.oup.com/femsle/article-abstract/238/2/345/490836)
strain has now been tested in human volunteers and was shown to be immunogenic [16]. Therefore both CD12 and WT05 represent interesting new Salmonella strains that warrant further evaluation as gene delivery vehicles. The results of our in vitro experiments indicate that the CD12 strain of S. typhimurium appears to be more invasive than WT05. The numbers of viable bacteria recovered from mice were higher than those of the SL7207 strain, which probably reflects differences in the background of the strains (TML vs SL7207). In addition, both CD12 and WT05 have been shown to effectively mediate gene transfer in vitro following infection of primary mouse macrophages.

This data suggests that both CD12 and WT05 show considerable promise as gene transfer vehicles that merit further evaluation. We are currently testing the new strains of S. typhimurium in an in vivo cancer model of murine mammary carcinoma.

![Graphs showing CFU counts over time for different strains](image)

References


[11] Dreher, D., Kok, M., Coohand, L., Kiama, S.G., Gehr, P., Pechere, J.C. and Nicod, L.P. (2001) Genetic background of mutated bacteria are unable to survive beyond Peyers patches [17]. However, we have shown it is possible to isolate live bacteria from MLN, liver and spleen of mice five days after oral inoculation. Interestingly the numbers of CD12 and WT05 bacteria recovered from mice were higher than those of the SL7207 strain, which probably reflects differences in the background of the strains (TML vs SL7207).

Fig. 5. Survival of S. Typhimurium strain CD12 (black bars), WT05 (grey bars) and SL7207 (white bars) within spleen (a), liver (b) and mesenteric lymph nodes (c). C57/BL/6J mice were inoculated orally with 1 x 10^9 bacteria, the spleen, liver and mesenteric lymph nodes were subsequently removed and homogenised. Viable bacterial CFU were determined by plating aliquots of dilution series onto LB agar. A total number of CFU from each organ was then compared.

![Graph showing survival over time for different strains](image)


