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A suicidal strain of *Listeria monocytogenes* is effective as a DNA vaccine delivery system for oral administration

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ABSTRACT

In this study we determined the *in vivo* activity of model ovalbumin vaccines delivered by direct intramuscular delivery of plasmid DNA or oral delivery using a recombinant suicidal *Listeria monocytogenes* strain (rsΔ2). In a previous report we described how rsΔ2 is capable of delivering luciferase, as protein or DNA, *in vitro*, into non-dividing intestinal epithelial cells (Kuo et al., 2009). This is achieved by engineering a dual expression shuttle vector, pDuLX-Luc, that replicates in *E. coli* and rsΔ2 and drives gene expression from the *Listeria* promoter (P*hly*) as well as the eukaryotic cytomegalovirus promoter (CMV), thereby delivering both protein and plasmid DNA to the cell cytoplasm. For the current *in vivo* study rsΔ2 containing pDuLX-OVA was used to deliver both ovalbumin protein and the mammalian expression plasmid by the oral route. Controls were used to investigate the activity of this system versus positive and negative controls, as well as quantifying activity against direct intramuscular injection of expression plasmids. Oral administration of rsΔ2(pDuLX-OVA) produced significant titres of antibody and was effective at inducing targeted T-cell lysis (approximately 30% lysis relative to an experimental positive control, intravenous OVA-coated splenocytes + lipopolysaccharide). Intramuscular injection of plasmids pDuLX-OVA or p3L-OVA (which lacks the prokaryotic promoter) also produced significant CTL-mediated cell lysis. The delivery of the negative control rsΔ2 (pDuLX-Luc) confirmed that the observed activity was induced specifically by the ovalbumin vaccination. The data suggest that the oral activity of rsΔ2(pDuLX-OVA) is explained by delivery of OVA protein, expressed in rsΔ2 from the prokaryotic promoter present in pDuLX-OVA, but transfection of mammalian cells *in vivo* may also play a role. Antibody titres were also produced by oral delivery (in rsΔ2) of the p3L-OVA plasmid in which does not include a prokaryotic promoter.

1. Introduction

Bacterial vehicles for vaccine delivery are of considerable interest due to their potentially low cost of production, and the possibility that they could achieve adequate vaccination by oral administration. These advantages could facilitate a large scale vaccination program against the global health threats caused by malaria, tuberculosis, emerging viruses or other infectious diseases. Several bacterial strains have been evaluated as delivery carriers for vaccines including non-pathogenic or attenuated pathogens using organisms including *Shigella flexneri*, *Salmonella* spp., *Escherichia coli*, *Vibrio cholerae*, *and Listeria monocytogenes* [1–5]. Of these *Listeria monocytogenes* (*L. monocytogenes*) has several unique features that make it a promising candidate for vaccine delivery. This organism is capable of invading, surviving and replicating in the majority of mammalian host cells, has a built-in mechanism for propelling itself from one cell to a neighbouring

Abbreviations: BHIC, charcoal-treated brain heart infusion broth; FACS, fluorescence-activated cell sorting; FCS, fetal calf serum; FBC, fetal bovine serum; CSFC, carboxyfluorescein diacetate succinimidyl ester; Luc, luciferase; MHC, major histocompatibility complex; OVA, ovalbumin; PBS, phosphate-buffered saline; pCMV, plasmid expression vector carrying cytomegalovirus promoter.

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cell, naturally infects intestinal epithelia, and can infect both phagocytic and non-phagocytic cells. The challenge with *Listeria* is how to build safety into the delivery system by reducing pathogenicity whilst retaining some of the natural advantages of the organism [5–10].

L. monocytogenes has the capacity to stimulate the major histocompatibility complex (MHC)-I and MHC-II pathways and induces the proliferation of antigen-specific T lymphocytes. It can induce therapeutic immunity against a wide range of tumour-associated antigens and infects and kills tumour cells directly. For this reason *Listeria*- mediated immunotherapy has been explored clinically using parenteral administration [11]. Paterson et al. reported that *L. monocytogenes* can effectively initiate an immune response by delivering tumour antigens, both using bacterially expressed protein and encoded in eukaryotic systems [12]. Chen et al. showed that live attenuated *L. monocytogenes* when administered parenterally can induce a T-cell response against tumour associated antigens [13]. *L. monocytogenes* has been reported to be an ideal system for delivery of fusion proteins to increase immune responses [11].

A number of studies have attempted to use engineered attenuated strains of *L. monocytogenes* as vaccines for parenteral use, using intramuscular, intradermal or subcutaneous injection as the route of administration [14–21]. The pioneering *in vivo* work of the Paterson group has shown that recombinant engineered *L. monocytogenes* can successfully express influenza virus nucleoprotein (NP) and subsequently provide systemic immunity after parenteral administration. The unique biological properties of attenuated *L. monocytogenes* have encouraged researchers to investigate this bacterium as a potential therapeutic vaccination vector to treat or detect a number of cancers [12,22–24]. These characteristics include (i) the potential to deliver intracellular DNA, RNA or protein to cancer cells both *in vitro* and *in vivo* [25] (ii) the ability to infect and survive in antigen presenting cells [19] and (iii) the potential to be used as a vector for expression of tumour-specific antigens [25–29]. *L. monocytogenes* has also been used as a parenteral delivery vehicle to vaccinate against a number of viruses infecting humans. These include HIV, where vaccines have been developed to drive strong and functional HIV-specific cellular immune responses [30–32], human papilloma virus-16 (HPV-16) [21,33,34], and Hepatitis C [35].

To reduce the virulence of *Listeria* species several attenuated strains have been produced. This raises the possibility that attenuation may compromise the potential of the bacterial delivery system. Goebel and colleagues showed that a mutation in a single gene has significant effects on the T cell responses in the infected host after intraperitoneal administration [36], suggesting that engineered *Listeria* need to be evaluated on a case-by-case basis. Nevertheless attenuated strains can still induce useful responses. Yang and colleagues showed that the replication-deficient *L. monocytogenes* strain ΔdalΔdat (Lmdd) was able to secrete the CD24 protein after parenteral administration and elicit specific immune responses which reduced tumour size and extend tumour-free survival in mice [23].

Intramuscular delivery of bacteria expressing proteins might be expected to be effective in inducing immune responses, the main limitation of such a strategy being safety and off-target immune responses. There are alternative approaches to vaccination when intramuscular delivery is acceptable. Indeed intramuscular injection of naked DNA expression plasmids causes gene expression in muscle cells and can induce generation of cytotoxic T lymphocytes. Wolff et al. showed that when plasmid DNA encoding reporter enzymes was injected, the intramuscular route resulted in expression of a significantly higher amount of protein than direct injection into other tissues [37].

The most interesting aspect of *L. monocytogenes* from a delivery viewpoint is its capacity to infect the host when administered orally [17,18]. Since this organism is capable of infecting the gastrointestinal epithelium, it is particularly attractive to explore its use as a generic delivery system for oral vaccination. However, little is known about the responses to *Listeria*-mediated oral delivery. The unique intracellular life cycle of *L. monocytogenes* has encouraged Goebel and colleagues to explore this species for cytoplasmic transfer of plasmid DNAs and the eukaryotic expression of exogenous genes in the nucleus of eukaryotic cells [1,8]. Building on this work we engineered a recombinant suicidal strain of *L. monocytogenes* using the attenuated Δ2 strain and have shown previously that the suicidal strain (rsΔ2) is lysed once it enters the cytoplasm and has the capacity to deliver proteins and genes in fully differentiated Caco-2 monolayers *in vitro* [9]. Vaccines can be delivered by rsΔ2 in the form of protein expressed by the bacteria, gene expression plasmids for transfection of mammalian cells, or both approaches simultaneously. Antigen delivery is facilitated by 'shuttle' plasmids that are engineered in *E. coli,* using the normal processes of recombinant DNA technology, and include DNA sequences that promote replication in *Listeria* or act as promoters to drive gene expression. The hypothetical mechanism for vaccination using a dual prokaryotic and eukaryotic expression plasmid (such as our pDuLX plasmid [9]: see below and Fig. 2) is shown in Fig. 1. pDuLX also includes five binding sites for NF-κB, which is activated by *Listeria* infection and has the potential to promote uptake of pDuLX into the mammalian cell nucleus by way of its nuclear localisation sequence. The present study was designed to evaluate the antigen delivery capacity of *L. monocytogenes* (rsΔ2) after oral delivery *in vivo* using a mouse model. We used ovalbumin (OVA) as a model vaccine to allow us to use established methods to assess both humoral and cell-mediated responses. Activity was benchmarked against a positive control, OVA-coated splenocytes injected intravenously with lipopolysaccharide (LPS). This positive control is not a practical option for human vaccination but is a useful experimental control. We also benchmarked activity against intravenous DNA vaccination using our shuttle plasmids injected as naked DNA dissolved in saline.

2. Experimental section

2.1. Animals, bacterial strains and plasmids

Female C57BL/6 mice (age 6–8 weeks) were used for *in vivo* experiments. The protocols were approved by the Animal Ethics Committee, Monash Institute of Pharmaceutical Sciences, Monash University, and experiments were conducted in accordance with the instructions published by the National Health and Medical Research Council of Australia. The strain of *L. monocytogenes,* designated as rsΔ2, was generated during our previous study by inserting a cell wall hydrolysin gene, "*ply118*" together with its associated *holin* gene from a Listeria-specific phage, into the attenuated *L. monocytogenes* genome of *L. monocytogenes* strain Δ2. The *hol118/-ply118* gene was placed under the control of the *Listeria* promoter P*actA*, inducing bacteria to undergo autolysis in eukaryotic cells [9]. *The* plasmids used in this study are listed in Table 1. *L. monocytogenes* was grown in charcoal-treated brain heart infusion broth (BHIC) or on agar plates supplemented with appropriate antibiotics.

2.2. Subcloning the ovalbumin gene into pDuLX

Ovalbumin is a chicken glycoprotein which is well-known to induce an immune response in mice. It is often used as a model antigen for *in vivo* vaccination studies because, depending on its mode of administration, it can generate antibody-mediated and cell-mediated immunity. cDNA encoding a secreted form of OVA [38] (provided by Dr. Andrew Lew, Walter and Elisa Hall Institute, Melbourne) was amplified by polymerase chain reaction (PCR) using the following primers: