Live bacterial vaccine vectors: An overview

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Abstract

Genetically attenuated microorganisms, pathogens, and some commensal bacteria can be engineered to deliver recombinant heterologous antigens to stimulate the host immune system, while still offering good levels of safety. A key feature of these live vectors is their capacity to stimulate mucosal as well as humoral and/or cellular systemic immunity. This enables the use of different forms of vaccination to prevent pathogen colonization of mucosal tissues, the front door for many infectious agents. Furthermore, delivery of DNA vaccines and immune system stimulatory molecules, such as cytokines, can be achieved using these special carriers, whose adjuvant properties and, sometimes, invasive capacities enhance the immune response. More recently, the unique features and versatility of these vectors have also been exploited to develop anti-cancer vaccines, where tumor-associated antigens, cytokines, and DNA or RNA molecules are delivered. Different strategies and genetic tools are constantly being developed, increasing the antigenic potential of agents delivered by these systems, opening fresh perspectives for the deployment of vehicles for new purposes. Here we summarize the main characteristics of the different types of live bacterial vectors and discuss new applications of these delivery systems in the field of vaccinology.

Key words: bacterial vector, vaccine delivery system, DNA vaccine, cancer vaccine, antigen presentation.

Introduction

The strategy of using live bacterial cells as vehicles to deliver recombinant antigens has emerged over the past two decades as an interesting alternative for the development of new vaccines. The evolution of genetic engineering techniques has enabled the construction of recombinant microorganisms capable of expressing heterologous proteins in different cellular compartments, improving their antigenic potential for the production of vaccines against viruses, bacteria, and parasites.

Intrinsic characteristics of these microorganisms, such as the lipopolysaccharides in Gram-negative bacteria, or lipoteichoic acid in Gram-positive bacteria, along with other pathogen-associated molecular patterns (PAMPs), are recognized by pattern recognition receptors (PRRs), which mediate different signaling pathways, resulting in the production of inflammatory cytokines and expression of other antimicrobial genes (Janeway and Medzhitov, 2002). This innate immune response to bacterial pathogens and its influence on the adaptive immune system makes attenuated live microorganisms extremely efficient vehicles for stimulation of specific and long-term immune responses against carried antigens. Hence, besides production and delivery of the antigens, the innate features of these vectors can enable them to act as useful immunostimulating adjuvants.

The goal of this review is to bring together the main features and the multiple different applications of LBVs as a highly versatile delivery system.

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Basic Features of LBVs

Microorganisms used as LBVs

Genetic engineering techniques have made it possible to identify and delete important virulence genes, enabling the attenuation of pathogenic bacteria and creating vectors unable to revert to their virulent forms. Several mutations have been described for different serotypes of Salmonella enterica (serovars Typhi and Typhimurium, hereafter referred to as S. typhi and S. typhimurium, respectively), with the most frequently used being the araA mutation (as well as araC and araD), which blocks the ability of the microorganism to synthesize aromatic compounds. This renders the bacteria unable to reproduce in the host, while retaining the capacity to invade the small intestine and to persist in infecting long enough to produce the antigen and elicit an effective immune response (Cárdenas and Clements, 1992). Other useful mutations that can attenuate pathogenicity affect biosynthesis of the nucleotides adenine (pur) and guanine (guaBA), and outer membrane proteins C and F (ompC, ompF), as well as expression of the cAMP receptor (cya/crp), the conversion of UDP-galactose to UDP-glucose (galE), DNA recombination and repair (recA, recBC), and regulation of virulence genes (phoP, phoQ) (Mastroeni et al., 2001).

Listeria monocytogenes infection (listeriosis) is a rare and preventable foodborne illness that can cause bacteremia, meningitis, fetal loss, and death, with the risk being greatest for older adults, pregnant women, and persons with immunocompromising conditions. Attenuation of Listeria monocytogenes for vaccine purposes has been achieved using auxotrophic mutants (Zhao et al., 2005) or deletion of virulence factors such as the genes actA and internalin B (inlB) (Brockstedt et al., 2004).

Lactic acid bacteria (LAB) such as Lactococcus lactis and some strains of Lactobacillus are attractive candidates for the delivery of heterologous antigens, not least due to their GRAS (Generally Regarded As Safe) status, as well as their ability to stimulate mucosal and systemic immune responses against recombinant epitopes associated with them (Wells and Mercenier, 2008). Moreover, studies have indicated that certain species of Lactobacillus show a non-specific immune system adjuvant effect that is related to activation of macrophages (Perdigón et al., 1988). However, the type of stimulus generated appears to be strain-dependent, and this is not well understood (Seegers 2002). Difficulties in the use of members of this class of bacteria as efficient vectors are related to a limited understanding of their mechanisms of action in the immune system, as well as to selection of the most suitable means of heterologous antigen expression (Pouwels et al., 1996). Immunization with Lactococcus lactis remains a challenge, as it generally fails to induce potent immune responses when administered by the oral route (Bahey-El-Din and Gahan, 2011).

The reemergence of whooping cough in places with wide vaccine coverage demands improved immunization techniques, and live attenuated Bordetella pertussis is an interesting candidate for intra-nasal vaccination (Feunou et al., 2010). Live B. pertussis is already undergoing clinical trials in adults, targeting new-born babies. Recombinant LBV based on attenuated B. pertussis has also been proposed, especially as a carrier for antigens from causative agents of respiratory diseases (Li et al., 2011).

Other bacterial species that have been studied for heterologous antigen delivery include Streptococcus gordonii (Lee 2003; Oggioni et al., 1995), Vibrio cholerae (Kaper and Levine 1990; Silva et al., 2008), Mycobacterium bovis (BCG) (Bastos et al., 2009; Nasser Eddine and Kaufmann, 2005), Yersinia enterocolitica (Leibiger et al., 2008), and Shigella flexneri (Barry et al., 2006; Sizemore et al., 1995). Relatively new species that have been investigated for use as vaccine vectors include Pseudomonas aeruginosa (Epaulard et al., 2006), Bacillus subtilis (Duc et al., 2003; Istitaco et al., 2001; Paczcz et al., 2007), and Mycobacterium smegmatis (Li et al., 2009). In the veterinary field, other bacteria have been used to develop a double protective immune response, against a heterologous antigen and against the vector itself; these include Erysipelothrix rhusiopathiae (Ogawa et al., 2009), Mycoplasma gallisepticum (Muneta et al., 2008), and Corynebacterium pseudotuberculosis (Moore et al., 1999). A number of live attenuated bacterial vaccines are licensed for veterinary use, including Lawsonia intracellularis, Streptococcus equi (deleted in the araA gene), Chlamydophila abortus, Mycoplasma synoviae, Mycoplasma gallisepticum (temperature-sensitive mutants), and Bordetella avium. Most of the strains were selected as attenuated, but were not precisely mutated to promote the attenuation and do not carry heterologous antigens (Meeusen et al., 2007).

Some examples of the main microorganisms used for the development of live bacterial vector vaccines are shown in Table 1, including some LBV vaccines that have reached Phase I clinical trials.

Bacterial spores as vaccine vectors

Recent research with Bacillus subtilis has demonstrated the possibility of antigen delivery and induction of an immune response using bacterial spores as vectors (Duc et al., 2003; Istitaco et al., 2001). Despite poor immunogenicity due to low levels of antigen expression in spores, and their short residence time in the gastrointestinal tract of the host after oral vaccination, their greater resistance to adverse conditions for long periods, heat resistance, probiotic effects, low production cost, and GRAS status make the spores of B. subtilis attractive for use in delivery of vaccine antigens (Duc et al., 2003; Ferreira et al., 2005).
Table 1 - Examples of live bacterial vaccine vectors under development for use against different classes of pathogens or tumors.

| Vector/ attenuation or complementation | Antigen/ target | Animal model /Inoculation route | Detected immune response | Reference |
|---------------------------------------|-----------------|---------------------------------|--------------------------|-----------|
| *Bacillus Calmette-Guerin* / rBCG30   | Overexpression Ag85b / *Mycobacterium tuberculosis* | Human / Intradermal          | CD4+, CD8+ T cell proliferation | (Hoft et al., 2008)* |
| *Bacillus subtilis* / probiotic       | LTB / *Escherichia coli* | Mouse / Oral                    | IgA, IgG                   | (Pacecez et al., 2007) |
| *Bordetella pertussis* BPZE1 / affected activity of three major toxins | SP70 / enterovirus 71 (EV71) | Mouse / Intranasal             | IgG                      | (Ho et al., 2008)* |
| *Lactobacillus casei* / non pathogenic | E7 / HPV16      | Mouse / Oral, subcutaneous, intramuscular | Th1, CTL                 | (Adachi et al., 2010) |
| *Lactococcus lactis* / non pathogenic | MSA2 / *Plasmodium falciparum* | Rabbit / Oral and nasal          | IgA, IgG, Th              | (Ramasamy et al., 2006) |
| *Listeria monocytogenes* /Deletions genes for D-alanine synthesis | Gag / HIV       | Rhesus monkey / Oral and intraperitoneal | IgG, Th1/Th2        | (Jiang et al., 2007) |
| *Listeria monocytogenes* BUG876 /ΔactA (actin polymerization) | LACK / *Leishmania major* | Mouse / Oral and intraperitoneal | Th1                     | (Soussi et al., 2002) |
| *Listeria monocytogenes* XFL7 / Culture Attenuated Chemically selected | HPV-16 E7 / Cervix carcinoma | Human / Intravenous             | HPV-16 E7-specific T cell responses | (Maciag et al., 2009)* |
| *Salmonella typhi* wild type Ty2 Vi /ΔgalE (galactose epitrase) | Gag, gp120 / HIV | Mouse/ Intranasal               | IgA, IgG, CTL             | (Feng et al., 2008) |
| *Salmonella typhi* Ty21a / galE | O-Ps / *Shigella dysenteriae* | Mouse/ Intraperitoneal          | IgG                      | (Xu et al., 2007)** |
| *Salmonella typhi* | Escherichia coli ETEC LT-B | Human / Oral | IgG or IgA                  | (Khan et al., 2007)* |
| *Salmonella typhi* Ty21a | OprF-Orp1 fusion / P. aeruginosa | Human / Oral, nasal and systemic/ | IgG, IgA                  | (Bumann et al., 2010)* |
| *Salmonella typhi* Ty21a | urease or HP0231 / *Helicobacter pylori* | Human / Oral | CD4+ T cell                 | (Aebisher et al., 2008)* |
| *Salmonella typhimurium* SL7207 /ΔaroA (aromatic synthesis) | Glycoprotein S (DNA vaccine) / Transmissible gastroenteritis virus | Mouse / Oral | IgA, IgG                   | (Yang et al., 2009) |
| *Salmonella typhimurium* SL3261 /ΔaroA mutant | Sm14 / *Schistosoma mansoni* | Mouse / Oral | IgG                      | (Pacheco et al., 2008) |
| *Salmonella typhimurium* /Δc28-36, ΔasdA16 | PspA / *S. pneumoniae* as secondary infection after influenza infection | Mouse / Oral | IgG (Th1/Th2), IgA       | (Seo et al., 2012) |
| *Shigella flexneri* 2a, S. sonnei and S. dysenteriae 1 /ΔguaBA (guaB guanylate synthase) | Multiple ETECs / Shigella and E. coli | Guinea pig / Intranasal     | IgG, IgA                  | (Barry et al., 2006) |
| *Streptococcus gordonii* RM4 / commensal | Pertussis toxin (PT) / *Bordetella pertussis* | Mouse / Oral | IgA                      | (Lee et al., 2002) |
| *Vibrio cholerae* CVD 103-HgR /ΔctxA (subunit A of cholera toxin) | Intimin / *Escherichia coli* | Rabbit / Oral | IgA                      | (Keller et al., 2010) |
| *Vibrio cholera* /ΔCTA | CT-B / *Escherichia coli* ETEC | Mouse, rabbit / Oral intranasal | IgG, IgA                  | (Roland et al., 2007)** |
| *Yersinia enterocolitica* /ΔYopP (outer membrane protein) | Listeriolysin (LLO) / *L. monocytogenes* | Mouse / Oral | Specific CD8 T cells            | (Leibiger et al., 2008) |

*Phase 1 clinical trial.
**Preclinical phase.
Pacecz and colleagues demonstrated that changes in the antigen expression system can increase the immune response (Pacecz et al., 2007). An episomal expression cassette using a promoter inducible under stress conditions increased the concentrations of specific IgG and S-IgA antibodies against the model antigen in mice. Zhou and co-workers reported that the use of spores of B. subtilis to deliver the antigen Tp22.3 by oral immunization conferred 45% protection in challenge assay with the parasite Clonorchis sinensis in mice (Zhou et al., 2008). Uyen and colleagues reported that use of the model antigen fragment C of tetanus toxin (FCTT) expressed on the surface of spores stimulated a Th1 response, while expression within the germinating spores led to a Th2 response (Uyen et al., 2007). Nasal immunization with the antigen expressed on the spore surface needed 10 times fewer spores to induce the same level of antibodies, compared to oral immunization (Uyen et al., 2007).

Systemic and local immune responses to LBVs

As antigen delivery systems, live vectors can readily induce a wide range of immune responses. Bacterial vectors exhibit a natural tropism for antigen-presenting cells (APCs), and therefore promote an antigen exposure to stimulate an immune response (Shata et al., 2000). The bacterium Salmonella enterica, which is the most widely studied LBV, invades the M cells of the intestine and then infects macrophages (Jones et al., 1995), so that the expressed heterologous antigens are presented to the host immune system. Dendritic cells, which represent another class of APCs, capture Salmonella in the lamina propria and can also sensitize the host immune system (Rescigno et al., 2001).

The conserved molecular patterns of microorganisms, such as LPS, specific nucleotide sequences, peptidoglycans, and flagellin are recognized by specific receptors (PRRs). The families of these receptors include the cell surface Toll-like receptors (TLRs), C-type lectin receptors (CLR), and families of cytoplasmic proteins (NLR, RLRs) (Neta and van der Meer, 2011). During pathogen invasion across the layer of epithelial cells, they are recognized by these cells or by the immune system cells underneath. The recognition of the microorganisms, whether intracellular or extracellular, occurs through the PRR receptors and mediates a series of immune system signaling processes. As result, the transcription factor NF-κB is activated, together with other mechanisms that induce the production of pro-inflammatory cytokines and chemokines. These molecules recruit the APCs and are the key molecules linking the innate and adaptive immune systems (Miyaji et al., 2011). Progress has been made in understanding the interaction of PAMPs with the intracellular host receptors and the role of autophagy as a mechanism of pathogen clearance. This results in immune system signaling and further presentation of antigens, ultimately stimulating cellular and humoral responses mediated by CD4+ and CD8+ lymphocytes (Kuballa et al., 2012; Yano and Kurata, 2011).

The chosen vector can directly influence the type of immune response that will be induced. Attenuated intracellular pathogens, such as Salmonella, Listeria, and M. tuberculosis, are able to stimulate a strong cellular immune response (Flesch et al., 1998) because they can survive within macrophages after being phagocytosed. Many of the molecular mechanisms used by these bacteria to survive within host cells are well known. They act by slowing the maturation of phagosomes and inhibiting their fusion with vesicles containing microbicidal substances (Jones et al., 1995), or by evading from inside the phagocytic vesicle directly to the cytoplasm, as shown by the bacterium L. monocytogenes (Portnoy et al., 1992).

An important advantage of using live bacteria as vaccines is the possibility of exploiting the immune response of a special physiological compartment, namely the mucosal system. Vaccines delivered by mucosal routes are designed to stimulate local and systemic immune responses, while formulations that employ other inoculation routes predominantly stimulate systemic immunity. Mucosal route vaccination strategies are generally associated with reduced side effects, offer easier administration, and can reduce the costs of production and implementation (Cortes-Perez et al., 2007; Gentschev et al., 2002).

Although the different mucosal sites are spatially compartmentalized, they are immunologically connected, so that immune responses induced in one site can also be observed in another distant mucosal tissue (Pavot et al., 2012). The secretion of IgA and IgM antibodies constitutes the major effector response exhibited by the mucosa-associated lymphoid tissues (MALT). Since more than 90% of infections in humans begin at mucosal sites (Bouvet and Fischetti, 1999), a line of defense in these tissues is desirable for a higher level of protection, and this can be more easily achieved by mucosal vaccination.

Pathogenic bacteria are particularly well adapted to the mucosal surface, where most of them initiate the infection process. Because of this, certain species of attenuated pathogenic bacteria have been extensively studied for the purpose of vaccine development. Amongst the most used are attenuated mutants of Salmonella enterica serovar Typhi or Typhimurium (Cárdenas and Clements, 1992; Galen et al., 2009; Spreng et al., 2006) and Listeria monocytogenes (Bruhn et al., 2007). Although some live attenuated vaccine strains have been licensed for oral administration, such as the typhoid vaccine S. typhi Ty21a and the Mycobacterium bovis vaccine BCG, Vibrio cholerae CVD103-HgR (Levine et al., 1988) remains as the only recombinant live oral vaccine licensed until now.

Administration of LBV vaccines is mostly via the nose or mouth, although the mucosa of the urogenital tract is also used. Some researchers argue that the respiratory
route offers advantages over the oral route, since it avoids the acidic and proteolytic environment of the stomach (Cortes-Perez et al., 2007; Locht 2000). In addition, intranasal vaccination generally induces stronger local and systemic immune responses when compared to oral vaccination (Locht 2000). For example, Cortes-Perez et al. (2007) reported higher production of IFN-γ after nasal administration of a vaccine based on lactic acid bacteria carrying the antigen E7 of human papillomavirus type 16, compared to immunization by the oral route (19). Nonetheless, concerns remain regarding use of the nasal immunization route employing live organisms or powerful adjuvants.

Heterologous Antigen Delivery by LBVs

Antigen expression systems and stability

The form of antigen delivery seems to have a major impact on the type and magnitude of the immune response of the vaccinated organism. Kaufmann and Hess reported that secretion of the antigen significantly increased the effectiveness of a vaccine used against intracellular pathogens (Kaufmann and Hess, 1999). A number of bacterial secretion systems have been successfully used for this purpose. The Type I secretion system, for which the main prototype is the alpha-hemolysin of E. coli, allows the direct secretion of the entire protein from the bacteria by using a plasmid that encodes the HlyC, HlyB, and HlyD proteins, secretion of the entire protein from the bacteria by using a less, concerns remain regarding use of the nasal immunization route employing live organisms or powerful adjuvants.

Several proteins with the LPXTG anchoring motif, found in a number of species, have been employed to display heterologous antigens on the surface of Gram-positive bacteria (Leenhouts et al., 1999). In the case of lactic acid bacteria, better results were achieved using cell surface antigens than secreted proteins, indicating the participation of the carrying cell for a more effective immune response to antigens (Pouwels et al., 1996; Seegers 2002).

An alternative secretion system composed of outer membrane vesicles in Gram-negative bacteria has been investigated. In this system, the antigen is targeted to the periplasmic space of the bacteria, and when the outer membrane vesicle is formed, it incorporates the antigen. Such vesicles guide the antigen to APCs, conferring high immunogenicity on them (Alaniz et al., 2007). An immunization test using the purified vesicles from recombinant S. typhimurium, producing a derivative of the pneumococcal protein PspA, conferred protection against challenge with a 10x 50% lethal dose (LD50) of Streptococcus pneumoniae in a mouse model (Muralinath et al., 2011).

Stable antigen expression is another crucial factor that affects the ability of an LBV to stimulate a protective immune response in the vaccinee. Furthermore, a stable expression of the antigen in the absence of selective pressure is required. For this purpose, chromosomal integration of expression cassettes can be used instead of plasmid-based gene expression. Ideally, this system enables stable antigen expression and concomitant production of multiple antigens by inserting multiple expression cassettes in the chromosome. It is free from selective pressure markers, and can be used to create attenuating mutations concomitantly with introduction of the antigen expression cassette(s) (Hussein and Hensel, 2008). The immune response elicited against antigens carried by LBV as the chromosomal expression system was lower than the response to plasmid-based antigen expression. This was supposedly related to the lower level of antigen expressed, due to fewer copies of the heterologous gene in the chromosomal expression system (Hussein and Hensel, 2009).

In vivo inducible promoters for antigen expression

In addition to the carrier and the nature of the immunogen, the promoter used to drive the expression of the antigen can also have a direct impact on the quality of the immune response (Medina et al., 2000). The literature
has described various expression promoters used in plasmids to control the expression of recombinant genes. In the case of live bacterial vaccines, a special feature is desirable for the promoters, namely in vivo induction. Constitutive expression of the heterologous antigen often causes metabolic burden, leading to a decrease in the fitness of the LBV that ultimately affects the immune response (Galen and Levine, 2001). The use of in vivo inducible promoters, where the antigen synthesis is driven by promoters that are activated by microenvironmental conditions encountered by the LBV in the host tissues, represents an alternative approach that could improve LBV expression performance.

Frequently-used in vivo inducible promoters include: ppagC, a promoter related to the invasion and survival of Salmonella inside macrophages (Miller et al., 1992); pkatG, a promoter of catalase, induced by exposure of the microorganism to hydrogen peroxide generated by macrophages during infection (Dunstan et al., 1999); phtrA, induced by increasing temperature, which controls the expression of Htra, required for survival of the microorganism in the macrophage (Roberts et al., 1998); pnirB, a promoter of NADH-dependent nitrite reductase, induced by anaerobiosis or by low oxygen pressure inside the host tissue (Oxer et al., 1991); pOmpC, an outer membrane protein regulated by osmotic and pH changes in the environment; and pssaG, a promoter located within the Salmonella Pathogenicity Island-2 (SPI-2), which encodes a type III secretion system involved in adapting the pathogen to the intravacuole environment within mammalian cells (McKelvie et al., 2004). Comparative studies have examined the effectiveness of promoters for induction of the expression of specific antigens, as well as the resulting immune responses. Comparing the promoters pnirB, ppagC, and pkatG in a S. typhimurium SaroAD strain, the ppagC promoter provided the best results in terms of the amount of heterologous protein expression and the level of the antigen-specific antibody response (Dunstan et al., 1999). Bullifent et al. (Bullifent et al., 2000) compared the phoP, ompC, pagC, and lacZ promoters for expression of the Y. pestis F1-antigen in a S. typhimurium aroA strain, and identified the phoP promoter as the most effective for induction of serum and mucosal antibody responses after intragastric immunization. The cytomegalovirus (CMV) promoter has proven ability to initiate gene transcription in many different mammalian cell types. This is the promoter commonly used for DNA vaccines, even when delivered by live Salmonella so that it can be recognized by the host transcription system (Weiss 2003). Investigations concerning promoters need to consider their stability in the microorganism during host invasion and maintenance, their ability to be activated in vivo, and the specific conditions of the environment for activation, all of which will be reflected in modulation of the immune response.

LBV Applications, Development, and Innovation

Cytokine and DNA delivery by LBVs

A new strategy to improve antigen presentation is related to the simultaneous expression and secretion of cytokines. These molecules are essential to determine the innate and adaptive immune responses, and for establishment of immunological memory (Chabalgoity et al., 2007). The in vivo production of IL-12 (Bermúdez-Humarán et al., 2005), IL-4, and IL-18 (Rosenkranz et al., 2003), as well as other cytokines, can modulate the type of immune response against a presented antigen. Chabalgoity’s group performed immunization with S. typhi and S. typhimurium harboring plasmids encoding FCTT and the cytokines IL-4 or IL-18. They concluded that the presence of both cytokines had pronounced effects on the immune response against bystander antigens, and also affected IFN-γ production (Rosenkranz et al., 2003).

Cytokines have been successfully employed for therapeutic purposes in several studies. The use of bacterial vectors for cytokine delivery is a useful alternative to other techniques such as direct injection of these molecules, as it increases the time of exposure of the host. This strategy was demonstrated by Xu et al. (1998), in treatment of Leishmania major infection using attenuated Salmonella expressing IL-2, IFN-γ, MIF, and TNF-α. Administration of the recombinant strains expressing the cytokines promoted the in vivo expression of inducible nitric oxide synthase, limiting the development of lesions and reducing parasite loads by up to three orders of magnitude. Although not used for vaccine purposes, it is worth mentioning here that a Lactococcus strain expressing IL-10 was successfully applied in a Phase I clinical trial for treatment of Crohn’s disease (Braat et al., 2006). Delivery of genes coding cytokines by bacterial vectors has also been used for tumor prevention and/or therapies. Two mucosal co-administered live Lactococcus lactis strains expressing cell wall-anchored E7 Ag and a secreted form of IL-12 were evaluated for treatment of HPV-16-induced tumors in a murine model. After challenge, immunized mice developed a CTL response and an E7-specific mucosal immune response that led to the prevention of inducible tumors. Therapeutic immunization induced regression of palpable tumors in mice (Bermúdez-Humarán et al., 2005).

The use of DNA vaccines carried by live bacterial vectors has been reported as a strategy for transfection of mammalian cells (termed bactofection) (Loessner and Weiss, 2004; Schoen et al., 2004; Weiss 2003). These vectors drive the DNA vaccine to mucosal surfaces and the antigens are expressed, processed, and presented by APCs, especially dendritic cells, resulting in activation of CD8+ T cells via MHC class I antigen presentation (Schoen et al., 2004).

In the process of bactofection, the release of plasmid DNA into the host cell occurs more effectively after lysis of
the bacterial vector in the cytoplasm or in the phagosome (Jain and Mekalanos, 2000; Pilgrim et al., 2003). Following release, the DNA should proceed to the cell nucleus to be transcribed. Immunization with plasmid DNA carried by some bacteria, especially enteroinvasive species such as Shigella flexneri, Salmonella spp., Yersinia enterocolitica, and Listeria monocytogenes, has shown good results (Schoen et al., 2004).

An alternative strategy that does not require transport of exogenous DNA to the cell nucleus is the delivery of functional mRNA molecules. Schoen et al. (2005) constructed a self-destructing Listeria monocytogenes strain able to release translation-competent mRNA directly into the cytosol of epithelial cells, macrophages, and human dendritic cells. With this system, mRNA molecules coding for GFP or ovalbumin and containing elements for recognition and translation by mammalian cells were produced in the carrier bacteria upon entry into the cytosol, and were immediately translated after being released from the lysed bacteria. According to the authors, the system using mRNA molecules offers many advantages over the plasmid DNA delivery technique, such as faster production of the desired protein in the infected cells and no risk of DNA integration into the chromosome of the mammalian host cells, amongst others (Schoen et al., 2005).

Cancer vaccines

A recent application of bacterial vectors is for vaccination and/or therapeutic purposes against various types of tumor, such as melanoma and cancers of the prostate, breast, kidney, and cervix (Paterson et al., 2010). Tumor antigens and antigens from viruses associated with oncogenesis (such as the human papilloma virus) were delivered by LBV as recombinant proteins or as DNA vaccine molecules.

The immunotherapeutic use of these vectors exploits their intrinsic immunostimulatory properties to try to circumvent a major obstacle in tumor immunotherapy, which is the common tendency of tumor associated antigens (TAAs) to induce immune tolerance instead of triggering active responses of T cells. This is related to the initial presentation of these antigens to the immune system by tumor cells, without the presence of co-stimulatory molecules (Pardoll 2003). A strong response of both innate and adaptive immunity against the recombinant bacterial vector is elicited, resulting in breaking of the tolerance pattern against the TAAs (Paterson et al., 2010).

The vast majority of studies of anti-tumor vaccines, using mouse models, have employed attenuated strains of S. typhimurium or Listeria to deliver TAAs (Hernández-Luna et al., 2013; Singh and Wallecha, 2011). In the latter case, the antigens are usually expressed fused to the virulence factors LLO or ActA, which possess motif sequences rich in proline, glutamic acid, serine, and threonine residues (PEST domains), flanked by clusters containing positively charged residues that direct the fused proteins to proteosomes for degradation and presentation of generated peptides via MHC I (Wood et al., 2008). Using this strategy in studies with the E7 antigen of HPV-16 in mice, Sewell et al. (2004) showed that regression of tumors was more pronounced when the antigen was fused to a fragment containing the LLO PEST domain. In 2009, this bacterial vector (L. monocytogenes expressing the E7 antigen fused to a fragment of listeriolsysin O, Lm-LLO-E7) was used in a safety study (Phase I) in patients with advanced carcinoma of the cervix. This constituted the first clinical trial of a live attenuated Listeria vaccine, which demonstrated its safety for human use (Maciag et al., 2009).

Xiang et al. (2008) reported four novel oral DNA vaccines delivered by S. typhimurium that caused marked suppression of tumor growth and dissemination by targeting the tumor vasculature and microenvironment. These vaccines were developed against melanoma, colon, breast, and lung carcinomas in mouse models, and targeted vascular endothelial growth factor receptor-2, transcription factor Fos-related antigen-1, and the anti-apoptosis proteins survivin and Legumain, respectively.

Strategies to improve this class of LBV vaccines include the associated delivery of DNA encoding cytokines to increase the immune response against the TAAs and then against cancer cells (Luo et al., 2003; Rosenkranz et al., 2003), and the use of the type III secretion system to deliver the tumor antigen directly into the host immune cell, inducing a cytotoxic mediated response (Epaulard et al., 2006; Nishikawa et al., 2006).

Another interesting strategy to improve cancer therapy is the use of attenuated Salmonella expressing single chain antibody fragments on the cell surface, which are specific against the carcinoembryonic antigen (CEA) presented by different tumor cells. Salmonella species already have the ability to invade tumor tissues, but this technique enables the recombinant LBV to be guided and concentrated in the tumor. The strategy is associated with the delivery of apoptotic proteins to conclude the elimination of the tumor cells (Bereta et al., 2007; Chorobik and Marcinkiewicz, 2011).

Delayed attenuation

A few years ago, pioneering work by Curtiss III and colleagues resulted in the creation of new recombinant Salmonella strains with a feature called “delayed attenuation”, which in mouse models was able to induce higher immunogenic responses against the carried heterologous antigens (Curtiss et al., 2010). These bacteria were programmed to have certain virulence genes turned off after colonization of the host tissues, as a result of which full attenuation of the strain could only occur in vivo (Curtiss et al., 2010). The system is based on the control of virulence genes by an arabinose-inducible promoter. The strains are cultured in the presence of arabinose, normally expressing...
the virulence genes, and the bacteria exhibit a fully virulent phenotype. Once in the host tissues, arabinose is no longer available, resulting in a progressive attenuation of the strains as cell division proceeds (Curtiss et al., 2009).

Another strategy uses a mutation that limits the capacity of cells to synthesize the LPS O-antigen side chains in the absence of mannose. The cells are grown in the presence of mannose, synthesizing wild-type levels of LPS O-antigen side chains. Under in vivo conditions, the synthesis of these molecules is terminated by the low availability (or absence) of free mannose, leading to attenuation of the Salmonella cells. A fragment of the PspA protein from Streptococcus pneumoniae was used as a model antigen to show that the delayed attenuation strains were able to induce stronger immune responses, and provided a higher degree of protection, compared to a “standard” attenuated Salmonella strain. Delayed attenuation strains exhibiting a wild-type phenotype during the initial stage of infection are supposedly able to colonize the host lymphoid tissues more efficiently than simple attenuation strains, leading to a more robust immune response (Li et al., 2009). Using a similar strategy, Kong et al. (2008) constructed strains that lysed after colonization of the host tissue. These regulated delayed lysis strains preclude both the persistence of Salmonella in the host and the survival of the bacteria if excreted, and hence act as biological containment systems.

A third development was the construction of a system for delayed antigen synthesis in order to avoid metabolic burden problems that might reduce colonization ability and thus immunogenicity (Xin et al., 2008). M. tuberculosis antigens (Juárez-Rodríguez et al., 2012) and influenza nucleoprotein (Ashraf et al., 2011) are other examples of antigens delivered by these modified Salmonella strains, which have yielded good levels of protective immune response. The delayed attenuation concept was also employed to create an attenuated Yersinia pestis strain that provided good levels of protection (Sun et al., 2010).

Heterologous prime-boost and vector priming

There are continuing concerns regarding reuse of the same bacterial vector to deliver different antigens to the same vaccinee, and conflicting results have been reported. The difficulty is that an immune response elicited against the vector itself in a first immunization can suppress or mask the expected response against the antigen delivered in a second immunization.

There is evidence that prior exposure of an organism to Listeria, used as a vector, may not affect subsequent boosters (Starks et al., 2004; Stevens et al., 2005). Despite faster clearance in animals with previous exposure to the vector, the vaccines were capable of stimulating functional T cells and inducing protective immunity (Bruhn et al., 2007). Similar results were described after tests in humans (Leong et al., 2009). Studies using Salmonella LBVs indicated that immunization with a particular strain did not hinder a response against a heterologous antigen carried by either the same or another immunologically related strain (Bao and Clements, 1991). A Phase I clinical trial for a S. typhi vaccine carrying the ETEC coli LT-B antigen showed no evidence of anti-carrier immunity preventing boosting, with anti-LT-B antibodies found in 67% of those vaccinated (Khan et al., 2007). On the other hand, Gahan et al. (2008) showed that prior exposure to Salmonella significantly decreased the ability of this vector to survive in the host cells, compromising the effectiveness of the vaccine. According to the authors, this negative effect did not diminish with time, and the same vector might not be suitable for delivery of multiple doses of the same vaccine.

Heterologous prime-boost vaccination seems to be a good strategy to overcome the live vector-specific immunity question. Originally, this strategy involved the administration of the same antigen by two different delivery methods, which generally induced higher levels of immune response than homologous boosting. Although the mechanisms underlying this process are still not fully understood, there is substantial evidence that the order of prime-boost administration, the nature of the antigen, the delivery vehicle, and the route of administration influence the immune response. Combining different antigen presentation forms seems to elicit higher quality immune responses, involving different subsets of T cells and modulation of cytokine profiles (Lu 2009). The prime-boost approach for LBVs, presenting the same antigen using two different Salmonella strains, was studied by Sevil Domènech et al. (2007). It was found that a second immunization using the same Salmonella vector reduced the maintenance period of the bacteria, while using a different Salmonella strain for boosting could effectively circumvent this limitation (Sevil Domènech et al., 2008). Nonetheless, according to Vinduramulle and Attridge (2003), the impact of prior immunity to the vector depends on the strain of Salmonella used, as well as the nature of the antigen delivered.

Although heterologous prime-boost generally employs live viruses, DNA vaccines, and recombinant purified proteins, LBV can act as one of the antigen delivery systems in this vaccination scheme. There have been several examples of this strategy. Tartz et al. (2008) reported the successful combination of a Salmonella-based LBV expressing the CD8+ epitope of the circumsporozoite protein (CSP), together with a purified recombinant Bordetella adenylate cyclase toxoid fusion (ACT-CSP), to construct a malaria vaccine that provided complete protection against Plasmodium berghei in murine model experiments. Pan et al. (2009) prepared a vaccine against the H9 subtype of the avian influenza virus using a DNA vaccine delivered by Salmonella typhimurium as prime, followed by a killed avian influenza vaccine as booster. An anthrax vaccine able to elicit strong antibody responses consisted of a prime with Salmonella typhi expressing the protective antigen (PA) of Bacillus anthracis, and boosting with recombinant PA or
the licensed US human alum-adsorbed anthrax vaccine (Bailie et al., 2008).

**Conclusions**

Viewed as a whole, the research field of live bacterial vectors (LBVs) has shown significant progress over the past two decades. Various LBVs have proved to be effective and powerful tools for use in human and animal health. New vectors, expression systems, and immunization strategies have gradually increased the potential of vaccines based on LBV platforms. In parallel with the development of LBVs, many questions have been raised about the safety of these genetically modified microorganisms, including the risk of environmental contamination, lateral transfer of genes conferring resistance to antibiotics, and reversion of the attenuation to a more virulent form. The need to address these issues has led to renewed efforts to combine the high immunogenicity of LBVs with low risk, a proper level of safety, and efficacy. Nowadays, research is also focused on the refinement of existing LBV vaccine candidates, as well as the development of specially designed new LBVs. A good example of a promising LBV candidate developed following this strategy is the Salmonella strain prepared by the Curtiss III group, which is under evaluation in clinical trials. This strain carries multiple genetic modifications that, besides increasing its immunogenicity, address issues such as the reduction of undesirable side effects by lowering its reactogenicity, and providing proper biological containment of the recombinant microorganism.

Research employing attenuated pathogens as carriers is notably more advanced than studies using commensal bacteria. As a consequence, genetically mutated pathogens, after being proven to be safe in clinical trials, are more likely to first be used as LBVs, even though they do not have GRAS status. It is also possible that, as has been seen for other innovative technologies in vaccine development, such as DNA vaccines or recombinant virus vectors, the first LBV-based vaccines carrying heterologous antigens will be licensed in the veterinary field, due to the less stringent regulatory requirements compared to products intended for use in human health.

The viability of LBV vaccine production is favored by its advantageous characteristics, as well as by its potentially lower production costs, since no complex purification is required and adjuvants are avoided, in contrast to other recombinant vaccine technologies. A further favorable point is the ability to use the mucosal route, which simplifies vaccine administration and promotes a special type of local immunity. This is a highly attractive feature of this class of vaccines, especially for mass vaccination programs in both developed and developing countries.

The successful development of this vaccine delivery technique enables it to be used in applications including the transport of cytokines, modulation of the immune response, and delivery of DNA vaccines to the interior of APCs. It provides new options for tumor treatment, which is encouraging research efforts to further improve the system, which is certainly expected to be an important player in a new generation of vaccines in the near future.

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