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Review

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 *aroA* mutation (as well as *aroC* and *aroD*), 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 flexnery* (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; Isticato *et al.*, 2001; Paccez *et al.*, 2007), and *Mycobacterium smegmatis* (Lü *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 *aroA* 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; Isticato *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	(Paccez *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 /Deleted genes for D-alanine synthesis	Gag / HIV	Rhesus monkey / Oral and intramuscular	IgG, Th1/Th2	(Jiang *et al*., 2007)
Listeria monocytogenes BUG876 /Δ*actA* (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^- /Δ*galE* (galactose epimerase)	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 -OprI 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	(Aebischer *et al*., 2008)*
Salmonella typhimurium SL7207 / $aroA^-$ (aromatic synthesis)	Glycoprotein S (DNA vaccine) / Transmissible gastroenteritis virus	Mouse /Oral	IgA, IgG	(Yang *et al*., 2009)
Salmonella typhimurium SL3261 /*aroA mutant*	Sm14 / *Schistosoma mansoni*	Mouse / Oral	IgG	(Pacheco *et al*., 2008)
Salmonella typhimurium / Δcrp-28, 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 / Δ*guaBA* (guanidine synthesis)	Multiple ETECs / *Shigella* and *E. coli*	Guinea pig / Intranasal	IgG, IgA	(Barry *et al*., 2006)
Streptococcus gordonii RJM4 / commensal	Pertussis toxin (PT) / *Bordetella pertussis*	Mouse / Oral	IgA	(Lee *et al*., 2002)
Vibrio cholerae CVD 103-HgR. /$ctxA^-$ A (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.

Paccez and colleagues demonstrated that changes in the antigen expression system can increase the immune response (Paccez *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* coworkers 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 *Clonorchissinensis* 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 (CLRs), and families of cytoplasmic proteins (NLR, RLRs) (Netea 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-κβ 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, along with the hemolysin secretion signal ($HlyA_s$) linked to the recombinant antigen (Gentschev *et al.*, 2002). It replicates stably in various Gram-negative bacteria, including *Salmonella*, *Shigella* spp., and *Vibrio cholerae* (Spreng *et al.*, 1999). Many vaccines have been developed for use against intracellular bacteria, parasites, and viruses, employing the hemolysin secretion system (Gentschev *et al.*, 2002).

The Type III secretion system has also been exploited, and has been proven to effectively deliver antigens of interest directly into the cytosol of APCs, leading to the activation of effectors and memory T-CD8+ lymphocytes (Rüssmann *et al.*, 1998). Attenuated pathogenic bacteria, such as *Salmonella* (Panthel *et al.*, 2008), *Yersinia* (Leibiger *et al.*, 2008), and *Pseudomonas* (Epaulard *et al.*, 2006) have already been used for delivery of antigens using this system.

The expression of heterologous antigens on the surface of the bacterial vector has been used to induce immune responses using Gram-positive and Gram-negative bacteria (Liljeqvist and Ståhl, 1999). For this exposure, the antigen of interest is usually expressed fused to surface proteins of the vector (Georgiou *et al.*, 1997; Lee *et al.*, 2002) and should mainly induce humoral immune responses (Cheminay and Hensel, 2008). Some examples of these fusion proteins include Lpp-OmpA, TolC, and FimH of *E. coli*, OprF of *Pseudomonas*, VirGβ of *Shigella*, IgAβ of *Neisseria*, FliC of *Salmonella*, and PulA of *Klebsiella* (Georgiou *et al.*, 1997; Kotton and Hohmann, 2004; Liljeqvist and Ståhl, 1999).

The AIDA (Adhesin Involved in Diffuse Adherence) auto-transporter system has also been used for the surface expression of model antigens that are important virulence factors of different pathogens. Some examples are the p60 antigen of *Listeria monocytogenes*, antigens OspA/OspG of *B. burgdorferi*, the LT-B subunit of *E. coli*, and Stx-B subunits of enterohemorrhagic *E. coli* (Buddenborg *et al.*, 2008).

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 vectors (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) (Husseiny 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 (Husseiny 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: p*pagC*, a promoter related to the invasion and survival of *Salmonella* inside macrophages (Miller *et al.*, 1992); p*katG*, a promoter of catalase, induced by exposure of the microorganism to hydrogen peroxide generated by macrophages during infection (Dunstan *et al.*, 1999); p*htrA*, induced by increasing temperature, which controls the expression of Htra, required for survival of the microorganism in the macrophage (Roberts *et al.*, 1998); p*nirB*, a promoter of NADH-dependent nitrite reductase, induced by anaerobiosis or by low oxygen pressure inside the host tissue (Oxer *et al.*, 1991); p*OmpC*, an outer membrane protein regulated by osmotic and pH changes in the environment; and p*ssaG*, 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 p*nirB*, p*pagC*, and p*katG* in a *S. typhimurium* Δ*aroAD* strain, the p*pagC* 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 listeriolysin 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 *E. 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 Domenech *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 Vindurampulle 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 (Baillie *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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