CHAPTER 5.10

LACTOBACILLI AS LIVE VECTORS FOR ORAL VACCINATION AGAINST TB

Beatriz del Rio, María Cruz Martín, María E. Sarmiento, Armando Acosta, Miguel A. Alvarez

‘In the best of all possible worlds, every person would have access to the best that science and medicine can offer.’

Dr Margaret Chan

‘Life is a little piece, little time …’

Manuel Mendive

Luminous Object
Kcho
Installation
Collection of the National Museum of Fine Arts, La Habana, Cuba
INTRODUCTION

TB caused by the bacterium MTB, is one of the world’s deadly infectious diseases with more than 9 million people becoming sick worldwide each year (Center for Diseases Control and Prevention, CDC; (1) and one third of the global population infected (2). The only licensed vaccine for TB prevention, the BCG (Bacille Calmette-Guérin) protects infants from the more invasive forms of TB (1), although it fails in protecting adolescents and adults which remain susceptible to MTB pulmonary disease. Hence, different strategies have been attempted to create a new generation of vaccines for BCG replacement, to increase its immunogenicity and to reduce safety problems related to BCG vaccination.

The scientific community is pursuing the generation of effective prophylactic/therapeutic vaccine candidates to eradicate the TB epidemic. At the moment, eleven pre-exposure vaccines candidates have entered in clinical trials to prevent the infection of healthy individuals (1). Other vaccine candidates have been formulated as post-exposure or therapeutic vaccines to target the dormant MTB and prevent a re-infection of the latently infected people (3). Some of the new vaccine candidates are based on new recombinant BCG with an increased antigenicity or immunogenicity (4, 5), recombinant bacteria (6), viruses (7, 8) and subunit vaccines (9).

The induction of an effective immune response may vary depending on the administration route of the vaccine (10) so in addition to new formulations for TB vaccine design, alternative routes for vaccine administration, such as mucosal vaccine delivery, have been suggested instead the intradermal immunization.
ADVANTAGES ASSOCIATED WITH VACCINE ADMINISTRATION BY THE MUCOSAL ROUTE

The mucosa contributes to the host defense against pathogens by acting as a barrier to prevent initial infections and initiating a specific adaptive immune response. Mucosal delivered vaccines would offer undeniable advantages over traditional systemic vaccines, improving the accessibility and cost-effectiveness and hence, increasing the coverage of vaccinated population. Syringes and needles are not needed for mucosal vaccination, which eliminates the risk of infection with other serious diseases (AIDS, hepatitis,...) and reduces the need of medical-trained personnel that in many parts of the world is a limiting factor. Moreover, delivery of vaccines through the mucosa could improve its efficacy to induce a specific and protective immune response compared to other routes of administration. In fact, respiratory and oral mucosal vaccinations not only have shown to induce a protective immune response, but they have improved the response compared to the parenteral immunization (11, 12). Vaccine formulations for oral, nasal, rectal and vaginal mucosal administration have been successfully developed.

Since MTB is a mucosal pathogen, it has been suggested that TB vaccine administration through the mucosal route could be more effective than the parenteral administration to induce an adaptive immune response. More optimal mucosal routes such as oral (13) and intranasal (14) administration are being investigated as an alternative to the parenteral route for TB vaccine administration.

After mucosal immunization, the strongest immune response occurs at the mucosa that was directly exposed to the vaccine and it decreases or is even absent in distal mucosal sites (10). However, oral vaccine administration is also able to elicit mucosal and systemic humoral and cellular responses (15, 16) even at anatomically distal mucosal sites, including the gastrointestinal, genital, and respiratory tracts (17–19).

A number of studies involving oral vaccination against TB have shown that the oral route for vaccine administration might be effective to induce a specific immune response. Oral vaccination of healthy individuals with BCG did not induce adverse reactions during an oral trial compared to the placebo group and it increased the mycobacterium-specific IFN-γ responses (20). Similarly, a boost on the cellular response represented by an increase of specific IFN-γ producing PBMCs (peripheral blood mononuclear cells) was detected when BCG was orally administered to healthy people that had received intradermal BCG in childhood or adolescence (21).
Therefore, oral immunization is an attractive system with several benefits as its immunogenicity, its non-invasive nature and the population acceptance. However, in order to design oral vaccines, a delivery system is needed to avoid the degradation of soluble antigens in the stomach and to skew the immune response towards an adaptive immune response rather than the induction of oral tolerance.

**VACCINE DELIVERY SYSTEMS FOR ORAL IMMUNIZATION**

Different synthetic (microparticules, liposomes, chitosan,…) and microbial (bacterial toxins, virus like particles, live vectors,…) vaccine delivery systems for oral immunization have been proposed (22, 23). Of them all, the use of bacteria as live vectors was considered very convenient because it is an inexpensive technology that has the potential for the production of protective antigens *in vivo*. Moreover, it is possible the simultaneous expression of multiple antigens to develop multivalent vaccines (24).

Attenuated pathogenic bacteria strains, such as *Salmonella enterica* (25, 26) have been used to generate oral vaccine candidates against different infectious diseases. Nonetheless, the use of attenuated pathogens as carriers of heterologous antigens for oral vaccine formulation could have safety problems associated with the risk of reversion to a virulent organism and the possibility of causing disease in immune compromised individuals.

A different approach to generate new vaccine candidates could be the use of live non-pathogenic bacteria as vaccine vectors. In the last years the commensal bacteria of the genus *Lactobacillus* have been described as a non-harmful microorganism for the human health that could be safely used to generate oral vaccine candidates against various infectious diseases (27, 28).

**ADVANTAGES OF LACTOBACILLI AS LIVE VECTORS FOR MUCOSAL IMMUNIZATION**

*Lactobacillus* have been suggested as good candidates to be used as mucosal delivery vehicles for oral vaccine design against different microbial pathogens such as bacteria e.g. enterotoxigenic *Escherichia coli* (29), *Salmonella enterica* serovar Enteritidis (30), *Borrelia burgdorferi* (infectious agent of the Lyme disease) (31), *Yersinia pestis* (infectious agent of plague) (32), and viruses e.g. Norwalk (33), foot and mouth disease virus (34), SARS-associated coronavirus (causative agent of the Severe Acute Respiratory Syndrome (35) and rotavirus (36).
Lactobacillus are non-pathogenic bacteria belonging to the Lactic Acid Bacteria group, which have been broadly used in the production of fermented food (37). They have GRAS (generally regarded as safe) status and some strains are normal constituents of the human gastrointestinal microbiota (38). These food-grade commensal bacteria have advantages as oral mucosal delivery vehicles in terms of safety and a lower risk of side effects such as those associated with live attenuated pathogenic bacterial vectors (39). In addition, many Lactobacillus strains are acid resistant (40), adhere to mucosal epithelium (41), and show intrinsic immunostimulatory properties (42–44). These properties could be beneficial for the use of Lactobacillus as mucosal delivery vehicles to be given orally and thus would be amenable to large-scale vaccination programs in populations at risk (28).

As stated previously in general, a TB oral vaccine based in Lactobacillus would have several advantages as the immunogenicity, does not need trained healthcare personnel and the logistic to dispose the infected needles and syringes used, and would be ideal for mass vaccination campaigns. Moreover, while severe complications on healthy people are relatively rare, some BCG-related skin lesions had been described (flat red scars) as result of tissue necrosis and destruction (45). Additionally, local complications on the injection site had been reported, including regional suppurative lymphadenitis, chronic ulceration, injection site abscesses, and rarely keloid and lupoid reactions (46, 47). The use of Lactobacillus as oral mucosal delivery vehicles for a TB vaccine would prevent these adverse effects associated to the BCG injection.

The immunological mechanisms of protection of the current BCG vaccine still unknown, although it seems to be clear that a strong cell-immune response of both CD4+ and CD8+ T cells is involved. CD4+ T cells polarize into different T cell subsets including Th1, which secretes cytokines as IL-2 for secondary expansion of CD8+ memory T cells, and both TNF-α and IFN-γ for macrophage activation (48, 49). Another memory CD4+ T cell subset that has a role in the vaccine protection is the Th17 cells. Th17 cells secret IL-17 that populate the lung and, after infection, trigger the production of CXCL9, CXCL10 and CXCL11 chemokines that recruit CD4+ T cells producing IFN-γ in the airways, which stops the pathogen growth (50). In addition, CD8+ T cells produce IFN-γ and TNF-α, which activate macrophages (51, 52) and also, they act as cytolytic T lymphocytes by secreting perforin and granulysin which lyse host cells and directly attack MTB (53). Hence, the crucial mechanism of action of a potential TB vaccine would be the generation of memory T cells, including Th1, Th17 and cytotoxic CD8+ T cells.
A good number of studies have shown the capacity of recombinant *Lactobacillus* expressing antigens to induce specific humoral and cellular immune-responses when it is orally administered, and hence has been considered a candidate as vehicle for mucosal immunization and a valuable strategy for TB vaccine development. Oral administration of vaccine based on *Lactobacillus* producing specific antigens results in induction of mucosal and systemic humoral and cellular responses at distal mucosal sites such as the gut and respiratory tract (31, 32). It had been also described that in animal models, oral administration of recombinant *Lactobacillus* may facilitate the polarization of the naïve immune system by skewing it away from Th2 toward Th1 responses (54), and promote humoral and cell mediated immunity (54, 31, 32). Moreover, it has been described that different oral vaccines based in *Lactobacillus* are able to elicit both humoral (serum IgG and mucosal IgA) and cellular immune-responses (31, 32, 55, 56). Further, oral administration of *L. casei* expressing β-lactoglobulin (BLG), one of the allergens on cow’s milk allergy, induced the production of cytokine IL-17 in BLG-reactivated splenocytes (57).

**EMERGING TECHNOLOGY TO GENERATE PHARMACEUTICAL-GRADE RECOMBINANT LACTOBACILLUS AS ORAL VACCINES AGAINST TB**

The design of genetically engineered *Lactobacillus* as oral vaccination delivery vehicle must ensure the stability of the gene encoding the antigen without selective pressure and the safety for human consumption. This essentially implies the absence of any antibiotic selection markers on the recombinant *Lactobacillus*. Martin et al. (58, 33) have developed a technology to generate pharmaceutical-grade recombinant *Lactobacillus* that expresses heterologous genes. The technology implies an integration system that allows the stable integration of the heterologous gene into the chromosome of *Lactobacillus*, and a depuration system to delete the non-pharmaceutical-grade DNA (i.e. antibiotic resistance genes, *Escherichia coli* DNA). Since the genes can be stably expressed in the recombinant *Lactobacillus* and the bacteria does not carry external non-pharmaceutical grade DNA, the system has been proposed as genetic tool to generate safe oral vaccines candidates against different infectious agents. In addition, we have developed different integrative vectors with signals that allow not only the cytoplasmic production of the proteins encoded by the integrated genes, but also can be secreted into the environment or attached to the external cell surface with either a covalent or non-covalent binding.
The expression and secretion of the selected protein has been directed by using the promoter and the secretion signal of the aggregation-promotion factor gene (apf) of *Lactobacillus crispatus* M247 (59). For cell surface attachment, a translational fusion has been made between the gene encoding the protein and a fragment of the proteinase P gene (prtP) that encodes the anchor region of the proteinase P (60).

A graphic representation of the steps needed to develop a pharmaceutical-grade *Lactobacillus casei* expressing a heterologous gene encoding an antigen is shown in Figure 5.10.1. The gene encoding the TB antigen of interest (*tb*) is cloned into pEM76, an expression vector that contains the integration cassette of the phage A2 (61), an origin of replication for *E. coli* and two antibiotic selection markers i.e. the erythromycin gene (*Em*) and the ampicillin gene (*Ap*) that work as selection markers for recombinant *Lactobacillus* and *E. coli*, respectively. pEM76 cannot replicate in lactobacilli but the integration cassette includes a gene (*int*) encoding for the integrase of phage A2, a recombinase that catalyzes the site-specific recombination between the *attP* sequence also present in the integration cassette and the *attB* sequence naturally present in the genome of lactobacilli. Once the *tb* gene encoding the antigen is cloned in a multicloning site located upstream the *int* gene, which can be done easily in *E. coli*, the resulting plasmid is transformed into *L. casei*. The integrase catalyzes then the site-specific recombination between the *attP* and the *attB* and the vector is integrated into the *L. casei* chromosome.

Afterwards, the recombinant *Lactobacillus* is transformed with pEM94, a replicative plasmid that carries a different selection mark — the resistant gene for chloramphenicol (*Cm*) and the β gene codifying for the β-resolvase. This enzyme catalyzes the site-specific recombination between two directly repeated recognition sites (six sequences) present in the integrative vector flanking the non-pharmaceutical-grade DNA. As a result of this recombination, the non-pharmaceutical-grade DNA is deleted. pEM94 is subsequently removed by increasing the incubation temperature, since it has an origin of replication sensible to the temperature. The newly generated recombinant *Lactobacillus* carries a *tb* gene that is stably integrated into the genome and therefore does not need selective pressure, and ensures the safety for human consumption since the integration/depuration technology applied, eliminates the entire hazard DNA.

The delivery system could be used to integrate heterologous genes in a range of lactic acid bacteria, since the integration machinery shows a degree of flexibility concerning the nucleotide sequence of the *attB* site into the chromosome (61, 36). Heterologous genes had been successfully integrated into other species of...
Figure 5.10.1 Mechanism of the integration/depuration system to generate pharmaceutical-grade recombinant Lactobacillus casei that produces heterologous proteins. The plasmid pEM76::tb carrying the gene of interest (tb), is integrated into the genome of L. casei to render L. casei EM76::tb. When the recombinant Lactobacillus is transformed with pEM94, the β-resolvase catalyzes the elimination of the non-pharmaceutical grade DNA (in red) contained between the two directly oriented six sequences, generating L. casei::tb (pEM94). A subsequent step with high temperature eliminates pEM94 from L. casei::tb (pEM94) rendering L. casei::tb which has stably integrated a tb gene and only carries pharmaceutical-grade DNA (in green).
Lactobacillus, e.g. L. rhamnosus and L. plantarum (unpublished data), and even bacteria belonging to a different genus, such as Lactococcus lactis (61).

So far, this technology had been successfully used to generate a food-grade L. casei that expresses the gene encoding VP60, the major viral capsid protein and main antigen of Norwalk virus (33). The generation of this recombinant Lactobacillus opens new avenues for producing a new oral vaccine for humans against this enteric virus, which is reported worldwide as the more common cause of outbreaks and sporadic cases of gastroenteritis in individuals of all ages (62, 63).

As we have seen so far, if the integrated gene codify for antigens against a defined pathogen, the recombinant Lactobacillus may serve as an oral vaccine for active immunization (33). Genes for VHH llama antibodies have been also integrated into the Lactobacillus genome to obtain oral vaccines for human passive immunization (36).

CONCLUSIONS

New approaches are needed to generate vaccine candidates against TB that could improve or replace the actual BCG vaccine. The oral route for vaccine administration appears to be an alternative to induce mucosal immunization instead the traditional parenteral route. The commensal bacteria of the genus Lactobacillus have been considered very good candidates as vaccine vectors for both active and passive immunization. However, a live vector for human immunization should not contain hazard DNA. An emerging integration/depuration technology has been suggested to generate safe recombinant Lactobacillus strains to be used as oral vaccine vectors against TB. These recombinant strains express genes encoding either antigens or antibodies, which can be accumulated in the cytoplasm, secreted or anchored externally to the cell wall. The application of such delivery system will hopefully help on the development of effective vaccines against TB for human active or passive immunization purposes.
REFERENCES


