Expression of two loops V3 from HIV-1 fused to cholera toxin A2B subunit using *Lactococcus lactis* as a vector to induce immunity in mucosa

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*Lactococcus lactis*, a nonpathogenic bacterium has been used widely as a vector to deliver antigens to the mucosa; one of the principal threats to world health is AIDS and HIV infection, involving the passage of the virus across a mucosal surface. We used *L. lactis* to express V3 loop from HIV-1, A2B subunit of cholera toxin and their fusion with one or two loops V3 (V3A2B and V3V3A2B). Six-weeks-old Balb/c mice were orally immunized with the recombinant bacteria. Samples of serum, intestinal washes and PBMC lymphocytes were collected to detect IgA and IgG anti-V3, and IL-2 respectively. The recombinants that express the V3 alone or fused to the A2B induced anti-V3 IgA antibodies and anti-V3 IgG antibodies in serum and intestinal washes but recombinant V3V3A2B was the most efficient and the only one that induced the expression of IL-2 in PBMC when compared with the controls (p<0.05). We induced anti V3 IgG and IgA and IL-2 in mice immunized with *L. lactis* recombinants that express the antigen V3 fused to A2B subunit of CT; which suggests that this strategy could be used to induce immune response specific of HIV.

Key words: HIV, vaccines, *Lactococcus lactis*, lactic acid bacteria, mucosal immunity.

INTRODUCTION

Candidate vaccines for HIV can be divided broadly into five categories: synthetic peptides or peptide subunits, live recombinant vectors, live attenuated vaccines, whole inactivated particles and DNA vaccines. Recently, over a hundred vaccine candidates in clinical trials were evaluated, but only three are in clinical phase III. Most vaccines include recombinants vectors, mainly adenovirus, adeno-associated, alphavirus, poxvirus and flavivirus; recombinants bacteria are also used, such as *Salmonella* (IAVI Report, 2014) and *Escherichia coli*, to express gp120 by their easy production and recently results are shown to elicit broad neutralizing sera against HIV-1 in small animals (Bhattacharyya et al., 2013). The use of commensal bacteria as a vector has certain advantages

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because it has been used in the food industry for years and can be administered orally.

*Lactococcus lactis* is a noninvasive, nonpathogenic, Gram-positive bacterium that has a long history of use in the production of fermented milk products. *L. lactis* lacks the ability to multiply *in vivo* except in gnotobiotic mice. However, genetically modified *L. lactis* have been effective in delivering antigens to the mucosal immune system and inducing a local immune response. Live *L. lactis* have been shown to pass rapidly through the gastrointestinal tract of animals and humans without colonization (Medina et al., 2008).

Recently, based on its suitable safety profile, *L. lactis* has been used as a vaccine vector to express different types of antigens (Xin et al., 2003; Groot et al., 2008; Yi-jing et al., 2010); this capacity was tested in a phase I study that employed *L. lactis* to express IL-10 in humans (Braat et al., 2006). Gastrointestinal, respiratory and genitourinary mucosal tissues serve as portals of entry for many pathogens, including sexually transmitted pathogens such as HIV. More than 90% of global HIV-1 transmission occurs across a mucosal surface (Griffin et al., 1996). Conserved antibodies epitopes located in the Env region on the viral protein gp120 have been identified based on their recognition by neutralizing monoclonal antibodies (mAbs). One of these epitopes is the V3 loop of gp120, contain conserved elements that play a role in virus attachment and which is critical for virus infectivity. Therefore, the induction of antibodies via the V3 loop is important to prevent infection (Hioe et al., 2010). Several strategies have been developed using the V3 loop as an antigen to induce immunity against HIV, in bacterial systems, including *Mycobacterium* (Joseph et al., 2010), *Salmonella* (Chin’ombe et al., 2009) and *Brucella* (Golding et al., 2002). Adjuvants are substances that enhance the immune response to a vaccine. Cholera toxin (CT) is an 86-kDa enterotoxin produced by *Vibrio cholera*, and this toxin is an exceptionally potent mucosal immunogenic (Vajdy and Lycke, 1992). CT can act as an adjuvant to promote the long-term immunological memory to unrelated immunogens in the gut mucosa after oral immunization. CT consists of five B subunits (CTBs), arranged as a pentamer and covalently linked to a single 33-kDa A subunit (CTA) (Kim et al., 1998). The use of CTB fused to the V3 region has been previously demonstrated in a transgenic potato (Kim et al., 2004). To date, few studies concerning the use of *L. lactis* for the presentation of HIV-1 antigens (Xin et al., 2003) have been published. The present study was focused on the strategy to use *L. lactis* to express HIV-1 antigen V3 loops fused to CTB to induce HIV-1 specific response of antibodies and cytokines through oral immunization.

**MATERIALS AND METHODS**

**Bacterial strains and growth media**

*E. coli* DH5 alpha was used as a host strain for plasmid construction and was grown in Luria-Bertani broth (Invitrogen, Carlsbad, CA, USA) at 37°C with shaking. *L. lactis* strain NZ29000 was cultured in M17 broth (Becton Dickinson/Difco, 7 Loveton Circle, Sparks, MD, USA) containing 5% glucose and incubated statically at 30°C. The antibiotic concentrations used to select the recombinant bacteria on agar medium were 100 μg/mL of ampicillin for *E. coli* or 5 μg/mL of chloramphenicol for *L. lactis*.

**DNA cloning**

All DNA cloning was performed as previously described (Sambrook and Russell, 2001). The secretion plasmid pSEC:Nuc was kindly provided by Dr. Philip Langella (Bermudez-Humaran et al., 2004), and V3 loop were amplified from pSFV-HxB2 kindly provided by Dr. Bernard Vernier (Dacheux et al., 2004), A2B subunit from El Tor, variant of *Vibrio cholera* was amplified and kindly provided by Microbiology Laboratory of Biological Sciences, UANL, Mexico. To generate recombinant *L. lactis* pSEC:V3 and pSEC:A2B, the gene fragments for the V3 loop from gp120 of HIV-1 subtype B and the A2B subunit from CT were generated using PCR. The oligonucleotide sequences for the amplification of the V3 loop were 5'- GCC ATG CATCCT GTA CAA GAC CCAAC - 3' and 5'- GCC ACT AGT CC ACA ATG TGC TTG TCT - 3' and the sequences for the amplification of the A2B subunit were 5'- CCG ATG CAT CCA GTA ATA CTG GCCAT - 3' and 5'- CGG ACT AGT ATT TGC CAT ACT - 3'. Each forward primer contained an NsiI site in the 5' region, and each reverse primer contained a SpeI site in the 3' region. PCR reactions were performed in a total volume of 50 μL in a cocktail containing 2X Taq polymerase master mix (Promega, Madison, WI, USA), 200 ng of template DNA and 10 pmol of each primer. The amplification was performed with 35 cycles at 94°C for 1 min, 62°C for 30 s, and 72°C for 2 min. The resulting 115- or 510-kb PCR products were cloned into the pGEM-T vector (Promega, Madison, WI, USA) and transformed into *E. coli*. The PCR products cloned were digested, extracted from an agarose gel and purified using QIAEX II kit (Qiagen, Hamburg GmbH, Germany) prior to insertion downstream of the nisin-inducible promoter of the pSEC vector. To obtain the fusion plasmid pSEC:V3A2B, and pSEC:V3V3A2B the V3 loop was inserted into the plasmid pSEC:A2B upstream of A2B using the NsiI site (Table 1). All the constructs were characterized by enzymatic digestion and PCR, after that they were sequenced and electroporated into *L. lactis* NZ29000 electrocompetent cells with a single pulse of 2,500 V in a 0.4-cm electroporation cuvette using a Micropulser (BIO-RAD, Hercules, CA, USA). After that, *L. lactis* cells were incubated for 2 h at 30°C and plated on GM17 agar with chloramphenicol. The transformants were visible after 48 h of incubation.

**Detection of V3 or A2B expressed in *L. lactis* recombinants by Western blots**

The *L. lactis* recombinant proteins were induced using 1 mg/mL of nisin for 2 h at 30°C, and the supernatants and pellets were separated by centrifugation at 8,000 xg for 2 min. The pellets from 1 mL of recombinant bacteria were suspended in TES buffer (25% sucrose, 1 mM EDTA, 50 mMTris-HCl pH 8.0 and 10 mg/mL lysozyme) with 1 mM of phenylmethyl-sulfonylfluoride (PMSF) and 1 mM dithiothreitol (DTT). The sample was incubated for 30 min at 37°C prior to the addition of 50 µL of 20% SDS and soul of loading buffer. The supernatants were precipitated with trichloroacetic acid with a single pulse of 2,500 V in a 0.4-cm electroporation cuvette using a Micropulser (BIO-RAD, Hercules, CA, USA). After that, *L. lactis* cells were incubated for 2 h at 30°C and plated on GM17 agar with chloramphenicol. The transformants were visible after 48 h of incubation.
gel electrophoresis, transferred to Hybond membranes (Amersham, Buckinghamshire, England), blocked in a 5% solution of skim milk powder in Tris-buffered saline containing 0.05% Tween 20 and incubated at room temperature with shaking for 1 h. The membrane was allowed to react overnight at 4°C with a mouse monoclonal anti-V3 (ImmuNox, Woburn MA, USA) or anti-A2B (AbDSerotec, Raleigh, USA) antibody at a 1:1,000 dilution in blocking buffer and then with anti-mouse antibody conjugated to HRP (Sigma Aldrich, Raleigh, USA) antibody at a 1:1,600, 1:3,200, 1:6,400 or 1:12,800. After an additional extensive washing step, the microplate was incubated for 2 h with an anti-mouse IgG-HRP conjugate at a final dilution of 1:8,000 or an anti-mouse IgA conjugated with alkaline phosphate (Sigma Aldrich, USA). Protein detection was performed using the chemiluminescent Super Signal West Pico ECL Substrate (Pierce, Rockford, IL, USA).

**Immunization schedule**

A total of 7 groups (5 mice per group) of 6-week-old female BALB/c mice were used for this study and immunized through oral administration with a single dose of 10⁷ CFU live recombinant *L. lactis* that express V3, A2B, V3A2B, V3V3A2B or the combination of V3 and A2B. *L. lactis* NZ9000 or PBS were used as a negative control. For oral immunization, 10⁹ UFC recombinant or the recombinant or the combination of V3 and A2B were suspended in 200 μL of PBS and intragastrically administered to the mice, according to Xu and Li (2007). The mice were sacrificed at ten days after immunization.

**Sample collection**

Samples of whole blood were collected by heart puncture and centrifuged at 600 xg for 10 min to obtain the serum. The intestinal washes were collected using 2 mL of TBS with PMSF, and then centrifuged at 600 xg for 10 min. The supernatant was stored at -20°C until analysis. PBMC were collected from whole blood heparinized by the method of Ficoll-Paque™ PLUS (GE Health care Bio-sciences AB, Sweden) employed according to the instructions of manufacturer.

**Detection of HIV-specific antibodies anti-V3 IgG or IgA by ELISA**

IgG or IgA antibodies against the V3 were detected in the serum and the intestinal wash samples by ELISA as follows: 5 μg of the V3 peptide RIQRGPGRAFTVIGK (Dalton Chemical Laboratories, Ontario, Canada) was directly coated onto the wells of a microtiter plate and incubated overnight at 4°C. The excess was removed, and the wells were washed three times with TBS. The wells were then blocked using 150 μL of TBS containing 3% BSA for 2 h at 37°C and washed as before. Each microplate well was incubated for 2 h with the sample at dilutions of 1:100, 1:200, 1:400, 1:800, 1:1,600, 1:3,200, 1:6,400 or 1:12,800. After an additional extensive washing step, the microplate was incubated for 2 h with an anti-mouse IgG-HRP conjugate at a final dilution of 1:8,000 or an anti-mouse IgA conjugated with alkaline phosphate (Sigma Aldrich, USA). Finally, the plate was washed three times with TBS and ABTS (2,2' azino-di-(3-ethylbenzthiozoline sulfonic acid)) or pNPP (p-nitrophenylphosphate) at 2 mg/mL (Sigma Aldrich,USA) was added as substrate. The absorbance in each well was measured at 405 nm using an ELISA microplate reader (Synergy HT, BioTek, Winooski, VT, USA).

**Detection of IL-2 cytokine by ELISA**

PBMC cells from mice immunized orally with *L. lactis* recombinants were adjusted to 1x10⁶ cells with RPMI (Invitrogen, Carlsbad, CA, USA) in 12 wells plate and sensitized with 5 μg of V3 peptide RIQRGPGRAFTVIGK, *L. lactis* NZ9000 and phytohemagglutinin (PHA) were used as a control. After 36 h of incubation at 37°C and CO₂ at 5%, supernatants were recover and IL-2 cytokine induced was detected by ELISA KIT (PEPROTECH, Rocky Hill, New Jersey, USA) following the manufacturers recommendations.

**Table 1. Bacterial strains and plasmid used in this work.**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype or phenotype</th>
<th>Source/reference</th>
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</thead>
<tbody>
<tr>
<td><em>L. lactis</em> NZ9000</td>
<td>Wild type, MG1363 (nisRK genes in chromosome), plasmid free</td>
<td>Langella et al. (2004)</td>
</tr>
<tr>
<td><em>E. coli</em> DH5 alpha</td>
<td>Wild type, fhuA2, Δ(argF-lacZ), U169, phoA, glnV44, q80, Δ(lacZ)ΔM15, gyrA96, recA1, relA1, endA1, thi-1, hsdR17.</td>
<td>Promega</td>
</tr>
<tr>
<td><em>Vibrio cholera</em> 01 El Tor</td>
<td></td>
<td>Microbiology Department, UANL</td>
</tr>
<tr>
<td>pSFV-HxH2</td>
<td>Amp&lt;sup&gt;+&lt;/sup&gt;, SP6 promoter, encoding the envelope glycoproteins of HIV</td>
<td>Bernard Verrier et al. (2004)</td>
</tr>
<tr>
<td>pGEM-T</td>
<td>Amp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Promega</td>
</tr>
<tr>
<td>pSEC:Nuc</td>
<td>Cm&lt;sup&gt;+&lt;/sup&gt;, gene expressed from PnisA encodes SPUsp-Nuclease</td>
<td>Langella et al. (2004)</td>
</tr>
<tr>
<td>pSEC:A2B</td>
<td>Cm&lt;sup&gt;+&lt;/sup&gt;, gene expressed from PnisA encodes SPUsp-A2B subunit CT</td>
<td>This work</td>
</tr>
<tr>
<td>pSEC:V3</td>
<td>Cm&lt;sup&gt;+&lt;/sup&gt;, gene expressed from PnisA encodes SPUsp-V3 loop HIV</td>
<td>This work</td>
</tr>
<tr>
<td>pSEC:V3A2B</td>
<td>Cm&lt;sup&gt;+&lt;/sup&gt;, gene expressed from PnisA encodes SPUsp-V3A2B fused</td>
<td>This work</td>
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<tr>
<td>pSEC:V3V3A2B</td>
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**RESULTS**

**Construction of plasmids for the expression of the V3 loop and A2B subunit of CT**

The genes for the V3 loop and the A2B subunit of the CT were amplified by PCR, to created pSEC: V3 and pSEC:A2B, and their fusions pSEC:V3A2B, pSEC:V3V3A2B. All the constructs were characterized
using enzymatic restriction (data not shown), PCR analysis, fusions were characterized using V3 loop as forward and A2B as reverse oligonucleotides (Figure 1) and sequenced (data not shown), prior to electroporation into *L. lactis* NZ9000 competent cells.

**Detection of expression of** *L. lactis* **recombinants proteins by Western Blot**

The nisin-induced proteins from the pellet and the supernatants of each *L. lactis* strain were analyzed using Western blots. The recombinant proteins were detected only in the pellets (Figure 2). The recombinant *L. lactis*: V3, *L. lactis* V3A2B and *L. lactis* V3V3A2B were detected using an anti-V3 monoclonal antibody to ensure that the small protein would be recognized (Figure 2A, C and D, respectively) and the recombinant *L. Lactis*:A2B were detected by anti-A2B monoclonal antibody (Figure 2B).

**Anti-V3 IgG and IgA antibodies titers in the serum and intestinal washes**

To analyze the humoral response induced by the different recombinant clones of *L. lactis*, we obtained sera and intestinal washes from the orally immunized mice. In orally immunized mice with *L. lactis* V3, *L. lactis* V3A2B, *L. lactis* V3V3A2B and the combination of *L. lactis* V3 and *L. lactis* A2B induced significant (p<0.05) anti-V3 IgA antibodies production in serum but not IgG, only *Lactis* V3V3A2B induced significant titers of anti-V3 IgG as compared to control *L. lactis* NZ9000 (Figure 3). All the recombinants, except A2B were efficient to induce significant (p<0.05) titers of anti-V3 IgA and IgG in intestinal washes, the most efficient was *Lactis* V3V3A2B as compared to the control NZ 9000 (Figure 4).

**Analysis of IL-2 in PBMC by ELISA**

To analyze the cellular response induced by the different recombinant clones of *L. lactis*, we obtained PBMC from the orally immunized mice. The recombinant *L. lactis* that express one loop V3 or their fusion to A2B as reverse oligonucleotides does not induce a significant (p<0.05) production of IL-2, only the recombinant *L. lactis* V3V3A2B that express two loops V3, was efficient to induce a significant production of IL-2 in PBMC (Figure 5).

**DISCUSSION**

Many strategies have been used to develop a HIV vaccine, recently, several replication-competent viral vectors based on vesicular stomatitis virus, vaccinia virus, measles virus and sendai virus were used in clinical trials. Viral vectors have proven to be well tolerated and immunogenic with evidence of efficacy in macaques (Parks et al., 2013). Another strategy is the bacterial vectors used to express HIV antigens to induce immunity (Charbit et al., 1993; Sirois et al., 2005). In this work, we used successfully, a non-pathogenic and non-colonizing candidate for delivering proteins by mucosal routes (Wang et al., 2008; Enouf et al., 2001): *L. lactis*, to express the V3 loop from gp120 of HIV-1, or the A2B subunit from CT as an adjuvant, expressed before by Sun et al. (2009) and their different fusions are showed in (Table 1). Our recombinant proteins were not detected in the supernatants of the recombinant *L. lactis* strains, probably by an inefficient secretion of the protein, or the method used to detected it, but have the potential to induce a significant production of antibodies and cytokines in vivo. Similar results were found by Zhang et al. (2011) in hepatitis B expressed in *Lactococcus* on antibodies production, and supported by several reports
Figure 3. Titers of anti-V3 IgG and IgA antibodies in serum of mice immunized orally with $10^9$ CFU live *L. lactis*. Serum from mice immunized with the recombinant that express loop V3 (V3), A2B Subunit CT (A2B), the combination of both (V3, A2B), and their fusion with one or two V3 loops (V3A2B), (V3V3A2B) or PBS or *L. lactis* wild type NZ9000 (NZ) as a negative control. Using an ELISA plate coated with 5 μg of V3 peptide in PBS, anti-V3 IgG and IgA antibodies were detected in serially diluted samples (1:200, 1:400, 1:800, 1:1,600, 1:3,200, 1:6,400 and 1:12,800) of serum. (*) P<0.05 as compared to control NZ.

Figure 4. Titers of anti-V3 IgG and IgA antibodies in intestinal washes from mice immunized orally with $10^9$ CFU live *L. lactis*. Intestinal washes from mice immunized with the recombinant that express loop V3 (V3), A2B Subunit CT (A2B), the combination of both (V3, A2B), and their fusion with one or two V3 loops (V3A2B), (V3V3A2B) or PBS or *L. lactis* wild type NZ9000 (NZ) as a negative control. Using an ELISA plate coated with 5 μg of V3 peptide in PBS, anti-V3 IgG and IgA antibodies were detected in serially diluted samples (1:200, 1:400, 1:800, 1:1,600, 1:3,200, 1:6,400 and 1:12,800) of intestinal washes. (*) P<0.05 as compared to the control NZ.
Figure 5. Analysis of IL-2 production by PBMC of mice immunized orally. Cytokine production of IL-2 by PBMC of mice immunized with 10^7 CFU live *L. lactis* wild type NZ9000 (NZ) as a negative control, or the recombinant that express loop V3 (V3), A2B Subunit CT (A2B), the combination of both (V3, A2B), and their fusion with one or two V3 loops (V3A2B), (V3V3A2B). PHA was used as a positive control. (*) P<0.05 as compared to the control NZ.

of the induction of humoral immunity using intracellular protein expression in lactic acid bacteria (Grangette et al., 2001; Shaw et al., 2000; Su-Jung et al., 2006). We found that the expression of the V3 loop protein alone or fused to A2B efficiently induced anti-V3 antibodies IgA or IgG as compared to the control (*L. lactis* wild type, NZ9000); these results correlate with Xin et al. (2003) and Siroiset al. (2005), indicating that the V3 loop is an effective antigen for inducing humoral immunity against antigen HIV.

An induction of anti-V3 IgA or IgG antibodies as compared to the control (NZ 9000) occurred when *L. lactis* recombinant express V3 loop and A2B subunit CT. It has been reported that CTB induces a high titer of IgA antibody to HIV-1 (Bukawa et al., 1995; Kang et al., 2003) and IgG antibodies are induced less efficiently (Kim et al., 2004), like our results the IgG in serum were not significant as compared to IgA antibodies production.

The double fusion V3V3A2B was the most efficient statistically to induce anti-V3 IgG, IgA and IL-2 cytokine production as compared to other *L. lactis* recombinants used. It has been reported that CTB induce a better adjuvant effect (Albu et al., 2003; Seder et al., 1993).

It is important to note that specific antibodies production with one dose of recombinant bacteria was obtained, similar to that obtained in a model of gp120 expression by our laboratory (unpublished); however other studies required several doses of viral antigens expressed in lactic bacteria.

We fused two loop V3 with the purpose of increasing its immunogenicity, because V3 loop is small peptide as compared to A2B; our results determine that our strategy to increase the antigenicity was performed with double fusion V3V3A2B being the most efficient to induce a specific anti-V3 IgA or IgG antibodies and IL-2 production. We hypothesized that this effect probably is because the second V3 loop could function as a bridge between the first V3 loop and the A2B subunit, resulting in better exposure of the V3 loop for the induction of an immune response by antigen capture and presentation as well as improved protein stability. This study may provide an innovative and potent strategy of immunization to induce a specific immune response against HIV antigen in the mucosa, the main site of viral entry, greater than 90% of global HIV-1 transmission occurs across a mucosal surface (Griffin et al., 1996) through sexual transmission or mother to child transmission by breastfeeding, however it should be mentioned that the virus also could be transmitted by mechanisms where the mucosa is not involved, such as through the blood in drug users and donors in lesser proportion. Finally, an important aspect to consider in the use of live vector vaccines is the risk arising from the nature of the organism, mutations that cause a reversal and produce disease in the host, especially in immunocompromised patients, where the immune system determines the magnitude of risk. Although *Lactococcus* is a microorganism that does not colonize, it could represent a risk.
because the recombinants are usually selected by resistance to antibiotics and plasmid may transform the patient's microflora and spread resistance genes. More experiments on the capacity to recognize and neutralize a broad range of antibodies and T cell specific response against HIV should be done and finally corroborate these results in a primate model to determine efficacy.

Conclusions

We found that \textit{L. lactis} may be used as an effective vaccine vector, and the strategy of fusing the V3 loop to an adjuvant, as the A2B subunit of the CT, could increase the immunity specific against HIV antigen. This study may provide an innovative and potent strategy of immunization to induce a specific immune response against HIV. Therefore, additional \textit{in vivo} experiments using these recombinant \textit{L. lactis} strains are necessary to determine whether the induced antibodies have a neutralizing capacity and whether this strategy may be used as an effective vaccine vector.

Conflict of interest

The authors declare that they have no conflict of interests.

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