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CD4 detected from *Lactobacillus* helps understand the interaction between *Lactobacillus* and HIV

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ABSTRACT

Human immunodeficiency virus (HIV) preferentially infects and destroys CD4+ cells and leads to a gradual decline in the number of CD4 cells. Despite evidence that probiotics increase CD4+ T lymphocytes in patients with HIV/acquired immunodeficiency syndrome (AIDS) and lower the risk of HIV transmission, little is known about the detailed mechanism underlying these effects. In this study, we investigated the cell surface protein of *Lactobacillus* and its role in blocking HIV-1 transmission by lactobacilli. Using reverse transcription–polymerase chain reaction (RT–PCR), immunofluorescence, and flow cytometry (fluorescence-activated cell sorting, FACS), we detected the CD4 receptor on the surface of *Lactobacillus*. Monoclonal antibody (mAb) for the CD4 receptor could partially inhibit HIV-1 binding to *Lactobacillus*. In addition, *Lactobacillus* could decrease HIV-1 pseudovirus infection of TZM-bl cells *in vitro* by 60–70%. Our data suggest that *Lactobacillus* can use this receptor to bind HIV and block HIV infection. This may in turn increase the CD4 T lymphocyte count in patients with HIV. These data provide direct evidence that *Lactobacillus* expresses the CD4 receptor and utilizes it to block HIV transmission.

1. Introduction

At present, there is an increased interest in using probiotic bacteria for the treatment of human immunodeficiency virus (HIV)-associated diseases and infection. *Lactobacillus* is a fermentative bacterium that resides in the gastrointestinal tract of humans and animals (Casas and Dobrogosz 2000). When utilized as a probiotic, it has many positive effects on health (Weizman et al. 2005). HIV transmits through the mucosal surfaces and causes severe damage to the gut, which has led some scientists to believe that the use of probiotics may help counter its devastating effects and infection. The use of probiotics has been shown to be safe for HIV patients (Wolf et al. 1998; Reid 2002). Clinical studies have demonstrated that probiotics have a beneficial effect on HIV-induced diarrhea (Cunningham et al. 2000). Recent randomized trials in Brazil (Trois et al. 2008) and Nigeria (Anukam et al. 2008) suggested that yogurt supplemented with *Lactobacillus* is significantly associated with an increase in the CD4 count in patients with HIV.

The CD4 receptor is the primary receptor for the entry of T-tropic HIV into its target cells *in vivo* (Planelles and Chen 1993). It has been shown that T-tropic isolates often appear in association with a decline in CD4+ T lymphocytes during disease progression. The CD4 count is therefore an important indicator of disease progression and treatment effect. An increasing number of studies indicate that probiotics can improve the CD4 cell count in HIV-positive individuals and lower the risk of HIV transmission; however, the mechanism by which probiotics increase the mean CD4+ cell count in HIV-positive individuals remains unclear (Irvine et al. 2010). Chang et al. (2009) showed that oral lactobacilli isolated from saliva of healthy humans can bind and capture HIV-1 *in vitro*. The present study was designed to investigate the extracellular proteins of *Lactobacillus* and their interaction with HIV and to test whether cell surface-associated proteins induce an increase in CD4 counts. Our results indicated that the HIV-1 CD4 receptor could be detected on the *Lactobacillus* cell surface. Furthermore, viral binding to *Lactobacillus* appeared to employ the CD4 receptor, and *Lactobacillus* could inhibit infection of cells by HIV-1 pseudovirus *in vitro*. This finding provides clear and direct evidence of the mechanism used by some lactobacilli to increase the CD4 cell count, bind HIV virus, and in turn block HIV transmission.

Abbreviations: CD4, cluster of differentiation 4; RT–PCR, reverse transcriptase/polymerase chain reaction; LAB, lactic acid bacteria; BSA, bovine serum albumin; mAbs, monoclonal antibodies; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate buffered saline; Taq, Thermus Aquaticus; TEM, transmission electron microscopy; MRS, de Man, Rogosa, and Sharpe broth; RNA, ribonucleic acid; β-gal, β-galactosidase; IgG, immunoglobulin G; FITC, fluorescein isothiocyanate; DC, dendritic cells; Th, T helper cells.

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2. Experimental procedures

2.1. Materials

Lactobacillus casei 393 was obtained from the American Type Culture Collection. *Lactobacillus* species were inoculated at 1% and propagated in de Man, Rogosa, and Sharpe (MRS) broth (Difco, Michigan, MI) at 37 °C for 15 h. CD4 and mAbs to CD4 were obtained from R&D Systems (Minneapolis, MN).

2.2. Observation of bacteria and HIV interaction by transmission electron microscopy (TEM)

For sample preparation, *L. casei* 393 was grown in MRS medium at 37 °C overnight. *L. casei* 393 was collected, washed three times with PBS to remove lactic acid. Then, a suspension of 1×10^8 bacterial cells was incubated with HIV pseudovirus in a total volume of 1 ml at 37 °C for 1 h. After washing three times with PBS and centrifuging at 6000 rpm for 5 min, *L. casei* 393 was fixed with paraformaldehyde (2%, v/v) at 4 °C overnight and diluted with 100 μl PBS. For TEM, copper slotgrids (Gröpl, Tulln, Austria) supported with Formvar film and coated with 0.5% bovine serum albumin (BSA; Sigma, Vienna, Austria) were used. Drops of the culture were directly added onto the grids. After 1 min, the drops were carefully drained off with filter paper, and the remaining cells were air dried and negatively stained with 2% uranyl acetate. Samples were observed using a TE microscope (JEM-2100EX, JEOL, Japan).

2.3. Confocal microscopic observation of receptor expression on the surface of lactic acid bacteria (LAB)

L. casei 393 cultures were collected and washed with PBS with 0.5% BSA, followed by staining with anti-CD4 mAb (R&D Systems) at a 1:500 dilution for 1 h at 4 °C. Alexa Fluor 488-conjugated goat antimouse IgG was used as secondary antibody (Invitrogen) at a 1:50 dilution for 30 min at 4 °C. Anti-GFP mAb was used as a negative control. Cells were visualized using a Leica Sp5X confocal fluorescence microscope with a 100× oil immersion objective.

2.4. Flow cytometric analysis of CD4 receptor expression on Lactobacillus

For flow cytometry (FACS), *L. casei* 393 cells were washed with PBS with 0.5% BSA and 0.1% NaN_3, incubated with mAb to CD4 (R&D Systems) at a 1:500 dilution for 1 h at 4 °C, and then incubated with FITC-labeled goat antimouse IgG Fc (Sigma) at a 1:100 dilution for 30 min at 4 °C. Samples were fixed with 0.1% paraformaldehyde and analyzed using a FACSCalibur four-laser cytometer (Becton Dickinson). Anti-GFP mAb was used as a negative control. At least 1×10^4 gated events were acquired in each case, and CD4 expression was evaluated by FACS analysis.

2.5. Reverse transcription–polymerase chain reaction (RT–PCR)

RT–PCR for CD4 was performed on cytoplasmic RNA extracted from *L. casei* 393. The RT–PCR reaction was performed in single tubes using the SuperScript III One-Step RT–PCR System according to the manufacturer's instructions (Invitrogen). In brief, serially diluted RNA for each chemokine receptor was reverse transcribed at 60 °C for 30 min, followed by 35 cycles at 94 °C for 1 min, 60 °C for 1 min, and 68 °C for 1 min, and a final extension at 68 °C for 7 min. Amplified DNA was analyzed on 1% agarose gel. Primers were designed to amplify the coding sequence based on the receptor sequences obtained from the GenBank database. The primers used were 5′-GAACCTGGTGGTGATGAGAG-3′ and 5′-GGGGCTACATGTCTTCTGAAACCGGTG-3′ (438 bp). In addition, control PCR reactions were conducted with primers on samples incubated in the absence of the template. The *Lactobacillus* LDH L gene (ldhL, 972 bp) was used as an internal control. PCR reactions for CD4 were also conducted with other lactobacilli (*Lactobacillus reuteri*, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*). The PCR products were confirmed by direct sequencing using the same primers as for those used the PCR reaction, in an ABI 377 DNA sequencer (PerkinElmer, Hünenberg, Switzerland).

2.6. HIV-1 pseudovirus preparation and antibody inhibition assays

HIV-1 pseudotyped with different envelope proteins was prepared by cotransfecting 293T cells with 10 μg of pNL-LucE^-R^- vector together with 20 μg of env-expressing plasmids (AD8, DH12, and LAI) using a standard calcium phosphate ($CaPO_4$) precipitation method. As a control, luciferase-expressing reporter viruses without an envelope protein were generated by cotransfecting 10 μg of pNL-LucE^-R^- vector and 20 μg of pCDNA3. Three days after transfection, supernatants of the transfected cultures were clarified by low-speed centrifugation at 3500 rpm for 10 min, followed by filtration of the supernatants through a 0.2-μm Acrodisc 25 filter (Gelman Sciences, Ann Arbor, MI). Pseudotyped virions were quantitated on 1 ml of clarified supernatant using a β-galactosidase (β-gal) staining assay.

For antibody inhibition experiments, *L. casei* 393 was incubated in the presence or absence of anti-CD4 mAb for 1 h at 37 °C. After binding, the lactobacilli were washed three times with 1.5 ml of PBS at 16,000 rpm for 4 min. Viral particles binding to the lactobacilli were lysed in ELISA lysing buffer (ZeptoMetrix) at room temperature for 2 h and then centrifuged at 16,000 rpm for 5 min. The resulting supernatant was used to perform HIV-1 p24 antigen ELISA according to the manufacturer's protocol.

2.7. In vitro infection inhibition by Lactobacillus

L. casei 393 was aerobically grown for 16–18 h in MRS broth at 37 °C and then diluted to a concentration of 1×10^4 cells/ml. Aliquots (1 ml) were mixed with HIV-1 AD8, DH12, or LAI. Pseudotyped virus that was not incubated with *Lactobacillus* was used as a control. After incubation, pseudotyped viruses were used to infect Tzmbl cells (1×10^4 cells per well in 96-well plates). After 2 days, the cells were washed with PBS and β-gal staining was performed to assay pseudovirus infection of the cells. Tzmbl cells contain the β-gal reporter gene, and the pseudovirus contains the tat gene. When the pseudovirus infects Tzmbl cells, the tat gene product activates β-gal expression. The β-Gal Assay Kit provides the reagents required to quickly measure the level of active β-gal expressed in the cells. Using β-gal quantitative assays, the number of stained cells was counted under a microscope.

3. Results

3.1. Viral capturing activity observed by TEM

To explore the interaction between *L. casei* 393 and HIV-1-pseudotyped virus (DH12), we used TEM to examine *L. casei* 393 after coincubation with HIV-1-pseudotyped virus (DH12) and three washes with PBS. Round virus particles approximately 80–100 nm in diameter were found to be distributed very closely around the *L. casei* 393 cells. This suggests there are interactions between the HIV-1-pseudotyped virus and *L. casei* 393 (Fig. 1).