Lactobacillus casei ssp. casei Induced Th1 Cytokine Profile and Natural Killer Cells Activity in Invasive Ductal Carcinoma Bearing Mice

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Received: 15 February 2011 ; Received in revised form: 30 July 2011 ; Accepted: 19 October 2011

ABSTRACT

Lactic acid bacteria which are used as probiotics have ability to modulate immune responses and modify immune mechanisms. It has also been indicated that some strains of this family can affect the immune responses against solid tumors.

In the present work, we proposed to study the effects of oral administration of L. casei ssp. casei on the NK cells cytotoxicity and also production of cytokines in spleen cells culture of BALB/c mice bearing invasive ductal carcinoma. 30 female In-bred BALB/c mice, were used and divided in two groups of test and control each containing 15 mice. Every day from 2 weeks before tumor transplantation 0.5 ml of PBS containing 2.7×10⁸ CFU/ml of L. casei ssp. casei was orally administered to the test mice and it was followed 3 weeks after transplantation as well with 3 days interval between each week. Control mice received an equal volume of PBS in a same manner.

Results showed that oral administration of L. casei significantly increased the production of IL-12 and IFN-γ (P<0.05) and increased the natural killer cells (NK) cytotoxicity in spleen cells culture of test mice (P<0.05). It has also been demonstrated that the growth rate of tumor in the test mice was decreased and their survival was significantly prolonged in comparison to the controls.

Our findings suggest that daily intake of L. casei can improve immune responses in mice bearing invasive ductal carcinoma, but further studies are needed to investigate the other involving mechanisms in this case.

Keywords: Invasive ductal carcinoma; Lactobacillus casei; NK cytotoxicity; Th1 cytokines
INTRODUCTION

Tumors usually emerge due to an imbalance between proliferation and apoptosis and in the breast tumors the deregulation between cell proliferation and apoptosis occurs in the early stages.\(^1\) The immune responses seem to play an important role in cancer development and investigations on immunological statues in patients with breast cancer or other types of cancers have demonstrated an ineffective immune responses, such as a decrease in delayed-type hypersensitivity (DTH), lytic function, and lack of cytokine production.\(^2\)

So in order to make the immune responses effective, using the immunomodulative agents may seem to be helpful in these patients.\(^3\) One of the most important types of immune responses against tumors which its triggering can have benefits for tumor development control is the natural killer cells response which are a type of cytotoxic lymphocytes that constitute the major component of the innate immune system.\(^4\) NK cells are also the first defense line of immune responses against tumors and infected cells by viruses, therefore play a key role in the rejection of tumors. Using of Lactic Acid Bacteria (LAB) as immunomodulator is a growing trend in many diseases in which efficient immune responses are needed.\(^5\) Lactic acid bacteria (LAB) are the most common probiotic microorganisms which can be useful for prevention of carcinogenesis and tumour growth.\(^6\) Although, the up-regulation of natural killer (NK) cell activity has also been observed in mice that were administered orally with \textit{L. casei shirota},\(^7\) but immunomodulatory effect of lactic acid bacteria is entirely strain specific.\(^8\) In other word each strain of these bacteria has its own effect on the pattern of immune responses. Beside NK cells, IL-12 has also been explained as a powerful anti-tumour factor which mediates the interferon (IFN)-\(\gamma\) production from T cells and NK cells, and augments their cytotoxic activity against tumor cells.\(^9\) Regarding the importance of the immunomodulative agent in the treatment protocol of tumor, in the present work, we aimed to evaluate the direct effect of oral administration of \textit{Lactobacillus casei} spp. \textit{casei} on the NK cytotoxicity and pattern of cytokines production in spleen cell culture of BALB/c mice bearing invasive ductal carcinoma.

MATERIALS AND METHODS

Animals

Six- to eight-week-old female inbred BALB/c mice, each weighing 25–30 g, were obtained from the Pasture Institute of Iran (Tehran, Iran). They were divided into two groups of test and control, each consisting of 15 mice. The mice were kept in plastic cages, allowed free access to water and maintained on a 12:12 h light and dark cycle during the study period. The temperature and humidity were controlled at 23±1°C and 55±10%, respectively and all mice were fed via standard mice pellet diet. Although the control mice in this study were kept separated from the test group but in the same temperature and humidity and fed with the same food.

Microorganism and Feeding Procedure

The \textit{L. casei} spp. \textit{casei} ATCC 39392 strains was purchased from the Persian type culture collection (Iranian Research Organization for Science and Technology, Tehran, Iran). It was inoculated in 10 ml of DeMan-Rogosa-Sharpe (MRS) broth (Merck, Germany) and cultivated for an overnight at 37.\(^\circ\)C under anaerobic conditions. Then it was collected (Approximately at the middle of log phase) by centrifugation at 4000 \(\times\)g for 30-min at 4\(^\circ\)C. They were washed three times with sterile phosphate buffer saline (PBS) and suspend in PBS. Then the suspension was diluted to obtain the appropriate number of \textit{L. casei} containing \(2.7\times 10^8\) CFU/ml. Finally 0.5 ml of this suspension was orally administered to mice using a standard gastric feeding tube\(^10\) according to the following procedure:

The test group of mice (\textit{L. casei} treated mice) were given 0.5 ml of this suspension daily for two consecutive weeks before tumour transplantation. Then the administration continued for 3 weeks with 3 days interval between each week. The control mice (PBS treated mice) were given an equal volume of PBS in a same procedure. To confirm the colonization of \textit{L. casei} in mice, their stools were collected 3 days after first administration. This strain was isolated and confirmed by \textit{lactobacillus} API biochemical kit.

Tumour Transplantation

The spontaneously adenocarcinoma breast tumour mice\(^11\) which were supplied by immunology division of
**Lactobacillus casei ssp. casei** Induced Th1 Cytokine

Pasteur institute of Iran (Tehran, Iran) and collected as tumour stock. After cervical dislocation of these mice, their tumours were removed aseptically, dissected into smaller 0.5 cm³ pieces using scalpel and they were washed three times with sterile phosphate buffered saline (PBS). The test and control mice were anesthetized with intra-peritoneal injection of ketamine and xylazine (10mg/kg of the body weight) and the tumour pieces were transplanted sub-cutaneously into their right flank.

**Measurement the Tumour Growth**

Tumor growth was measured twice a week using the calliper measurement for the tumour length. The volume was determined using the formula; 
\[ V = 0.5 \times d \times D ^{10} \]
where, V is the tumour volume (cm³), d is the shorter diameter and D is the longer diameter.

**Tumour Antigen Preparation**

In order to prepare tumor antigen, one of the tumour stock mouse was used and the tumor was extracted from its body and dissected in to small sections (1mm³) and homogenized in sterile PBS. Next it was washed by sterile PBS and sonicated. Then the protein dialysis method was used in order to extract the proteins from this lysate suspension.

Dialysis tubing (sigma) with a cut of 14 kD against PBS was used for 24 hours and the fresh PBS was replaced every 8 hours. The concentration of protein was measured using Bradford method. Finally it was applied to stimulate the splenocytes in Cytokine production tests and in DTH assay.

**Evaluation of Delayed Type Hypersensitivity Response (DTH)**

DTH response was carried out according to Jin et al. Briefly, 14 days after tumor transplantation, 7 mice from each group were challenged with the tumor antigen in the left footpad and with the PBS in the right footpad. Footpad induration was measured at 48h later using calliper measurement.

**Cytokine Determination in Spleen Cell Culture**

Three weeks after tumour transplantation, spleens of 8 mice from each group were removed aseptically and dissected then the spleen cells were prepared. RBCs were lysed with lysis buffer. The spleen cells were counted to 2.5 ×10⁶ cell/ml in RPMI1640 (Gibco Life Technologies, Germany) supplemented with 10% fetal bovine serum (Invitrogen, Paisley Germany), 100 µg/ml streptomycin and 100 IU/ml penicillin (Sigma, Germany) .Then cells were stimulated with 20 µg/ml of the tumor antigen (This amount was obtained from our pervious study/unpublished data) for 72h at 37°C in a humidified atmosphere of 5% CO₂. IL-12 and IFN-γ from the spleen cell cultured supernatants were detected using ELISA kit (R&D system) according to the manufacture instruction.

**NK Cell-mediated Cytotoxicity by Lactate Dehydrogenase (LDH) Assay**

In order to evaluate the NK cytotoxicity, 8 mice of each group were used and 2.5×10⁶ cells/well from their splenocytes were added to K562 cells in 1:50 and 1:100 (Target:Effector) ratio in 96-well U bottomed culture plates (Corning, Corning, N.Y.) in a total volume of 0.2 ml of RPMI1640 containing 2% BSA. The plates were gently centrifuged for 5 min at 250 g and then incubated for 4 h at 37°C in 5% CO₂. Then, the plates were centrifuged for 10 min at 250 g, and supernatant was removed from each well for LDH assay (Takara kit, Japan). The percentage of specific release of LDH was calculated using the experimental value- low control/high control-low control × 100.

**Survival Rate**

At the end of the study period (3 weeks after tumor transplantation) 7 mice of each group (Those were used for DTH assay as well) were kept in standard condition and fed with standard diet, free access to water, and maintained on a 12:12 h light dark cycle until they died. Daily death was recorded and after last death in both groups, data were analysed with Kaplan-miere test.

**Statistical Analysis**

All of the statistical analyses except for survival rate were conducted by SPSS 15.0, using student t-test. The survival rate data were analysed with Kaplan-miere test. The values are presented as mean±SD

**Ethical Approval:**
All experimental procedures involving animals were approved by the Ethics Committee of Tehran University of Medical Sciences.

Table 1. Tumour size and DTH results in experimental groups

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<th>Topics</th>
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<tr>
<td>L.casei</td>
<td>2.95**</td>
<td>1.00</td>
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<tr>
<td>PBS</td>
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a. Tumour size: Tumour growth was measured twice a week just after transplantation using the calliper measurement of the tumour length and the tumor volume was calculated with the text formula. The data are shown with ** (P<0.01) significance. 15 mice were used in this assay and all data were shown with mean ± SD.

b. DTH responses of the mice 48 h after tumour antigen rechallenge. The mice were challenged with the tumour antigen 14 days after tumour transplantation on the left footpad and with the PBS on the right footpad. The results showed a significant * (P<0.05) difference in the left footpad induration between the test and control mice. 7 mice were used in this assay and all data were shown with mean ± SD.

RESULTS

Measurement of Primary Tumour Growth
To evaluate the growth of tumor 15 mice of each group were used and tumor growth was measured twice a week with calliper. The tumor related volume of each mouse was calculated by the pervious mentioned formula. The data analysis showed a significant (P<0.01) decrease in the growth rate of tumor in the test mice in comparison to the controls (Table 1).

Evaluation of Delayed Type Hypersensitivity Response (DTH)
To asses the antigen-specific Th1 response, DTH reaction was evaluated in the tumor antigen rechallenged mice. They were challenged with tumor antigen in the left footpad and with sterile PBS in the right footpad 14 days after tumor transplantation. The results showed a significant (P<0.05) increase in the induration of left footpad in the test group after 48h of the tumor antigen challenge compared with the control group (Table 1).

Cytokine Determination in Spleen Cell Culture
After 72h of specific stimulation of spleen cells with 20 µg/ml of the tumor antigen the level of IL-12 and IFN-γ in the spleen cell culture supernatants were measured using a sandwich ELISA assay (R&D system). As figure 1 shows the level of IL-12 was significantly higher in the test group than control group (P<0.05). IFN-γ level also was significantly higher in L.casei group (P<0.01).

LDH Assay for NK Cell-mediated Cytotoxicity
To examine the effects of oral administration of Lactobacillus casei ssp casei toward NK cells, we investigated NK cell activities using K562 cells as target cells and evaluate the release of LDH from these cells after 4h of being close to NK cells among splenocytes. Results showed that in the L.casei administered mice the level of NK cytotoxicity significantly increased (P<0.05) in comparison to the control mice (Figure 2).

Figure 1. IL-12 and IFN-γ induction by L.casei administration in the spleen cell culture. The spleen cells were cultured for 72 h with tumor antigen stimulation and the levels of IL-12 and IFN-γ in the supernatants were determined with ELISA. 8 mice
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were used in this assay. The data are the means ± SD of triplicate cultures. *: Degree of significance for IL-12 was \( P < 0.05 \) and for IFN-\( \gamma \) was \( P < 0.01 \)

![Graph showing NK cytotoxicity](image)

**Figure 2.** NK cytotoxicity was measured by LDH assay. 2.5 \( \times 10^6 \) cells/well from the splenocytes were added to K562 cells in 1:50 and 1:100 (Target: Effectors ratio) and plates were gently centrifuged for 5 min at 250 \( g \), then in RPMI1640 containing 2% BSA the plates were incubated for 7 h at 37°C in 5% CO2 finally the percentage of specific release of LDH was calculated. The results showed a significant increase \( P < 0.05 \) in the NK cytotoxicity in **L.casei** group for both ratio of effector to target in comparison to the control group. The data are the means ± SD of triplicate cultures.

![Graph showing survival rate](image)

**Figure 3.** After the administration was completed 7 mice of each group were kept in standard condition until they died. Daily death was registered and after last death in both groups the data were analysed with Kaplan-miere test. Result showed a significant increase \( P < 0.01 \) in the survival rate of **L.casi** administered mice in comparison to the controls. **Survival Rate**

The results of survival analysis showed a decrease \( P < 0.01 \) in the rate of death in the **L.casei** administered mice compared to the controls (Figure 3).

**DISCUSSION**

There are many studies which have demonstrated that intake of some strains of LAB can affect the immune responses with different manifestations.\(^{14,15}\) We have previously reported that oral administration of **Lacidophilus** can induce the IL-12 production in spleen cells culture of BALB/c mice bearing tumor.\(^{16}\) In this report we evaluated the effect of oral administration of **L.casei** spp **casei** on the production of
the Th1 cytokines as well as NK cytotoxicity in the BALB/c mice bearing invasive ductal carcinoma. The results showed that the oral administration of L. casei spp casei not only increased the production of IL-12 and IFN-γ in the mice spleen cells culture, but also significantly increased the cytotoxicity of NK cells which can very likely be related to the high level of IL-12 and IFN-γ. NK cells are the first defense line of immune responses and play a key role in the rejection of tumors and infected cells by viruses. Although the results of several studies have also indicated the augmentation of NK cells activity by administration of some probiotics, nonetheless this type of effect does not belong to all strains of probiotic bacteria, rather each strain has its own effect on the immune responses. As it was indicated before, in the present work our findings demonstrated that oral administration of L. casei spp. casei can induce the production of IL-12 by mouse splenocytes.

IL-12 which is produced by the antigen-presenting cells (APCs), such as dendritic cells (DCs) and macrophages (MQ) is well known due to its important role in stimulating both NK and Th1 cells to produce the IFN-γ. According to this fact the increased level of IL-12 besides to the high level of IFN-γ which has been demonstrated in our study may be related to each other. IFN-γ is known for its antiviral, immunoregulatory, and anti-tumor properties. IFN-γ is also a major Th1 cytokine which can affect the up-regulation of major histocompatibility complex (MHC) expression in tumour cells. Furthermore, it has an ability to inhibit tumour angiogenesis and consequently suppress tumor growth. On the other hand IFN-γ has an effect on the NK cells regulation. Although Ogawa et al have indicated the activation of NK cells via oral administration of L. casei but in their study in addition of L. casei, dextran (glucose polysaccharide) was used. In other word they evaluated the symbiotic effects of this strain with dextran. While in the present work we studied the direct effect of oral administration of L. casei spp. casei alone. IL-12 and IFN-γ play a crucial role in modifying the balance between Th1 and Th2 cells and trigger the immune responses with Th1 bias.

Besides the raising of IL-12 and IFN-γ production in the spleen cells culture of mice in our study, the results of delayed type hypersensitivity (DTH) as a Th1 assay, in L. casei administered mice, when the tumor antigen was used into recalling immune response, the significant increase in the induration of left footpad has observed, which can also show that administration of L. casei may affect the improvement of the Th1/Th2 balance toward Th1 dominance that is more needed against tumor. Moreover the high level of IFN-γ participated in this balance as well. Nevertheless, IFN-γ has also other effects on the immune responses and tumour surveillance. Finally the decrease in the tumor related volume of the L. casei administered mice and the results of the survival rate in this group also confirm that a proper cytotoxic immune response might have been involved against transplanted graft of breast tumor in this group in comparison to the controls. In conclusion our findings suggest that oral administration of L. casei spp casei can affect the stimulation of Th1 cytokine production in spleen cells culture of mice and also affect the NK cells cytotoxicity. But more studies are needed to find out the exact mechanisms of these effects.

ACKNOWLEDGMENTS

This work was supported by the Vice-Chancellor for Research of Tehran University of Medical Sciences (Tehran, Iran). The authors have no conflicts of interest.

REFERENCES


