Probiotic Yogurt Culture Bifidobacterium Animalis Subsp. Lactis BB-12 and Lactobacillus Acidophilus LA-5 Modulate the Cytokine Secretion by Peripheral Blood Mononuclear Cells from Patients with Ulcerative Colitis

Abstract

Background: There are some evidences for the immunomodulation disorders in the response to intestinal microbiota in inflammatory bowel disease. Yogurt is a fermented milk product made with a starter culture consisting of different probiotics which could be colonized in intestine. However, the role of probiotics in the aetiology of ulcerative colitis (UC) has not been clarified. To determine how the immune system responds to these bacteria this study was planned.

Methods: Bifidobacterium lactis BB-12 (B. lactis) and Lactobacillus acidophilus LA-5 (L. acidophilus) were cultivated on MRS broth. PBMCs of 36 UC patients were separated by Ficoll-Hypaque centrifugation and co-cultured with different concentrations of UV killed bacteria in RPMI-1 640 plus 10 % FCS for 48/72 h. IL-10, TGF-β, IFN-γ and TNF-α were measured in supernatant of PBMCs by ELISA.

Results: Both bacteria significantly augmented IL-10, TGF-β, IFN-γ and TNF-α compared to control (p < 0.001). The secretion levels of IL-10 and TGF-β by B. lactis- compared to L. acidophilus-stimulated PBMCs were significantly higher (p < 0.05, p < 0.01 respectively). The secretion levels of TNF-α and IFN-γ by PBMCs after 72 h were significantly lower compared to 48 h stimulation by B. lactis (p < 0.001, p < 0.035 respectively).

Conclusion: These data show that both probiotics may trigger the pro- and anti-inflammatory immune response of UC patients. It seems that IL-10/TGF-β uprising by B. lactis could be the reason of TNF-α/IFN-γ reduction. Therefore albeit B. lactis still stimulates the effector Th cells but because of more stimulatory effect on Tregs, it could be a good potential therapeutic candidate for further investigation.

Introduction

Ulcerative colitis (UC) as one of the 2 major forms of inflammatory bowel disease (IBD) is a chronic and relapsing inflammatory condition that is characterized by colonic tissue edema, increased colonic epithelial permeability and extensive infiltration of leukocytes in the colon and rectum [1]. It is a cause of significant morbidity worldwide and its incidence and prevalence appear to be increasing with time. Patients with UC frequently experience episodes of bloody diarrhea with or without mucus, abdominal pain, fever and weight loss [2].

UC is assumed to be a result of a breakdown of tolerance to intestinal environmental antigens such as resident enteric bacteria. Several species of bacteria are living in human gastrointestinal lumen [3], which main sources of them are fermented foods containing probiotics, such as yogurt [4]. Some studies suggest that different species have variable potential to stimulate regulatory or effector T cells in the mucosal immune system [5, 6]. Indeed, cytokine profiles in co-cultures of the probiotic bacteria with peripheral blood mononuclear cells (PBMC) show marked differences between strains [7].

Upon antigenic stimulation, the T CD4+ cells differentiate into several subsets such as Th1, Th2, Th17 and regulatory T (Treg) cells which are characterized by distinct cytokine profile [8,9]. Treg cells are poised with regulatory activity limiting the development of T-cell responses to intestinal commensal bacteria and the development of pathologic intestinal inflammation. Evidence indicate the Th1 (such as IFN-γ and TNF-α), Th2 (IL-4, IL-5, IL-13) and Th17 (IL-17A) (all effector Th)-related cytokines could lead to UC disease progression and worsening of symptoms whereas the Treg-related cytokines (TGF-β and IL-10)
have been associated with the reduction and improvement of symptoms in UC patients [10, 11]. Recent evidences have indicated that probiotic treatment has a great influence on the host intestinal immune system to control inflammation [12, 13]. The aim of this study is to find whether B. lactis BB-12 and L. acidophilus LA-5, as probiotic strains used as dietary supplements in yogurt, differ in their capacity to induce Treg- or effector Th related cytokines in PBMCs from UC patients.

Materials and Methods

Patients

The study included 36 inactive UC patients (21 male and 15 female; 32.5 ± 10.4 years of age). Diagnosis of UC had been established based upon endoscopic and histological criteria as well as clinical symptoms. The patients have no complications or history of immunological disorders other than UC. None of the patients had received glucocorticoids, cyclosporine, or tacrolimus for at least 2 months prior to the study. The study was approved by Ethical Committee of Dezful University of Medical Sciences.

Isolation of peripheral blood mononuclear cells

After informed consent was obtained, 10 ml of heparinized venous blood was taken from patients with UC. The heparinized blood was loaded on 3 ml of Ficoll-Hypaque (Pharmacia), centrifuged at 1300 × g for 20 min, and PBMCs were separated as described previously [14–17]. Briefly, the cells were washed 3 times with phosphate buffered saline (PBS), resuspended in RPMI 1640 media (Gibco), supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco), and cultured at 37°C in a humidified 5% CO2 incubator to a final density of 2 × 10⁶ cells/ml.

Preparation of bacterial strains

Lactobacillus (L) acidophilus LA-5 and Bifidobacterium (B) lactis BB-12 were kindly provided by Sherkat Pegah Khuzestan, Dezful, Iran. Strains were cultured in de Man, Rogosa and Sharpe (MRS) broth (Merck, Darmstadt, Germany) at 37°C under anaerobic jars for 48 h. All bacteria were harvested by centrifugation (3000 g for 15 min) during stationary growth phase. Pelleted bacteria were then washed 3 times in PBS, concentration was determined by colony-forming unit (CFU) counting, and diluted to a final working concentration of 10⁸ CFU/mL in RPMI 1640. This stock suspension was aliquoted and stored at −20°C. To inhibit uncontrolled bacterial growth, ultraviolet (UV) inactivation was done by placing an aliquot in wells of a 48-well culture plate and placing the plate 4 cm from a UV light source for 40 min. Samples were then tested for inactivity by culturing on MRS agar compared to live bacteria sample. UV-killed bacteria have a preserved structural integrity, in contrast to heat-inactivated bacteria.

The measurement of TNF-α, IFN-γ, IL-10 and IL-1β secretion

After the incubation of PBMCs (2 × 10⁶ cells/well) with different concentrations of (in a cell:bacteria ratio of 1:100 and 1:50) killed L. acidophilus LA-5 and B. lactis BB-12 strains (or RPMI-1640 as control) for 48 and 72 h, the supernatant was aspirated and the TNF-α, IFN-γ, IL-10 and TGF-β levels were measured using sandwich ELISA (Quantikine, R&D Systems, Minneapolis, MN).

Statistical analysis

Results were expressed as mean ± SD. Differences in mean values between groups were analyzed with Student’s t-test to reveal significant differences between cytokine production in response to different strains of bacteria. Differences were considered to be significant at P < 0.05. Statistical calculations were performed with SPSS 19 (SPSS Inc., Chicago, IL, USA).

Results

Effect of B. lactis on pro-inflammatory and regulatory cytokines

As shown in Table 1, B. lactis significantly upraised IL-10 and TGF-β secretion by PBMCs in a dose- and time-dependent manner compared to control cultures (p < 0.001). The secretion levels of TNF-α and IFN-γ by B. lactis stimulated PBMCs at 72 h was significantly lower as compared to 48 h time (TNF-α: p < 0.001; IFN-γ: p < 0.035). This reduction could be due to immunomodulatory effects of the augmented IL-10 and TGF-β after 72 h compared to 48 h incubation time (Fig. 1a–d). The secretion levels of TNF-α and IFN-γ after stimulation of PBMCs by B. lactis at 100:1 bacteria:cells ratio was significantly lower than 50:1 bacteria:cells ratio (p < 0.001) (Fig. 2a–d). As it is shown in this reduction could be due to the immunomodulatory effects of the augmented IL-10 and TGF-β at 100:1 compared to 50:1 bacteria:cells ratio. At 48 h of incubation time, the secretion levels of IFN-γ by PBMCs in the presence of the 50:1 bacteria:cells ratio of B. lactis were significantly lower compared to 100:1 bacteria:cells ratio.

<table>
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<th>IFN-γ (pg/ml) Mean ± SD</th>
<th>TNF-α (pg/ml) Mean ± SD</th>
<th>IL-10 (pg/ml) Mean ± SD</th>
<th>TGF-β (pg/ml) Mean ± SD</th>
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<tr>
<td>Bacteria:Cell</td>
<td>48h</td>
<td>72h</td>
<td>48h</td>
<td>72h</td>
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<tr>
<td>Bifidobacterium</td>
<td>100:1</td>
<td>1.320 ± 341 1.556 ± 382</td>
<td>2.071 ± 452 2.463 ± 484</td>
<td>510 ± 253 824 ± 275</td>
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<td>Bifidobacterium</td>
<td>50:1</td>
<td>1.046 ± 322 1.177 ± 292</td>
<td>1.561 ± 381 2.072 ± 426</td>
<td>317 ± 171 498 ± 268</td>
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<td>Bifidobacterium</td>
<td>100:1</td>
<td>588 ± 228 505 ± 213</td>
<td>1.486 ± 380 822 ± 366  756 ± 191</td>
<td>1198 ± 487 1422 ± 401</td>
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<td>Bifidobacterium</td>
<td>50:1</td>
<td>833 ± 270 648 ± 177</td>
<td>1.888 ± 561 1.607 ± 362</td>
<td>630 ± 254 953 ± 302</td>
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<tr>
<td>Lactobacillus</td>
<td>100:1</td>
<td>1.444 ± 365 2.061 ± 388</td>
<td>2.171 ± 531 2.687 ± 553</td>
<td>473 ± 214 563 ± 250</td>
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<tr>
<td>Lactobacillus</td>
<td>50:1</td>
<td>1.073 ± 305 1.419 ± 438</td>
<td>1.754 ± 476 2.173 ± 546</td>
<td>315 ± 167 416 ± 233</td>
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<tr>
<td>Control</td>
<td>–</td>
<td>1.525 ± 59 2.16 ± 91</td>
<td>2.29 ± 109 3.35 ± 118  97 ± 61</td>
<td>132 ± 76 59 ± 35</td>
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SD = standard deviation.
significantly lower (P<0.05) whereas the secretion levels of IL-10 and TGF-β were significantly higher (P<0.001 and P<0.0001, respectively) as compared with the stimulation of PBMCs with L. acidophilus in the same time and same bacteria:cells ratio.

Similarly, at 48 h of incubation time, the secretion levels of IFN-γ by PBMCs in the presence of the 50:1 bacteria:cells ratio of B. lactis were significantly lower (P<0.05) whereas the secretion levels of IL-10 and TGF-β were significantly higher (P<0.002 and P<0.001, respectively) as compared with the stimulation of PBMCs with mixed probiotics in the same time and same bacteria:cells ratio.

At 48 h of incubation time, the secretion levels of IFN-γ by PBMCs in the presence of the 100:1 bacteria:cells ratio of B. lactis were significantly lower (P<0.0001 and P<0.001, respectively) whereas the secretion levels of IL-10 and TGF-β were significantly higher (P<0.003 and P<0.0001, respectively) as compared with the stimulation of PBMCs with L. acidophilus in the same time and same bacteria:cells ratio.

The same results were obtained with mixed probiotics in the same time and same bacteria:cells ratio.

At 72 h of incubation time, the secretion levels of IFN-γ by PBMCs in the presence of the 50:1 bacteria:cells ratio of B. lactis were significantly lower (P<0.001) whereas the secretion levels of IL-10 and TGF-β were significantly higher (P<0.003 and P<0.001, respectively) as compared with the stimulation of PBMCs with L. acidophilus in the same time and same bacteria:cells ratio.
IL-10 and TGF-β were significantly higher (P<0.0001) as compared with the stimulation of PBMCs with L. acidophilus or mixed probiotics in the same time and same bacteria:cells ratio. The same results were obtained in the presence of the 100:1 bacteria:cells (Fig. 1,2).

Effect of L. acidophilus on pro-inflammatory and regulatory cytokines
L. acidophilus upraised TNF-α, IFN-γ, IL-10 and TGF-β by PBMCs in a dose- and time-dependent manner in comparison with control cultures (p<0.001) (Table 1). The secretion levels of IL-10 and TGF-β after stimulation of PBMCs by L. acidophilus were significantly lower as compared to B. lactis and mixed bacteria, totally (at 2 different incubation times and 2 different cells:bacteria ratios) (p<0.05) (Fig. 1a–d). The secretion levels of TNF-α and IFN-γ after stimulation of PBMCs by L. acidophilus were significantly higher in comparison to B. lactis and mixed bacteria, totally (at 2 different incubation times and 2 different cells:bacteria ratios) (p<0.05) (Fig. 2a–d). This difference could be a result of the lower secretion of the mentioned immunoregulatory cytokines by L. acidophilus compared to B. lactis.

Effect of mixed bacteria on pro-inflammatory and regulatory cytokines
The mixed bacteria significantly influenced the secretion of TNF-α, IFN-γ, IL-10 and TGF-β by PBMCs in a manner of dose- and time-dependent manner in comparison with control cultures (p<0.001) (Table 1). The secretion levels of TNF-α and IFN-γ were significantly higher after stimulation of PBMCs by mixed bacteria, as compared to B. lactis, totally (at 2 different incubation times and 2 different cells:bacteria ratios) were significantly lower as compared to L. acidophilus (p<0.05 and p<0.05 respectively) (Fig. 2a–d). Furthermore, the secretion levels of IL-10 and TGF-β after stimulation of PBMCs by mixed bacteria was significantly lower in comparison to B. lactis, but were higher as compared to L. acidophilus (p<0.05 and p<0.05 respectively) (Fig. 1a–d).

Discussion
Although the immunopathogenesis of ulcerative colitis (UC) remains unclear, current evidence indicates that altered T cell response to intestinal environmental antigens such as resident commensal bacteria plays an important role [18–20]. So that an increased mucosal infiltration of activated CD4+ lymphocytes, dysfunctional dendritic cells, dysregulated macrophage-induced immune responses and abnormalities in regulatory pathways have been demonstrated [21,22]. Conventional therapies of UC, including corticosteroids, 5-ASA and thiopurines [23], were performed to reduce inflammation but do not change the natural course of disease, and many patients become refractory to conventional therapies during the course of the disease.

Recently, probiotic therapy has been considered to be potentially effective and safe in patients with UC. It’s been reported that individual probiotic species have variable potential to stimulate regulatory or effector T cells in the mucosal immune system. Indeed, cytokine profiles in co-cultures of the probiotic bacteria with peripheral blood mononuclear cells (PBMC) show marked differences between strains [24]. In the present study we found that the mixed (B. lactis plus L. acidophilus) bacteria strongly modulate the secretion of IL-10, TGF-β, IFN-γ and TNF-α in a manner of dose and time-dependent in comparison with control PBMCs. The amount of secreted TNF-α and IFN-γ by PBMCs after stimulation by mixed bacteria were less than L. acidophilus but more than B. lactis. These results indicate that B. lactis acts as a weak stimulator of PBMCs as compared to L. acidophilus.

Although the secretion of IL-10 and TGF-β after stimulation by L. acidophilus were upraised, however, the secretion of IL-10 and TGF-β by B. lactis stimulated-PBMCs were significantly higher in comparison to L. acidophilus and mixed bacteria. Higher levels of IL-10 and TGF-β may be a reason for the lower secretion levels of TNF-α and IFN-γ after stimulation of PBMCs by B. lactis, compared to L. acidophilus and mixed bacteria. Although we did not use blocking antibodies to IL-10 and/or TGF to demonstrate whether these cytokines are suppressing IFN and TNF production, these results still as a valuable data indicate that B. lactis could be a better candidate for Treg stimulation in comparison with L. acidophilus and mixed bacteria in UC patients. Our results are in accordance with some previous studies. Akemi Imaoka et al. showed the secretion levels of IL-10 in the cultures of PBMCs isolated from the UC patients with heat-killed Bifidobacterium were higher as compared control cultures in the absence of probiotic bacteria [25]. Kato et al. showed that administration of Bifidobacterium-fermented milk for patients significantly reduced the clinical activity index after 12 weeks as compared with the control group [26]. Moreover, Peran L et al. in an experimental study to compare the preventative effects exerted by B. lactis and L. acidophilus, in the trinitrobenzenesulfonic acid model of rat colitis showed when B. lactis was administered to colitic rats, a significant reduction in colonic TNF-α production was observed, whereas L. acidophilus administration significantly reduced colonic LTB4 production in the inflamed colon in comparison with untreated colitic rats [27]. Although still incompletely characterized, several reports indicate that the cytokine profile in UC patients is complex and seems to involve a variety of cytokines, as demonstrated by increased mRNA expression of IFN-γ, IL-13, IL-17A, IL-4, IL-5, IL-1b, IL-6, IL-8 and TNF-α which are categorized under 3 kinds of effector Th: Th1 (IFN-γ, TNF-α), Th2 (IL-4, IL-5, IL-13) and Th17 (IL-17A) and innate immune responses (TNF, IL-1b, IL-6 and IL-8) [28–32].

Studies have demonstrated that anti-TNF therapy resulted in down-regulation of mucosal TNF-α and IFN-γ mRNA expression in UC patients but therapy failure was positively associated with high mucosal mRNA expression of IFN-γ [33,34]. Increased expression of TNF-α and IFN-γ mRNA has been detected not only in active UC but also in inactive UC, suggesting that these cytokines play a pivotal role in relapse of UC [11,35]. Thus, it seems that high IFN-γ and TNF-α as pro-inflammatory cytokines have important role in mucosal inflammation and it suggests that suppression of them could have important role in the successful treatment of UC. So, in the present study we measured these 2 pro-inflammatory cytokines as the sign of effector Th stimulatory potential of B. lactis and L. acidophilus. On the other hand, evidence has shown that non-functional, absent Tregs or genetic mutations in Foxp3 induces hypersensitivity to intestinal bacterial antigens [36,37], and impairs the balance of intestinal mucosal immunity to inflammatory injury followed by lymphocytic infiltration of the intestinal mucosa [38,39]. CD4+CD25+Foxp3+ T cells increased in the colon of patients with UC [40]. These data indicated that Tregs would be a promising target for treating UC, because increasing the activ-

ity of appropriate Tregs in the gut should help to restore inflamed colonic tissues [41]. Then, although IL-10 and TGF-β are good representative of Tregs, to support the conclusions concerning the effects of the mentioned probiotics on Treg cells in this study it could be good to show increasing the frequency of the Treg population by known markers, such as Foxp3.

Furthermore, evidence has suggested that probiotic bacteria may have, in a species- or even strain-dependent manner, a potential use as anti-inflammatory agents which exert their effect by stimulation of Tregs [42]. Hence, in this study we tried to check if B. lactis, L. acidophilus or both have any effect on the Tregs of UC patients by measuring IL-10 and TGF-β. Although the results indicate that B. lactis, compared to L. acidophilus, had more stimulatory effect on Tregs of UC patients but it still stimulates, even less than Lactobacillus acidophilus, the effector Th cells (by augmenting TNF-α and IFN-γ) compared to control. Since it has been shown that each metabolic product or body fraction of probiotic bacteria could have its individual effect on immune system [43], it is suggested to fractionate B. lactis and check the individual effect of each metabolic product or trunk of the bacterium on Tregs of UC patients.

In conclusion, each of these 2 probiotics or their mixed could have individual modulatory impact on immune system including Treg/effector Th of UC patients. Because UC patients suffer from an aggressive intestinal mucosal inflammatory response, researchers are seeking for probiotic bacteria which potentiate Treg activity to suppress the effector Th cells in UC patients. Thus from these 2 selected probiotics in this study, B. lactis as one of the probiotic sources of yogurt, which had more stimulatory effect on Tregs of UC patients, albeit still stimulates the effector Th cells could be a good potential therapeutic candidate for further investigation.

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Declaration of Interest
The authors declare no conflicts of interest.

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