Humoral and cytokine responses induced by probiotic
*Lactobacillus casei* and *paracasei* strains in children
with atopic dermatitis

Bożena Cukrowska¹, Ilona Rosiak², Aldona Ceregra², Joanna Freszel³,
 Grażyna Zakrzewska¹, Elżbieta Klewicka⁴, Ilona Motyl⁴, Zdzisława Libudzis⁴

¹ Department of Pathology
² Department of Paediatrics
The Children’s Memorial Health Institute
Warsaw, Poland
³ Warsaw Agricultural University
Warsaw, Poland
⁴ Institute of Fermentation Technology and Microbiology
Technical University of Lodz
Lodz, Poland

Abstract

Probiotic bacteria have been shown to be useful in both prevention and treatment of atopic dermatitis (AD) in children. Recently we indentified novel probiotic *Lactobacillus casei* and *paracasei* stains which improved clinical syndromes of AD in children with cow’s milk (CM) allergy. The aim of the study was to analyze the effect of those strains on production of secretory and circulating anti-*Lactobacillus* antibodies as well as on modulation of serum cytokine profile. The study included 60 children with recognized AD caused by CM allergy. Patients were randomized in a double-blind design to receive either placebo or the mixture of *Lactobacillus casei* LOCK 0900, *L. casei* LOCK 0908 and *L. paracasei* LOCK 0919 for 3 months in daily dose $5 \times 10^9$. Antibody and cytokine responses were measured using immunoenzymatic methods before bacteria application, just after finishing bacteria in-take and 5 months later. We observed an increase in antibody secretion mainly of IgA isotype in stools, but without statistical significance ($p=0.08$). Cytokine profile analyses showed activation of proinflammatory IL-12 ($p=0.06$) and IL-18 ($p=0.03$) after 8-month lasting observation. We conclude that the improvement of clinical syndromes after *Lactobacillus* bacteria in-take is dependent on modulation of cytokine balance, and in this process IL-18 seems to play the most important role.

Key words: allergy, antibody response, cytokines, IL-18, *Lactobacillus*, probiotics

Introduction

Probiotics are live microorganisms that when ingested might have a positive effect on disorders in which immunological disturbances occur [7]. Experimental and clinical studies have indicated that probiotics can significantly influence the immune responses of host in promoting the production of secretory and circulating antibodies (Abs), and altering the balance of proinflammatory (Th1)/proallergic (Th2) responses and the cytokine production profile [2, 7, 15]. Recently, a role of probiotic bacteria, especially *Lactobacillus rhamnosus* strain GG (LGG), both in prevention

Address for correspondence

Bożena Cukrowska, MD, PhD
Department of Pathology
The Children’s Memorial Health Institute
Aleja Dzieci Polskich 20
04-736 Warsaw, Poland
fax: +48 22 8151975
e-mail: b.cukrowska@czd.pl
and treatment of allergic diseases was demonstrated [8, 9, 13, 18, 19]. LGG administrated to pregnant women reduced the incidence of allergy in their children after 2 and 4 years. Application of LGG to infants with atopic dermatitis (AD) decreased the severity of the disease. This strain induced an increase the proinflammatory cytokine response in peripheral lymphocytes in infants with IgE-associated AD and activated production of secretory IgA [11, 12, 16, 17]. It seems that antibodies, especially occurring on mucosa could react with allergens protecting their translocation through mucosal barrier. On the other hand improvement of disease symptoms could be induced by activation of Th1 cytokines which maintain Th1/Th2 balance in allergic children [2, 7].

We identified novel probiotic Lactobacillus casei and \textit{paracasei} strains which given to children with cow’s milk (CM) allergy demonstrating AD improved clinical syndromes in IgE-allergic patients [1, 5]. The aim of this study was to analyze the effect of probiotic Lactobacillus casei and \textit{paracasei} strains on production of secretory and circulating anti-Lactobacillus Abs in sera and stools as well as on modulation of cytokine profile in children with AD.

**Patients and methods**

**Patients’ characteristics and study design**

The study included 60 children aged 3 months – 18 months (mean age 10 months) with atopic dermatitis caused by CM allergy, who did not received antibiotics and probiotics for at least 3 months before the study. CM allergy was proved by challenge with CM formula. During the study children received hydrolyzed milk formula or were breast fed. In case of breast feeding women were on CMP-free diet. Patients were randomized in a double-blind design to receive either placebo or a mixture of three probiotic strains: Lactobacillus casei LOCK 0900, L. casei LOCK 0908, L. paracasei LOCK 0919 for 3 months in daily dose $5 \times 10^9$.

Clinical improvement was evaluated using Severity Scoring of Atopic Dermatitis (SCORAD) before bacteria application (time 0), at the end of their in-take (3 m) and 5 months later (8 m). At the same time sera and faeces were collected for measurement of antibody and cytokine levels. Finally, 44 children finished the whole study and determination of Ab levels were done in 33 children. Faeces were diluted with PBS in concentration 1 g/ml, vortex and centrifuged. Fecal supernatants were collected and frozen in $-20^\circ$C.

The infants participated in the study with the informed consent of their parents and the study was approved by the Ethics Committee of the Children’s Memorial Health Institute.

**Detection of specific anti-Lactobacillus Ab**

The Ab response was estimated in the sera by enzyme linked immunosorbent assay (ELISA) as previously described by Cukrowska et al [4] using heat-inactivating mixture of probiotic \textit{Lactobacillus} bacteria as a antigen. Microplates were coated with bacteria suspended in PBS at a concentration of $10^9$/ml. After overnight incubation bacteria were fixed to the plates by a 10-min incubation with 0.025% glutaraldehyde. The plates were then washed with tap water and phosphate saline buffer (PBS) pH 7.4, and blocked with 5% normal goat serum (Sigma Chemical Co, St. Louis, MO, USA) for 30 min in room temperature. After washing samples duplicates were applied. Serum samples were diluted in 1% bovine serum albumin (BSA) /PBS in following dilutions: for IgA Ab 1:50 and 1:200, for IgM 1:100 and 1:500, for IgG 1:200 and 1: 800. Fecal samples were applied without dilution. Standard normal human adult serum was always added in a five-fold dilution starting from 1:100 as a positive control, 1% of BSA being used as a negative control. After overnight incubation at 4°C and washing, polyclonal goat anti-human IgA, IgG or IgM conjugated to peroxidase (Jackson Immunoresearch) diluted 1:2000 in 1%BSA/PBS were added. Finally, the plates were washed and 50 µl of solution containing o-phenylenediamine dihydrochloride (Sigma Chemical Co, St. Louis, MO, USA) was added. The reaction was stopped with acid sulphuric and the plates were read on micro-ELISA reader at 450. The level of Ab activity was expressed as arbitrary units (AU) calculating from the calibration curve, in which standard normal human serum diluted 1:100 contained 100 AU.

**Cytokine determination**

In sera proinflammatory interleukin-18 (IL-18), IL-12, interferon-gamma (IFN-gamma), regulatory transforming growth factor –beta 1 (TGF-beta1) and proallergic IL-4, IL-5 were measured. The cytokine level was determined as previously described by Rosiak et al using R&D System Kits [14]. Briefly, 50 µl of supernatants was added to a microtitter well coated with the specific monoclonal antibody and left for 24 hour in 4°C. After incubation the wells were washed and 100 µl of detection antibody was added to each well and the plates were incubated for 2 hours in room temperature. Then the wells were washed again and 100 µl of streptavidin was added for 20 minutes. Finally, the plates were washed and 50 µl of solution containing o-phenylenediamine dihydrochloride (Sigma Chemical Co, St. Louis, MO, USA) was added. The reaction was stopped with acid sulphuric and the plates were read on micro-ELISA reader at 450. The amounts of cytokines were calculated from the standard curve. The results were expressed in pg/mL as arithmetical means.

**Statistical analysis**

The results were statistically analyzed using parametric t-test and nonparametric Mann Whitney test. P<0.05 was considered as statistically significant.

**Results**

**The level of specific anti-Lactobacillus antibodies**

In sera specific anti-\textit{Lactobacillus} Abs of IgM, IgG and IgA isotypes were found in both groups of children. An increase
in Ab levels mainly of IgA and IgM isotopes was detected during 8-month lasting observation in patients receiving Lactobacillus strains as well as in placebo group. Although Lactobacillus bacteria in-take induced slightly higher amounts of IgA anti-Lactobacillus Abs in sera as compared with sera of children receiving placebo the statistical significance was not achieved (Fig. 1). In stools IgA anti-Lactobacillus Abs dominated, and only minimal amounts of IgM and IgG Abs were found (Fig. 2). In contrast to circulating Abs which increased during 8-month observation in both groups, an increase in Ab level in stools was observed only in children receiving Lactobacillus strains. An increase was found only just after finishing the bacteria in-take (3 m). After next 5 months Ab amounts decreased to the level observed in placebo group. An increase was found in all isotypes, but did not achieve the statistical significance in patients supplemented with probiotics in comparison with placebo group, although for IgA Ab p=0.08.

Fig. 1 Specific anti-Lactobacillus Abs in sera of children receiving Lactobacillus strains (black bars) and placebo (white bars) Abs were measured by immunoenzymatic assays. There was no statistical significance between Lactobacillus and placebo groups.

Fig. 2 Specific anti-Lactobacillus Abs in stools of children receiving Lactobacillus strains (black bars) and placebo (white bars) Abs were measured by immunoenzymatic assays. *statistical significance between children receiving Lactobacillus strains and placebo.
Cytokine level in sera

IL-4 and IL-5 was not detected in most (>90%) sera, and IFN-gamma was found in 5 children with group receiving probiotics and in 4 from control placebo group. IL-12, IL-18 and TGF-beta1 were detected in sera of all children. The statistical significance between studied groups was found only for IL-18 (Fig. 3). IL-18 continuously decreased in placebo group after 3 and 5 months. In *Lactobacillus* group it maintained at the same level after finishing bacteria in-take (3 m), and increased 5 months later (p=0,03). Likely to IL-18, IL-12 slightly increased after 8-month observation in children receiving probiotics in comparison with placebo group, but without statistical significance (p=0,06) (Fig. 4). TGF-beta1 maintained at similar levels in both groups during the whole study (Fig. 5).

Discussion

Different probiotic strains have been shown to be useful in the treatment of AD in children [7, 13, 18, 19]. As atopic diseases are characterized by imbalance of Th1/Th2 cytokine profile, it is believed that probiotics affect the immune system by enhancing of regulatory and/or proinflammatory cytokines production and by reducing Th2 cytokine release [2]. In addition, probiotics are able to induce a production of secretory and circulating Abs which create the first line of defense against external antigens including allergens.

Our group presented that novel probiotic *L. casei* and *L. paracasei* strains markedly reduced the severity of AD in infants in randomized placebo controlled study [5]. After 3 months of treatment SCORAD index significantly decreased only in group receiving probiotics, and in IgE-dependent allergy was significantly lower in comparison with placebo group. In *vitro* analyses of those strains using blood cell cultures of atopic children have shown that they are potent inducer of both pro-inflammatory and regulatory cytokines, and on the other hand they do not trigger pro-allergic responses [14]. In present *in vivo* study we have shown that *L. casei* and *paracasei* strains activate both humoral and cytokine responses in children with CM allergy demonstrating AD. Bacteria induced production of anti-*Lactobacillus* Abs mainly of IgA isotype in gut. Although the level of secretory IgA Abs increased after finishing of bacteria in-take it was not statistically significant as compared with placebo group, and then after 5 months the amounts of Abs decreased to the level found in placebo group. We supposed that children response to probiotic bacteria by production of anti-*Lactobacillus* Abs, but this humoral activation is limited at time. Our earlier studies performed on germ-free piglets associated with non-pathogenic *Escheichia coli* O86 as well as with newborn children to whom probiotic strains of *E. coli* O83 were given presented that probiotic bacteia induced production of specific Abs, but this response occurred in short period after bacteria application [3, 4]. In piglets *E. coli* colonization induced production of specific IgA Abs in gut as early as 4 days after bacteria in-take. The level of these Abs rapidly decreased after 15 days, but increased in sera. In newborn we observed a significant increase in secretory IgA Ab level starting from 2 weeks after bacteria in-take, but after 8 weeks the level decreased although still it was
Lactobacillus casei/paracasei strains induced not only humoral responses but also activated cytokine production in children. In in vitro studies they activated blood cells of children with AD to production of IL-12, IL-18, TNF-alpha and IFN-gamma in higher amounts than known polyclonal activator PHA [14]. Simultaneously, Lactobacillus strains inhibited IL-5 secretion. Now we showed that in vivo they slightly induced proinflammatory IL-12 and significantly modulated production of IL-18. We observed that serum IL-18 in placebo group decreased during the whole study and this decrease correlated with clinical improvement. In placebo group SCORAD decreased after 3 month and then after 5 months as well [5]. In contrast, in children receiving probiotics in spite of better clinical improvement than in placebo group, IL-18 did not decrease. Probiotics activated IL-18 production at the same level after finishing of bacteria in-take, and even slightly increased its secretion 5 months later. It was shown that monocytes from patients with AD secreted reduced amounts of IL-18 [6], and it is known fact that IL-18 is a potent proinflammatory cytokine able to induce IFN-gamma, TNF-alpha and IL-1 [10]. This proinflammatory effect is the result of co-operation with IL-12, which in our study was slightly higher in children receiving probiotics. On the other hand IL-18 in the absence of IL-12 induces naive T-cell into Th2. Thus, IL-18 is a unique cytokine that stimulates both Th1 and Th2 responses depending on its cytokine milieu [10]. We supposed that probiotic Lactobacillus casei and paracasei strains used in our study exert the beneficial effects in allergic patients by regulation of Th1/Th2 cytokine profile by IL-18, i.e. cytokine which plays an important role in the overall immune response.

Acknowledgment

This study is supported by the State Committee for Research (project 2P05E 067 26).

References