Journal of Pediatric Gastroenterology and Nutrition
49:1–5 © 2009 by European Society for Pediatric Gastroenterology, Hepatology, and Nutrition and
North American Society for Pediatric Gastroenterology, Hepatology, and Nutrition

Lactobacillus Reuteri Modulates Cytokines Production in Exhaled Breath Condensate of Children With Atopic Dermatitis

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ABSTRACT

We measured the concentration of interferon-γ and interleukin-4 in the exhaled breath condensate of children with atopic and nonallergic dermatitis receiving a probiotic supplementation (*Lactobacillus reuteri* ATCC 55730) or placebo for 8 weeks. We demonstrated that the levels of these cytokines increased and decreased respectively only in atopic subjects receiving active treatment. Our data suggest that the oral administration of a specific probiotic strain in patients with atopic dermatitis, can modulate in vivo the cytokine pattern at a different site from intestine. ***JPGN 49:000–000, 2009.*** **Key Words:** Atopic dermatitis—Cytokine—Exhaled breath condensate—Probiotics. © 2009 by European Society for Pediatric Gastroenterology, Hepatology, and Nutrition and North American Society for Pediatric Gastroenterology, Hepatology, and Nutrition

In the last decade the interactions between intestinal microbiota and the host have prompted active research, and it is now clear that the close interaction between commensal bacteria and gut-associated lymphoid tissue plays a key role to maintain body health, providing positive stimuli for the immune system (1). Evidences suggest that childhood allergy development could be related, at least in part, to an imbalanced gut microbiota (2,3). In this light, probiotics, living microorganisms that when ingested in certain numbers are able to positively influence human health, have been proposed as potential preventive or therapeutic option for allergic disorders (4,5). The exact mechanisms of these possible clinical effects are still under investigation. The vast majority of the available data derives from in vitro or animal models. But, if this approach could be used to define singular aspects of probiotic activity, the possible adaptation of these results in humans is problematic (5). The lack of in vivo techniques to easily investigate, in a noninvasive way, the mechanisms used by these microorganisms limit our understanding in the definition of the probiotic effects in humans.

Atopic eczema/dermatitis syndrome (AEDS) is a common chronic inflammatory skin disease that usually begins in early infancy (6). At least 2 forms of AEDS have been identified one being an allergic form (atopic dermatitis [AD]) characterized by immunoglobulin (Ig)E-sensitization, observed in about 70% to 85% of patients, and second a nonallergic form that affects 15% to 30% of the subjects with AEDS (7). Atopic dermatitis is one of the most common allergic diseases in pediatric patients worldwide (6). We have recently demonstrated that the interleukin (IL)-4/interferon (IFN)-γ ratio was significantly higher in children with AD when compared with controls in their exhaled breath (8), and that these cytokines could be easily measured in exhaled breath condensate (9). *Lactobacillus reuteri* is a probiotic strain that has been proposed for the treatment of AD (10,11), but the effect of this microorganism on cytokine production in these patients is still largely undefined. The aim of our study was to evaluate the effects of oral supplementation of *L reuteri* on EBC cytokines expression in children with AEDS.

MATERIALS AND METHODS

Study Design and Patients

A prospective double-blind randomized placebo-controlled study was planned. From October 2007 to May 2008, all of the patients ages 4 to 10 years were referred to our tertiary Pediatric Allergology Center because AEDS were considered eligible for the study. We excluded children with active tuberculosis, autoimmune diseases, immunodeficiency, chronic inflammatory bowel diseases, cystic fibrosis, metabolic diseases, malignancy, malformation of the gastrointestinal tract, severe neurological impairment, and history of probiotics, antibiotics, or immunosuppressive agents (including systemic or topical

Received November 14, 2008; accepted August 3, 2009.

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AQ2 The authors report no conflicts of interest.

steroids) use in the last 8 weeks. Approval for the study was obtained from our Institution Ethics Committee and written informed consent was obtained from the parents of each child enrolled in the study. All of the enrolled patients underwent a full diagnostic evaluation including clinical and laboratory examinations. Atopic eczema was diagnosed in each patient by expert pediatric allergologists according to the UK working party's criteria (an itchy skin condition plus greater than or equal to 3 of the following; history of atopic disease in the family, dry skin during the last year, history of eczema, or visible eczema involving typical sites) (12). The clinical severity of eczema was assessed with the severity scoring index atopic dermatitis, developed by the European task Force for Atopic Dermatitis (13), at the beginning and end of the intervention period. Patients were classified as having moderate eczema, if the score was 25 to 50 points, or severe eczema, if the score was >50 points. Data on allergic sensitization were obtained from the results of skin prick tests performed with standardized extracts (Stallergenès S.A., Antony, France) of wheat, cow's milk, hen's egg, soy, and common airborne allergens. Total serum IgE levels were also analyzed by using a chemiluminescence assay (Immunolite, Diagnostic Products Corporation, Los Angeles, CA).

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Active treatment consisted of *L reuteri* (ATCC 55730) administered as commercial available chewable tablets (Nóos, BioGaia AB, Sweden). Each tablet contained a dose of 10^8 colony-forming units of the bacterial strain. The placebo group received chewable tablets with the same shape, size, taste, and appearance as those containing the viable *L reuteri*, which ensured that the study was blinded for investigators and patients. Moreover the boxes containing *L reuteri* or placebo were identical, and the 2 regimens were prescribed as 1 tablet, once per day (2 hours before meals) for 8 weeks. Adherence to treatment was evaluated by counting the tablets returned by the parents; a minimum tablet intake of 95% was considered as acceptable. The group assignment was performed according to a randomization list.

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All of the clinical and laboratory procedures were performed or evaluated by expert pediatric allergists unaware of the study group assignment. Data were collected in clinical charts and evaluated by statisticians unaware to the study aim.

Exhaled Breath Condensate Analysis

The EBC was collected using a condenser (TURBO. DECS, ItalChill, Parma, Italy) at baseline after the enrolment and after 8 weeks of therapy. The subjects breathed through a mouthpiece and a two-way nonrebreathing valve that also served as a saliva trap. Condensate, mean 1.2 mL (range 0.8–2.1 mL), was collected as ice at -20°C, transferred to eppendorf tubes and immediately stored at -80°C. A stable pH was achieved in all cases after deaeration of breath condensate specimens by bubbling with argon (350 mL/min) for 10 minutes (pH meter with a pH range of 0.00–14.00 and a resolution/accuracy on the order of 0.01 ± 0.02 pH), as previously reported (14).

Measurement of interferon-γ and interleukin-4

A quantitative enzyme-linked immunosorbent assay kit (Bender MedSystem Bouty, Wien Austria), with a detection limit of 1.5 pg/mL, was used to measure cytokines concentrations in EBC samples. The intra- and interassay variations were less than or equal to 10%. The analysis of 2 aliquots of the same sample was repeated by the same operator with 2 different kits in the same day. For each assay, a standard curve was designed with highly purified recombinant cytokines and analyzed based on the international reference standards.

Statistics

Statistical analyses were performed by using the Statistical Package for Social Sciences software version 11.0 (SPSS, Inc, Chicago, IL). For each parameter, the differences between posttreatment and postplacebo values were calculated by paired *t* test, and were expressed as mean ± standard error. Nonparametric Mann-Whitney test was used for comparison of continuous data if not normally distributed. Correlations were analyzed by using the Spearman bivariate correlation test. P values <0.05 were considered significant.

RESULTS

During the study period 63 patients were considered eligible for the study, and a total of 51 subjects (28 males, median age 5.8 years) were enrolled and randomly assigned to receive either active treatment or placebo. Twelve patients were excluded because of the lack of informed consent. Of the total 51 enrolled patients, 26 were assigned to the active treatment (16 with AD and 10 with nonatopic eczema), whereas 25 received placebo (14 with AD and 11 with nonatopic eczema). Main demographic and clinical characteristics of the study population were reported in Table 1. No significant differences were observed considering the major demographic and clinical characteristics of the groups evaluated in the study, except for total serum IgE levels.

Active probiotic treatment or placebo was well accepted by the patients and all of the subjects showed a sufficient grade of adherence (minimum tablet intake >95%). All of the children were able to successfully complete the EBC procedure, which was well accepted by all of the patients. Cytokine pattern in EBC were examined in all of the subjects at the enrolment and after 8 weeks of treatment. At baseline, the EBC IFN-γ concentration was significantly lower in patients with AD compared with patients with nonatopic eczema (0.25 ± 0.14 vs 0.40 ± 0.23, $P = 0.01$), and it significantly increased only in children with AD after probiotic treatment compared with patients who received placebo or with subjects with nonatopic eczema (Fig. 1). At enrolment no differences were found in the EBC IL-4 concentration between the groups ([AD vs non-AD] 2.50 ± 0.71 vs 2.10 ± 0.67, $P = 0.7$), whereas the concentration of this cytokine decreased significantly in patients with AD after probiotic treatment compared with children receiving placebo or subjects with nonatopic eczema (Fig. 1).

TABLE 1. *Characteristics of the patients enrolled in the study*

	Patients with AD receiving *Lactobacillus reuteri*	Patients with AD receiving placebo	Patients with nonatopic eczema receiving *Lactobacillus reuteri*	Patients with nonatopic eczema receiving placebo
Age (y), median (range)	5.5 (4.5–10)	6.0 (5–9)	5.8 (4.8–8.5)	6.2 (5.5–10)
AQ6 sIgE at baseline (kIU/L)*	121 (12–1077)	118 (7–895)	36 (9–100)	21 (7–89)
SCORAD index at inclusion	27 (21–44)	31 (23–50)	35 (23–47)	33 (27–50)
SCORAD index after intervention	25 (21–42)	27 (22–47)	31 (21–43)	34 (25–49)
Results of SPTs, n				
Wheat	1	0	0	0
Cow's milk	3	4	0	0
Hen's egg	4	2	0	0
Soy	1	0	0	0
Pollens	4	2	0	0
Dust mite	5	3	0	0
Molds	1	0	0	0
Pets with fur	3	2	0	0

AD = atopic dermatitis; SCORAD = severity scoring index atopic dermatitis; SPT = skin prick test.
* $P = 0.01$.

After 8 weeks of treatment, no significant changes in the severity scoring index atopic dermatitis index mean values were observed in patients who received the probiotic supplementation compared with children who received placebo (Table 1).

DISCUSSION

Several trials had already proven that probiotics would have few chances to improve AD; more recently, a meta-analysis of 6 prevention and 4 treatment clinical trials, indicates that current evidence is more convincing for probiotics' efficacy in prevention than treatment of pediatric AD (15). In our study we adopted AD as a model of a common allergic disease to investigate whether a short course of oral *L reuteri* supplementation could be able to positively modulate in vivo the cytokine pattern at a different site from intestine. Immune responses, mostly mediated by type 2 helper T cells (T_H2), play an important role in the pathogenesis of AD (6). Impaired T_H1 responses in early life have been associated with future risk and subsequent development of allergic disease. Appropriate immunoregulatory responses, induced by intestinal microorganisms, can invoke the counter-balancing T_H2 responses and the up-regulation of T_H1-type cells (2,3). Exhaled breath condensate cytokine pattern was used as useful measure of the functional capacity and maturational status of T_H1 pathways. The principal finding of our study was that *L reuteri* oral administration is associated with an increase in T_H1-type cytokine IFN-γ response in the EBC of children with AD. Although the pathogenesis of AD is still not completely elucidated, a number of observations suggest that this condition could be the initial cutaneous manifestation of a systemic disorder that also leads to other atopic diseases including food allergy, asthma, and allergic rhinitis (6). Investigations suggest that this disease is initiated, maintained, and

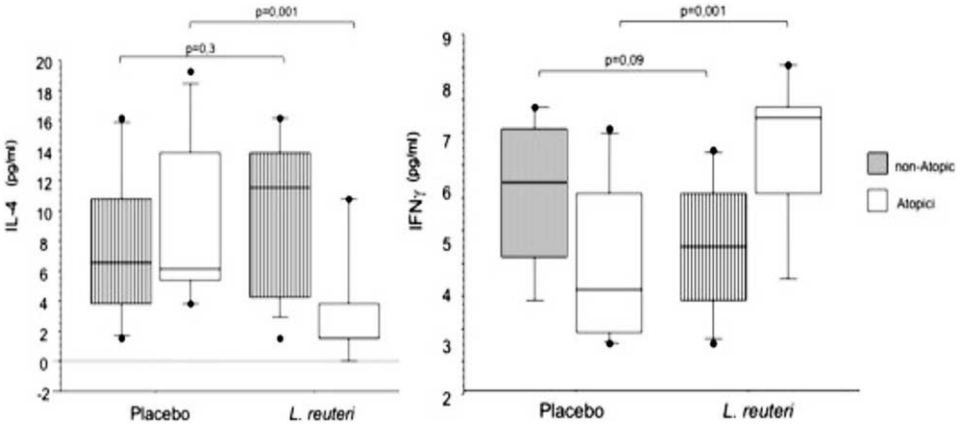

FIG. 1. IL-4 and IFN-γ concentration in children with nonatopic (lined boxes) and atopic (empty boxes) eczema after treatment with *Lactobacillus reuteri* or placebo. IFN-γ = interferon-γ; IL-4 = interleukin-4.

perpetuated by the actions of cytokines, chemokines, T cells, antigen-presenting cells, and other inflammatory cells. Recent studies on the pathogenesis of allergic diseases suggest the importance of intestinal microflora and its possible modulation by probiotics in regulating several function of the immune system (2,16). There are a number of trials that have evaluated the clinical benefits of probiotics in infants and young children with AD (5). Specific bacterial strains, most of them belonging to *Lactobacillus* or *Bifidobacterium* species, may aid in creating optimal conditions to redirect the polarized immunological memory to a healthy balance before such immune responsiveness to environmental antigens is consolidated (2,17,18). It is well recognized that T cells mature in the gut mucosa before seeding to distal mucosal site, such as respiratory tract, and that probiotics may influence their polarization (17). Interestingly, the probiotic's administration was associated with increased IFN-γ responses in children with AD (19).

At baseline, the EBC IFN-γ concentration was significantly lower in allergic than in nonallergic patients with eczema. Decreased capacity to generate IFN-γ is a hallmark of the atopic state (20). Dunstan et al (21) demonstrated that children with AD had significantly lower spontaneous (unstimulated) production of IFN-γ compared with the unaffected children. In a prospective study, secretion of IFN-γ was found reduced at birth in infants who later developed symptoms of atopic disease (22). Our results are consistent with a recent report by Pohjavuori et al (19) showing an increase of in vitro polyclonal IFN-γ response of peripheral lymphocytes of children with cow's milk allergy and AD after 4 weeks of supplementation with *L rhamnosus* GG. Our findings suggest that *L reuteri* could stimulate in pediatric patients with AD, IFN-γ production and lower IL-4 levels at sites in the body distant to that of the initial interaction of the probiotic. We have previously noted that children with AD show an exhaled pattern of T_H2/T_H1 cytokines similar to that of patients with asthma (8), and the results of the present study suggest the *L reuteri* could be able to positively modulate this pattern. Forsythe et al (17) recently investigated the immunomodulating effect of *L reuteri* in a mouse model of airway inflammation and hyper-responsiveness. In addition to the effects in the gut, the authors suggested that oral administration of specific probiotic strains can modulate immune responses in the lung. The common embryonic matrix of entodermic derivation of gastrointestinal and respiratory tissues confers immunological peculiarities regarding the early sensitization and activation of tolerance mechanisms to the digestive system (23). Specific probiotic strains may have a controlling influence on systemic allergic inflammation before altered structure and function of the target organ develop. In this regard, the cytokine profile elicited by a probiotic microorganism could play an important role in determining the immunologic outcome.

Although our preliminary data do not show any short-term clinical effect of *L reuteri* ATCC 55730, we believe that further studies on a larger sample of patients are needed to explore its potential clinical application before any possible use in clinical practice.

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