Lactobacillus GG effect in increasing IFN-γ production in infants with cow's milk allergy

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Background: Probiotic bacteria are potentially beneficial to maturation of the infant's immune system.

Objective: To examine the role of probiotic bacteria in treatment of cow's milk allergy (CMA) and IgE-associated dermatitis, we investigated the immunologic effects of *Lactobacillus rhamnosus* GG (LGG) and a mixture of 4 bacterial species (MIX).

Methods: In a randomized, double-blind study design, concomitantly with elimination diet and skin treatment, LGG, MIX, or placebo was given for 4 weeks to infants with suspected CMA. After anti-CD3 (OKT3) and anti-CD28 stimulation of PBMCs, IFN-γ, IL-4, IL-5, and IL-12 levels were measured in culture supernatants by ELISA. Intracellular IFN-γ, IL-4, and IL-5 production on CD4 lymphocytes was analyzed with fluorescence-activated cell sorting.

Results: Secretion of IFN-γ by PBMCs before the treatment was significantly lower in infants with CMA (*P* = .016) and in infants with IgE-associated CMA (*P* = .003) than in non-CMA infants. Among the infants who received LGG, the level of secreted IFN-γ increased in those with CMA (*P* = .006) and in those with IgE-associated dermatitis (*P* = .017) when compared with the placebo group. Secretion of IL-4 increased significantly in infants with CMA in the MIX (*P* = .034) but not in the LGG group.

Conclusion: Deficiency in IFN-γ response appears to be related to CMA. LGG raises IFN-γ production of PBMC in infants with CMA and in infants with IgE-associated dermatitis and may thus provide beneficial T$_H$1 immunomodulatory signals. MIX, although containing LGG, appears to modulate the immune responses differently. (J Allergy Clin Immunol 2004;114:131-6.)

Key words: Cow's milk allergy, CMA, IgE-associated, Lactobacillus *GG, probiotic, peripheral blood mononuclear cells, IFN-γ, IL-4, IL-5*

Abbreviations used
cfu: Colony-forming units
CMA: Cow's milk allergy
CM: Cow's milk
EHF: Extensively hydrolyzed whey formula
FACS: Fluorescence-activated cell sorting
LGG: *Lactobacillus rhamnosus* GG
MIX: Mixture of 4 bacterial species
SPT: Skin prick test

Experimental studies have provoked the suggestion that intestinal microbes are important regulators of immune responses and oral tolerance.[1,2] In the human intestine, microflora have also been suggested to affect the development of the immune system and atopic sensitization in early infancy. A reduced ratio of bifidobacteria to clostridia has been observed to precede the development of atopy.[3] Furthermore, patients with allergic diseases are less often colonized by enterococci and bifidobacteria but have more clostridia and staphylococci than the non-allergic patients.[3-5]

Cow's milk allergy (CMA) is a manifestation of failure of oral tolerance and is usually spontaneously resolved by age 2 years. Appropriate exposure to food-borne and orofecal microbes may downregulate the T$_H$2 polarization predominant in the neonatal period[6] and thus contribute to the development of a more balanced cytokine profile and response to food antigens.[7] CMA develops when proteins in cow's milk (CM) induce an immune-mediated reaction leading to clinical symptoms in susceptible infants.[8] A failure of tolerance induction is associated with an exaggerated immune-deviation process toward the T$_H$2 cytokine pattern and delayed maturation of T$_H$1 cells. The T$_H$1 cytokine IFN-γ plays an important role as an activator of antigen-presenting cells and T cells. Studies report that atopic infants show decreased IFN-γ production and delayed postnatal maturation of cellular immune functions.[9-12]

Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host.[13] These bacteria may provide important local and systemic immunoregulatory signals. In the current study, we followed the cytokine production of PBMCs in infants with suspected CMA who were treated with *Lactobacillus rhamnosus* GG (LGG) alone or with a mixture of 4 bacterial species (MIX).

METHODS

Subjects

Patients in this study were infants participating in a clinical study of the effects of probiotics on symptoms of atopic dermatitis in infants between November 1999 and March 2002 in the Skin and Allergy Hospital of Helsinki University Central Hospital. Of 230 infants with atopic dermatitis who were suspected to have CMA and who completed the study, 119 were taken for analysis (age, 1.4-11.5 months; mean, 6.5; 61% boys). Families gave written informed consent. The local ethics committee approved the study protocol.

On the first visit, infants and their breast-feeding mothers began a strict CM-free diet. All infants received extensively hydrolyzed whey formula (EHF; Peptidi-Tutteli, Valio, Helsinki, Finland). Other foods suspected to cause symptoms were also eliminated. The skin was treated topically.

Infants were randomized at the first visit—according to computer-generated block randomization of 6 infants—to receive 1 of 3 products in a double-blind manner: (1) the LGG group (n = 42) received capsules containing LGG (ATCC 53103) 5×10^9 colony-forming units (cfu); (2) the MIX group (n = 41) a mixture of 4 bacterial species: LGG 5×10^9 cfu, *L rhamnosus* LC705 5×10^9 cfu, *Bifidobacterium breve* Bbi99 2×10^8 cfu, and *Propionibacterium freudenreichii* ssp *shermanii* JS 2×10^9 cfu; and (3) the placebo group (n = 36), only the inert matrix material, microcrystalline cellulose. These products (supplied by Valio) were mixed with food twice a day for 4 weeks, and all looked, smelled, and tasted identical. Parents were urged not to feed the infants any other probiotic preparations during the study. Clinical improvement was evaluated at the second visit, after the 4-week treatment, and 4 weeks after the end of treatment.

After a successful 8-week elimination, 4 weeks after the end of probiotic treatment, we started a double-blind, placebo-controlled CM challenge. The active formula was an adapted CM formula (Tutteli, Valio) mixed with the EHF (1:2) to make it indistinguishable from the placebo formula (EHF alone). The challenge formula was given orally in increasing doses of 2, 10, 50, and 100 mL. The next day, symptom-free infants continued to receive the same formula daily at home for the next 4 days. After a washout period of 2 to 9 days, the challenge formula was changed, and this procedure was repeated. Thereafter, a decision of the symptomatic challenge period was made, and the milk code was opened. CMA was diagnosed in 65 infants who showed urticaria, clear worsening of atopic dermatitis, vomiting, diarrhea, wheezing, allergic rhinitis, or conjunctivitis during the challenge with CM-containing formula. In 54 infants, CMA was excluded, because the challenge was negative.

At the first visit, skin prick tests (SPTs) were performed with commercial allergen extracts of egg white (Alyostal prick test; Stallergenes SA, Antony, France), cat, dog, and birch (Soluprick; ALK-Abelló, Hørsholm, Denmark) according to the standard technique.[14] Duplicate tests were performed with fat-free CM; a panel of 10 widely used commercially available adapted CM, extensively hydrolyzed, amino acid–based, and soy protein–based infant formulas; cereal grains; and purified gliadin.[15] Any mean wheal diameter ≥3 mm greater than the negative control was considered positive. Concentrations of serum CM and wheat-specific IgE were measured by the Pharmacia CAP system RAST fluoroenzyme immunoassay (Uppsala, Sweden).

Infants with a positive SPT to any antigen tested or any specific IgE concentration >0.7 kU/L were considered to have IgE-associated dermatitis (n = 72). Infants with positive CM challenge and a positive SPT to any antigen tested or any specific IgE concentration >0.7 kU/L were considered to have IgE-associated CMA (n = 42). CMA was non−IgE-associated if CM challenge was positive and none of SPTs were positive or all specific IgE concentrations were <0.7 kU/L (n = 23).

Stimulation of PBMCs

Pretreatment blood samples were collected at the first visit and posttreatment samples after the 4-week treatment, on the second visit. Of the 119 infants' 63 blood samples paired, 40 first visit samples and 16 second visit samples were available for analysis. PBMCs were obtained by Ficoll-Hypaque (Pharmacia) centrifugation of the heparinized blood. PBMCs (2×10^6/2 mL) in RPMI 1640 medium (GibcoBRL, Gaithersburg, Md) containing 1% L-glutamine (GibcoBRL) and 5% AB serum (Finnish Red Cross Blood Transfusion Service, Helsinki) were cultured and stimulated with plates (Nunc 24 wells, Roskilde, Denmark) coated with monoclonal OKT3 (anti-CD3) antibody (1 μg/well; R&D Systems, Minneapolis, Minn) in the presence of soluble anti-CD28 antibody (2 μg/well; Becton Dickinson Immunocytometry Systems, San Jose, Calif). Control cultures were incubated similarly without stimulation (negative controls). After 24-hour incubation, a supernatant sample was collected for further ELISA analysis and Brefeldin A (10 μg/mL; Sigma, St Louis, Mo) was added to the culture media. Cells were collected for fluorescence-activated cell sorting (FACS) analysis after a further incubation of 16 to 18 hours.

Flow cytometry

Cells were fixed with 4% paraformaldehyde and suspended in 0.5% BSA in PBS. PBMCs were incubated with antibodies to surface markers such as CD4-peridinin chlorophyll protein (Becton Dickinson) or CD69-fluorescein isothiocyanate (Becton Dickinson). Cells were permeabilized with Becton Dickinson FACS Permeabilizing Solution and stained with phycoerythrin-conjugated mAbs to IFN-γ (Becton Dickinson), IL-4 (Becton Dickinson), or IL-5 (Serotec Co, Oslo, Norway). Ten thousand CD4 lymphocytes were collected in a FACScan flow cytometer (CellQuest software, Becton Dickinson). Analysis gates were set on CD4+ lymphocytes according to forward and sideward scatter properties. Results are expressed as percentage of cytokine-producing cells in CD4 cells.

ELISA

Quantities of IFN-γ, IL-5, IL-4, and IL-12 in the culture supernatants were measured with ELISAs: 96-well microplates (Nunc) were coated with monoclonal antihuman IFN-γ antibody (lot CD48432; Endogen, Woburn, Mass) at a concentration of 2 μg/mL (50 μg/well). Dilutions of recombinant human IFN-γ (lot M070524; BD Pharmingen, San Diego, Calif) were used to create a standard curve. Supernatant samples (100 μL/well) and standards (100 μL/well) were incubated for 2 hours at 37°C. Biotinylated antihuman IFN-γ monoclonal antibody (lot CD47529; Endogen) was added at a concentration of 0.25 μg/mL (50 μL/well). AP-streptavidin conjugate (Zymed Laboratories, San Francisco, Calif) served as the substrate. The detection limit of the assay was 8 pg/mL. IL-5 concentrations in the culture supernatants were measured with the same protocol by using a purified rat antimouse/human IL-5 monoclonal antibody at 1 μg/mL (lot M049743; Pharmingen) for coating and biotinylated rat antihuman IL-5 monoclonal antibody to measure bound IL-5 at 0.5 μg/mL (lot M057221; Pharmingen). A standard curve was created with dilutions of recombinant human IL-5 (lot M021519; Pharmingen). The detection limit of the assay was 15 pg/mL. IL-4 concentrations in the culture supernatants were measured with a commercial human IL-4 ELISA kit (Pelikine Compact, Amsterdam, The Netherlands) with a detection limit of 0.6 pg/mL. Total IL-12 protein levels were determined by commercial

high-sensitivity sandwich ELISA (Quantikine HS; R&D Systems) according to the manufacturer's instructions, with a detection limit of 0.6 pg/mL. All assays were performed in duplicate, and the intensity of the color was measured with multiscan MS version 8.0 (Labsystems Oy, Helsinki).

Statistical analysis

The nonparametric Mann-Whitney U test was used for comparisons of all variables, because no variable was normally distributed, and the Wilcoxon signed-rank test was used to compare the follow-up samples. All statistical tests were 2-tailed. Data were analyzed with SPSS for Windows (version 10.0; SPSS Inc, Chicago, Ill). P values $< .05$ were considered statistically significant.

RESULTS

Infants' clinical characteristics

Cow's milk allergy was diagnosed in 65 of the 119 infants. IgE-associated CMA was diagnosed in 42 infants and non−IgE-associated CMA in 23. In 54 infants, the CM challenge was negative, and CMA was excluded. IgE-associated dermatitis was diagnosed in 72 infants.

Cytokine secretion of PBMCs before the treatment

In infants with CMA, OKT3-stimulated IFN-γ secretion of PBMCs was significantly lower than in non-CMA infants (median, 478 pg/mL vs 857 pg/mL; $P = .016$). Decreased IFN-γ production was found in infants with IgE-associated CMA, and the decrease was significant in comparison with the non-CMA infants with IgE association (median, 418 pg/mL vs 931 pg/mL; $P = .001$; Fig 1). Infants with non−IgE-associated CMA had lower IL-4 and IL-5 secretion of PBMCs than did non-CMA infants without IgE association (median, 10 pg/mL vs 24 pg/mL; $P = .008$; and median, 26 pg/mL vs 95 pg/mL; $P = .002$) or with IgE association (median, 10 pg/mL vs 28 pg/mL; $P = .001$; and median, 26 pg/mL vs 90 pg/mL; $P = .038$). These infants with non−IgE-associated CMA also had lower IL-4 and IL-5 secretion than infants with IgE-associated CMA (median, 10 pg/mL vs 20 pg/mL; $P = .002$; and median, 26 pg/mL vs 84 pg/mL; $P = .004$; Figs 2 and 3). Secretion of IL-12 (median, 0, and range, 0-5 pg/mL vs median, 0, and range, 0-15 pg/mL) and IFN-γ (median, 601 pg/mL vs 857 pg/mL; Fig 1) was similar in infants with non−IgE-associated CMA and in non-CMA infants. Secretion of IL-4, IL-5 (medians, 20 pg/mL vs 25 pg/mL and 84 pg/mL vs 95 pg/mL; Figs 2 and 3) and IL-12 (median, 0, and range, 0-8 pg/mL vs median, 0, and range, 0-15 pg/mL) was similar in infants with IgE-associated CMA and in non-CMA infants.

Effects of probiotic treatment on cytokine secretion

IFN-γ secretion of PBMCs was measured before and after treatment with probiotics or placebo, and results were analyzed with the Wilcoxon signed-rank test. For 33 infants with CMA, in the LGG group, secretion of IFN-γ increased significantly ($P = .023$), but no changes oc-
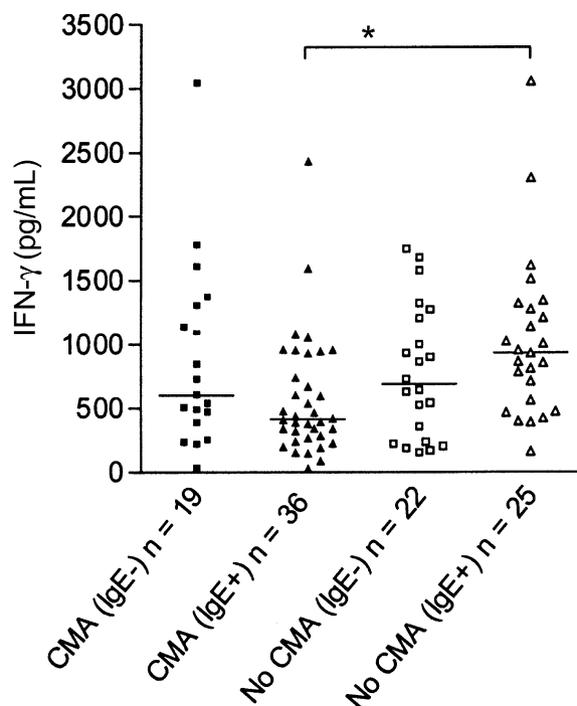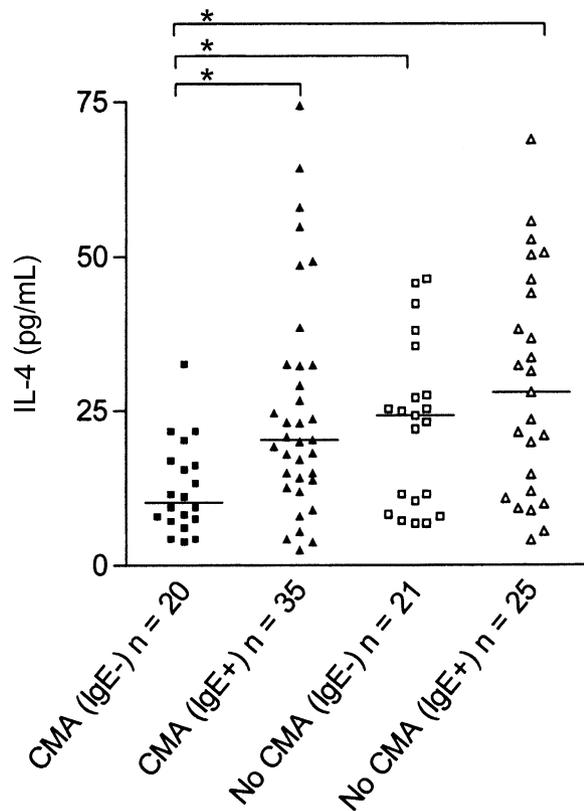

FIG 1. Secretion of IFN-γ by stimulated PBMCs before treatment in infants with non−IgE-associated CMA *(CMA IgE−)*, IgE-associated CMA *(CMA IgE+)*, no CMA without IgE association *(no CMA IgE−)*, and no CMA with IgE association *(no CMA IgE+)*. Horizontal lines show median values. *P* value by Mann-Whitney *U* test; **P* < .01.

curred in follow-up samples in the MIX ($P = .239$) or placebo groups ($P = .086$; Table I). Individual changes in IFN-γ concentrations were calculated to validate the results. Changes in concentrations (after-before) were compared between treatment groups with nonparametric Mann-Whitney U test. The level of IFN-γ increased in the LGG group more than in the placebo group ($P = .006$). In the MIX group, the increase in IFN-γ secretion was not significant in comparison with the placebo group ($P = .058$; Table I).

Stimulated PBMCs of infants with CMA produced more IL-4 after the treatment with MIX ($P = .034$), and the change (after-before) in IL-4 secretion was significant in comparison with the placebo group ($P = .028$). In the LGG and placebo groups, IL-4 secretion remained at the same level (Table I). IL-5 secretion in culture supernatants was similar in all groups (Table I). IL-12 levels were low in all samples, with no changes during follow-up (data not shown). The small number of follow-up samples did not allow subgroup analyses (IgE-associated CMA and non−IgE-associated CMA).

IgE-associated dermatitis was diagnosed in 72 infants, with follow-up samples available from 38. IFN-γ secretion in stimulated PBMCs of infants with IgE-associated dermatitis differed significantly among the LGG, MIX, and placebo groups. The LGG group showed an increase in IFN-γ secretion ($P = .048$), but MIX ($P = 1.000$) and placebo ($P = .158$) treatments had no effect on the IFN-γ response of stimulated PBMCs (Table I).

FIG 2. Secretion of IL-4 by stimulated PBMCs before treatment in infants with non−IgE-associated CMA *(CMA IgE−)*, IgE-associated CMA *(CMA IgE+)*, no CMA without IgE association *(no CMA IgE−)*, and no CMA with IgE association *(no CMA IgE+)*. *Horizontal lines show median values. P* values by Mann-Whitney *U* test; *$*P < .01$.
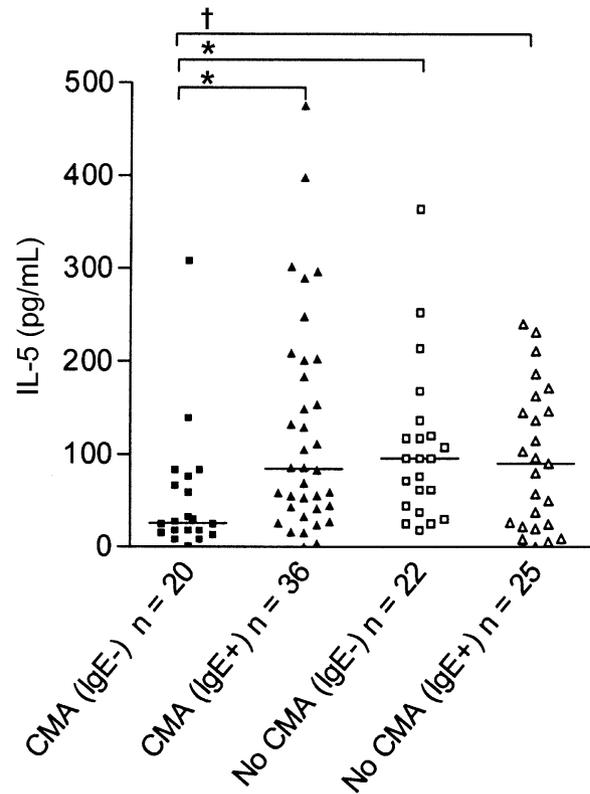
FIG 3. Secretion of IL-5 by stimulated PBMCs before treatment in infants with non−IgE-associated CMA *(CMA IgE−)*, IgE-associated CMA *(CMA IgE+)*, no CMA without IgE association *(no CMA IgE−)*, and no CMA with IgE association *(no CMA IgE+)*. *Horizontal lines show median values. P* values by Mann-Whitney *U* test; *$*P < .01$; $†P < .05$.

TABLE I. OKT3 and anti-CD3−induced cytokine secretion of PBMCs before and after treatment, medians (ranges), pg/mL

	IFN-γ			IL-4			IL-5		
	n*	Before	After	n	Before	After	n	Before	After
CMA									
LGG	12	403 (90-1780)	829 (298-3501)§	12	19 (9-74)	19 (8-46)	11	51 (0-302)	93 (0-281)
MIX	12	437 (23-2431)	681 (331-1681)	12	15 (4-49)	22 (13-65)§	9	47 (0-289)	83 (0-245)
Placebo	9	836 (286-1595)	579 (316-1136)	9	21 (7-32)	18 (4-30)	8	56 (0-138)	45 (0-108)
Dermatitis (IgE)†									
LGG	14	506 (90-1006)	840 (298-3501)§	14	22 (3-74)	24 (8-46)	13	91 (0-302)	99 (0-281)
MIX	12	952 (31-2431)	672 (289-1658)	12	19 (4-53)	25 (8-50)	11	113 (0-289)	82 (0-246)
Placebo	12	1042 (286-3059)	860 (316-2044)	12	24 (9-51)	24 (11-46)	11	70 (0-211)	66 (0-225)
Dermatitis‡									
LGG	24	780 (90-1780)	859 (298-3501)	24	19 (3-74)	19 (7-46)	23	51 (0-302)	79 (0-281)
MIX	21	492 (23-2431)	663 (59-1681)	21	18 (4-53)	23 (8-65)	18	69 (0-289)	73 (0-246)
Placebo	17	955 (231-3059)	765 (316-2044)	17	23 (7-51)	14 (4-46)	16	57 (0-211)	65 (0-225)

*Number of samples.

†IgE-associated dermatitis.

‡All infants with dermatitis.

§Increase in comparison to placebo group: $P < .05$.

The secretion of IFN-γ increased in the LGG group in comparison with the placebo group ($P = .017$). No differences in IFN-γ secretion appeared in the MIX group in comparison with the placebo group ($P = .319$; Table I). In infants with IgE-associated dermatitis, probiotic treat-

ment had no significant effect on IL-4, IL-5 (Table I), or IL-12 secretion (data not shown).

Probiotic treatment had no effect on IL-5, IL-4, IL-12, or IFN-γ secretion of OKT3 antibody-stimulated and CD28 antibody-stimulated PBMCs in infants with

non−IgE-associated dermatitis (data not shown). Similarly, non-CMA infants showed equal IL-4, IL-5, IL-12, and IFN-γ responses to OKT3 and CD28 antibody stimulation despite probiotic treatment (data not shown).

Intracellular cytokines

The expression of the CD69 activation marker appeared in >95% of stimulated CD4$^+$ lymphocytes. In unstimulated lymphocytes, this expression appeared in <1% of CD4 T cells. The number of CD4 cells expressing IL-4, IL-5, IFN-γ was similar in CMA and non-CMA infants, and also in infants with IgE-associated dermatitis and in infants with non−IgE-associated dermatitis (data not shown). Probiotic treatment had no effect on intracellular cytokine activation in lymphocytes stimulated with OKT3 and CD28 antibodies (data not shown).

DISCUSSION

Our results demonstrate, apparently for the first time, that LGG augments IFN-γ secretion of stimulated human PBMCs in infants with CMA and in infants with IgE-associated dermatitis. Results were parallel with our clinical findings. LGG alleviated skin symptoms in IgE-sensitized infants with atopic dermatitis.[16] Moreover, previous clinical studies have demonstrated beneficial effects of probiotic bacteria in intestinal inflammation and atopic dermatitis.[17-20] Cohort and cross-sectional studies have indicated a lower incidence of atopic skin and respiratory tract hypersensitivity reactions among infants with intestinal microflora populations of lactobacilli and bifidobacteria than among those in whom these bacteria are sparse.[3-5]

Oral induction of probiotic bacteria can influence the inflammatory response in healthy and hypersensitive infants differently. In a clinical study, the LGG treatment downregulated inflammatory response by decreasing receptors in neutrophils in milk-hypersensitive subjects. By contrast, in healthy subjects, LGG induced neutrophil activation.[21] In the current study, probiotic products did not have an effect on cytokine response in non-CMA infants. However, LGG augmented T$_H$1 cytokine IFN-γ secretion in infants with CMA and in infants with IgE-associated dermatitis, with the groups partly overlapping.

IFN-γ concentration in stimulated mononuclear cell culture supernatants was significantly lower before the treatment in infants with IgE-associated CMA than in non-CMA infants. Our results support observations that peripheral blood mononuclear cells in patients with atopic disease have a reduced T$_H$1 cytokine IFN-γ secretion capacity.[10,12] The non−IgE-associated CMA, which is considered a cell-mediated disease, appears to demonstrate different signal mechanisms than IgE-mediated CMA. Interestingly, we did not find increased IFN-γ in non−IgE-associated CMA, but rather decreased IL-4, which is a counterregulator of IFN-γ.

Neonatal immune responses are dominated by T$_H$2 cytokines in virtually all infants.[7] A transition from weak neonatal T$_H$2-skewed responses to environmental antigens to a pronounced T$_H$2 cytokine profile occurs during the first year of life in atopic infants. Nonatopic infants show a gradual fall in T$_H$2 responses to allergens. This process starts within the first 6 months of life and deviates toward T$_H$1-biased immune reactions by the age of 18 months.[12,22,23] This selective stimulation may be explained by the hygiene hypothesis, in which T$_H$1 responses are suggested to be induced by external factors such as adequate exposure to microbes during early infancy.[22,24]

Bacterial strains that are able to generate T$_H$1-cell activating signals might be beneficial downregulators in overactive T$_H$2-mediated allergic responses.[25] In our study, MIX effected the stimulation of mononuclear leukocytes differently than did LGG alone. The mixture of different strains tended to stimulate the IFN-γ response, but not significantly (P = .058). However, IL-4 production was augmented in the stimulated PBMCs of infants with CMA after treatment with the mixture of bacterial strains. LGG alone had no effect on IL-4 levels. The reason for this might be competition between different bacterial strains in the intestinal flora or their different action on the immune system of the gut. These immunologic differences between effects of LGG and the mixture of different strains are in accordance with the observation that the mixture of different strains did not have beneficial effects on clinical symptoms in infants with CMA or IgE-associated dermatitis.[16]

IL-12, secreted by monocytes, and IFN-γ cause the immunity to deviate toward the T$_H$1 response. In this study, we found no disease-associated differences in IL-12 secretion of the PBMCs in the infants. IL-12 secretion was low: 63% of the samples were below the detection limit. Because we stimulated T cells with anti-CD3 treatment, which does not activate monocytes, our results of low IL-12 responses are reasonable. Stimulation of the PBMCs with specific allergens might have given more information about immunologic mechanisms in CMA than did nonspecific stimulation with OKT3 and anti-CD28 antibodies.

Methodologic problems related to measurement of intracellular cytokines such as high intra-assay and interassay variation may explain the fact that we found no differences between the groups. In addition, the detection of cytokine-expressing cells does not necessarily correlate with the amount of protein secreted, which may be high even when secreted by a limited number of cells.

In conclusion, intestinal microflora may modify the polarization of immune cells and antigen-processing mechanisms. Some probiotic strains such as LGG may act as proinflammatory mediators and are potent inducers of the T$_H$1 cytokine profile and cell-mediated inflammatory reaction. LGG bacteria induced IFN-γ secretion in infants with CMA and in infants with IgE-associated dermatitis, but interestingly, not in infants with no CMA. This supports the view that the pattern of intestinal microflora may be aberrant in infants with an atopic predisposition, and the beneficial effects of probiotics are evident only in this group. MIX had no effect on IFN-γ levels but led to an IL-4 response. *Lactobacillus* strains

may offer clinical benefit mediated by immunologic mechanisms in treatment of allergic diseases.

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