Lactic acid bacteria inhibit T_H2 cytokine production by mononuclear cells from allergic patients

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Background: Among factors potentially involved in the increased prevalence of allergic diseases, modification of the intestinal bacteria flora or lack of bacterial stimulation during childhood has been proposed. Lactic acid bacteria (LAB) present in fermented foods or belonging to the natural intestinal microflora were shown to exert beneficial effects on human health. Recent reports have indicated their capacity to reduce allergic symptoms.

Objective: The purpose of this investigation was to determine the effect of LAB on the production of type 2 cytokines, which characterize allergic diseases.

Methods: PBMCs from patients allergic to house dust mite versus those from healthy donors were stimulated for 48 hours with the related *Dermatophagoides pteronyssinus* allergen or with a staphylococcal superantigen. The effect of LAB preincubation was assessed by measuring the type 2 cytokine production by means of specific ELISA.

Results: The tested gram-positive LAB were shown to inhibit the secretion of T_H2 cytokines (IL-4 and IL-5). This effect was dose dependent and was observed irrespective of the LAB strain used. No significant inhibition was induced by the control, gram-negative *Escherichia coli* TG1. Interestingly, LAB reduced the T_H2 cytokine production from allergic PBMCs specifically restimulated with the related allergen. The inhibition mechanism was shown to be dependent on antigen-presenting cells (ie, monocytes) and on the involvement of IL-12 and IFN-γ.

Conclusion: The tested LAB strains were demonstrated to exhibit an anti-T_H2 activity, and thus different strains of this family might be useful in the prevention of allergic diseases. (J Allergy Clin Immunol 2002;110:617-23.)

Key words: *Allergy, type 2 cytokine, lactic acid bacteria, immunomodulation, superantigen, dust mite*

Recent studies demonstrate a significant increase in the prevalence of allergic diseases in industrialized countries and underline the multifactorial cause of allergy.[1-3] Genetic inheritance was reported as one of the potential causes of allergy. The allergic reaction is characterized by a disruption of the T_H1/T_H2 balance toward a pronounced T_H2 profile. Indeed, T_H2 lymphocytes, by producing IL-4, IL-5, IL-9, or IL-13, might play a pivotal role in the development and maintenance of the allergic response.[4] Moreover, newborn babies express a constitutive predominant T_H2 cytokine profile because of the in utero environment, in which high IL-4 and prostaglandin concentrations have been detected.[5] The abnormal maintenance of the T_H2 profile, which usually decreases with age, was considered as a lack of a sufficient T_H1 response development.[5] Modern hygiene rules, intensive food sterilization,[1] and modification of the gut flora of newborn babies[6] caused by feeding with artificial formula were reported as potential factors involved in the altered establishment in T_H1 regulatory response.[7,8]

Dietary lactic acid bacteria (LAB) are well known for their use in the preparation of fermented food products. Moreover, some species or strains have been attributed to potential health benefits or probiotic effects.[9-14] These bacteria have been generally recognized as safe and have been consumed by persons or animals from time immemorial. Experiments carried out in mice sensitized to ovalbumin have clearly indicated that after gastric administration of LAB, the specific IgE and T_H2 profile–dependent inflammatory responses were inhibited.[15,16] Moreover, recent reports suggest that *Lactobacillus rhamnosus* GG can reduce allergic disease symptoms in human subjects. The administration of this strain to breast-feeding mothers and to newborn babies led to a high inhibition (50%) of the risk of atopic eczema in babies.[17,18] Recent in vitro experiments[19,20] demonstrated that the stimulation of PBMCs or monocytes from healthy donors by a variety of LAB species enhanced the secretion of IL-12, which is a pivotal pro-T_H1 cytokine involved in the control of allergic disease development. However, the mechanism by which LAB might affect the production of T_H2 cytokines responsible for the initiation of the development of allergic disease remains to be

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Abbreviations used
LAB: Lactic acid bacteria
SEA: Staphylococcal enterotoxin A
STAT: Signal transducer and activator of transcription

specified. In this report we have analyzed the effect of 4 LAB strains on the T_H2 cytokine production by PBMCs from allergic patients sensitized to *Dermatophagoides pteronyssinus* or from healthy donors after stimulation with staphylococcal enterotoxin A (SEA) or the relevant aeroallergen *D pteronyssinus*. The studied LAB strains were shown to exhibit anti-T_H2 activities involving the participation of antigen-presenting cells (APCs) as monocytes and the pro-T_H1 cytokines IL-12 and IFN-γ.

METHODS

Reagents

SEA was obtained from Sigma. Total extract of house dust mite (*D pteronyssinus*) was provided by Stallergenes SA.

Patients and healthy donors

Blood was collected from allergic patients sensitive to house dust mite. The allergic patients presented with a history of asthma and had specific IgE antibodies and positive skin prick test responses toward *D pteronyssinus* (RAST class >2). Their total IgE levels were greater than 150 kU/L. Healthy donors displayed total IgE levels of less than 50 kU/L and no *D pteronyssinus*–specific IgE in their sera (<0.35 kU/L).

Cell preparation

Blood was depleted from platelet-rich plasma and diluted in RPMI 1640 medium (Life Invitrogen). Human PBMCs were isolated by means of centrifugation (400*g* for 30 minutes) over a Ficoll density gradient (Pharmacia). After washing, cells were resuspended at a concentration of 2×10^6 cells/mL in RPMI 1640 medium containing 10% heat-inactivated FCS (Bio Media), 2 mmol/L L-glutamine, and 0.2 μg/mL Ticarpen (ticarcilline; SmithKline Beecham) to prevent bacterial overgrowth.

For T-cell, B-cell, and monocyte preparations, PBMCs were incubated on ice for 30 minutes with CD14 or CD19 antibodies coupled to magnetic microbeads (Miltenyi Biotec), washed, and applied onto a column placed in the magnetic field of a Macs separator (Miltenyi Biotec), according to the manufacturer's recommendations. $CD14^+$ monocytes or $CD19^+$ B cells were collected, washed, and conserved at 4°C before use. $CD4^+$ T cells were purified by using the $CD4^+$ T-cell isolation kit (containing CD8, CD11b, CD16, CD19, CD36, and CD56 antibodies), according to the manufacturer's recommendations.

Preparation of bacteria

Four types of LAB were used: *Lactobacillus plantarum* NCIMB8826 (LAB1), *Lactococcus lactis* MG1363 (LAB2), *Lactobacillus casei* ATCC393 (LAB3), and *Lactobacillus rhamnosus* GG (LAB4). Lactobacilli strains were grown at 37°C in MRS broth (Difco), and the lactococcal strain was cultured at 30°C in M17 supplemented with 0.5% glucose. *Escherichia coli* TG1 (TG1) was grown at 37°C in Luria-Bertani broth medium and used as the gram-negative bacteria strain control, as already reported.[21]

LAB strains and TG1 were pregrown overnight in appropriate medium. Exponential phase cultures were obtained by means of dilution of the preculture at 1:20 for LAB and 1:50 for TG1 in fresh medium and further incubation under appropriate conditions until the logarithmic phase (as measured by OD at 600 nm). Bacteria were then washed 3 times with PBS and adjusted at a final concentration of 10^{10} colony-forming units per milliliter. The bacterial suspensions were stored at –80°C in PBS solution containing 20% glycerol. The concentration of bacteria was determined by plating serial dilutions on the appropriate medium.

Heat-killed bacteria were prepared by means of a 1-hour incubation at 70°C. Alternatively, LAB were inactivated by a 15-minute incubation in paraformaldehyde (4%), followed by 3 washes in PBS. The absence of viable bacteria was checked by plating on the appropriate growth medium.

Activation of mononuclear cells

PBMCs (2×10^6/mL) were stimulated with SEA (2 μg/mL) or *D pteronyssinus* allergen (1 IR/mL [index of reactivity—equivalent to approximately 2 μg of protein]). Three protocols were used to evaluate the effects of LAB on PBMCs: live LAB strains were added either (1) 3 hours before, (2) at the same time, or (3) 3 hours after the stimulation. In each case centrifuged supernatants were collected after 48 hours' incubation and stored at –20°C until use. It was verified that the low concentrations of the used antibiotic did not kill the bacteria but permitted maintenance of a constant number of viable bacterial cells throughout the incubation period with PBMCs. Moreover, some experiments were performed with killed LAB by using the first protocol. Purified monocytes or B lymphocytes were preincubated with LAB strains for 3 hours at a ratio of 10 bacteria per cell (10:1) in the presence of purified $CD4^+$ T cells (2×10^6/mL corresponding to a ratio of 10 T cells/monocyte), and then SEA or *D pteronyssinus* were added to the coculture to evaluate the involvement of APCs in the effect of LAB on lymphokine production. Supernatants were treated as described above. Control experiments were performed with anti-IL-12 and anti-IFN-γ antibodies (obtained from R&D Systems) to evaluate the involvement of IL-12, IFN-γ, or both on the effect induced by LAB.

Cytokine assays

IL-4 and IFN-γ were quantified in the supernatants by means of specific ELISA (Diaclone), whereas IL-5 ELISA was performed by using Pharmingen products, according to the manufacturer's recommendations. The sensitivity of the IL-4, IL-5, or IFN-γ assay was 0.5, 5, or 5 pg/mL, respectively.

Statistical analysis

Nonparametric statistical analyses were performed with the Mann-Whitney test. *P* values of less than .05 were considered statistically significant.

RESULTS

LAB specifically inhibit the IL-4 production from SEA-stimulated PBMCs of healthy donors and amplify IFN-γ secretion

Preliminary experiments have shown that PBMCs from healthy donors stimulated with a streptococcal superantigen (2 μg/mL) produced high levels of IL-4 and IL-5, 2 T_H2 cytokines usually associated with allergic disorders. Heat-killed LAB were able to downregulate this type 2 cytokine profile secretion.[22] In the present work we have confirmed these observations with a different superantigen: staphylococcal SEA.

When live strains of LAB were preincubated with PBMCs, the subsequent IL-4 production induced by SEA

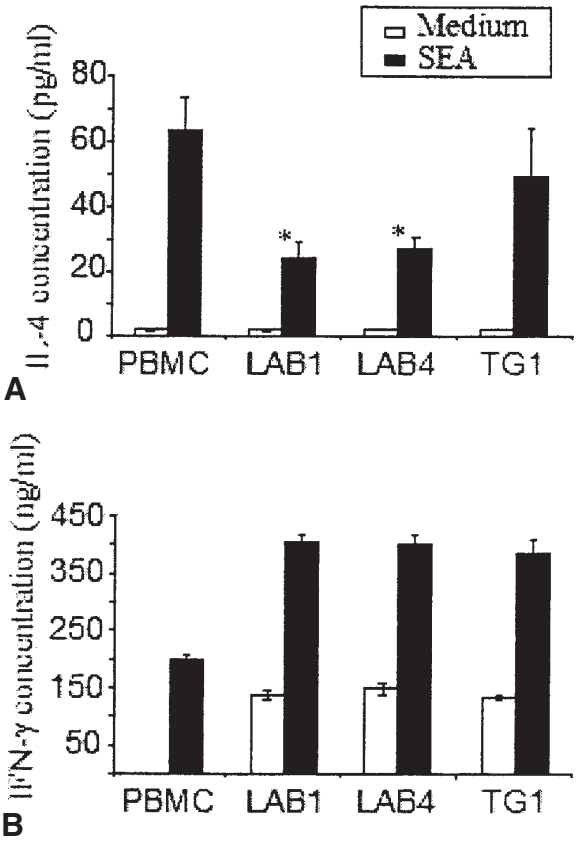

FIG 1. LAB reduce IL-4 production and increase IFN-γ production. PBMCs from healthy donors (n = 11) were preincubated for 3 hours (at 2×10^6/mL) with 10^7 live colony-forming units of 2 gram-positive LAB strains (LAB1 and LAB2) and one gram-negative control bacterium (TG1) and further stimulated with SEA (2 μg/mL). IL-4 **(A)** and IFN-γ **(B)** were quantified after 48 hours' incubation by means of specific ELISA. The *asterisk* indicates significant inhibition ($P \leq .05$) of T_H2 cytokine production in response to SEA stimulation.

was highly reduced when compared with that induced by positive controls (no preincubation with LAB). Interestingly, no significant inhibition was observed when PBMCs were preincubated with the gram-negative control strain TG1 (Fig 1, *A*). Although neither the 2 LAB strains nor TG1 induced basic IL-4 production, all 3 strains triggered the secretion of IFN-γ. Moreover, when PBMCs were stimulated with SEA, the level of IFN-γ release was greatly increased, and a synergistic effect was observed not only with the LAB but also with TG1 (Fig 1, *B*). Thus LAB appeared to be able to alter both T_H1 and T_H2 cytokine secretion in an opposite manner.

Parameters influencing cytokine production

Further experiments carried out with 4 LAB strains demonstrated that T_H2 cytokine inhibition was dependent on several parameters. When PBMCs were stimulated with LAB before adding SEA, the inhibitory effect on T_H2 cytokine production increased with the LAB/PBMC ratio (Fig 2, *A* and *B*). This effect was similarly observed for IL-4 and IL-5 production. For example, for the strain LAB1 *(L plantarum)*, the percentage of

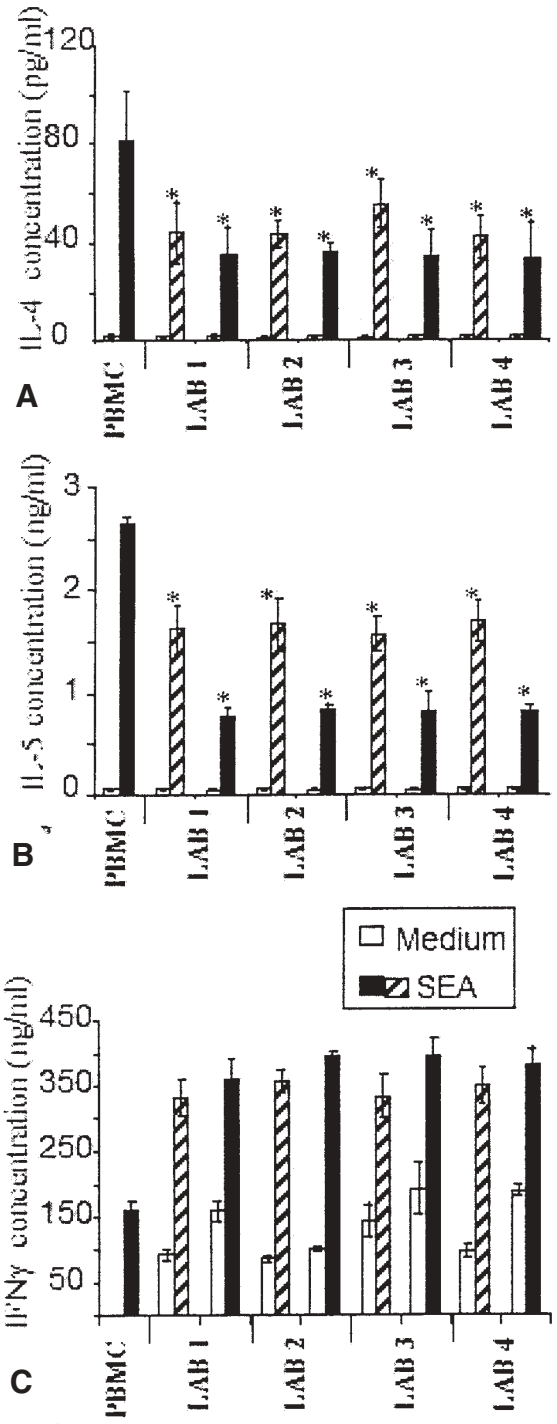

FIG 2. Influence of the concentration of LAB on their immunomodulatory properties. PBMCs from healthy donors (n = 4) were preincubated for 3 hours (at 2×10^6/mL) with 4 live strains of LAB (LAB1, LAB2, LAB3, and LAB4) at 2 bacteria/cell ratios (1:1 *[striped bars]* or 10:1 *[solid bars]*) before either being stimulated with the SEA superantigen (2 μg/mL) or not. IL-4 **(A)**, IL-5 **(B)**, and IFN-γ **(C)** were quantified in 48-hour supernatants by means of specific ELISA. The *asterisk* indicates a significant inhibition ($P \leq .05$) of T_H2 cytokine production in comparison with the SEA-induced response.

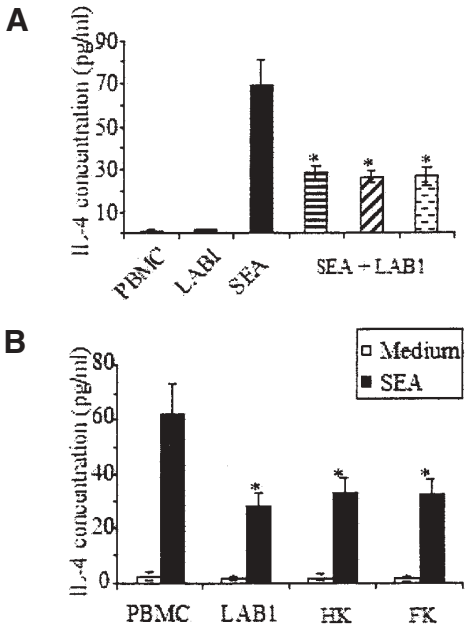

FIG 3. Parameters influencing the production of cytokines. PBMCs from healthy donors (n = 4, at 2×10^6/mL) were stimulated with SEA (2 μg/mL). **A,** Mononuclear cells were either preincubated for 3 hours with LAB1 (10:1 ratio) before SEA stimulus (▭) or stimulated simultaneously with the LAB strain and the superantigen (▨), or LAB strains were added 3 hours after SEA stimulus (▭). **B,** Mononuclear cells were preincubated for 3 hours with live LAB1 (10:1), heat-killed LAB1 *(HK)*, or paraformaldehyde-fixed LAB1 *(FK)* before stimulation with SEA. IL-4 concentration was measured at 48 hours by means of ELISA. The *asterisk* indicates a significant ($P \leq .05$) inhibition of T_H2 cytokine production in comparison with the SEA-induced response.

inhibition of IL-4 production in response to SEA increased from 46% ± 3% (1:1 ratio) to 57% ± 5% (10:1 ratio), and the percentage of inhibition of IL-5 production increased from 42% ± 9% (1:1 ratio) to 71% ± 1% (10:1 ratio). However, no significant difference was noticed among the 4 LAB strains used. In contrast to T_H2 cytokines, these LAB strains induced a basic production of IFN-γ, which seems to depend on the LAB/PBMC ratio. It is noteworthy that LAB were shown to strongly potentiate the IFN-γ production in response to SEA stimulation (Fig 2, *C*).

The reduction of IL-4 release was equivalent when the LAB were added simultaneously or subsequently to the SEA stimulus (Fig 3, *A*). Moreover, the level of inhibition was not affected by the physiologic state of the bacteria because live bacteria induced the same effect as heat-killed or paraformaldehyde-treated LAB (Fig 3, *B*).

LAB inhibit the T_H2 cytokine production of allergen-stimulated PBMCs from allergic patients

The ability of LAB to correct the imbalance between T_H1 and T_H2 cytokines was next evaluated in a more relevant model of stimulation by allergen. When PBMCs from patients allergic to *D pteronyssinus* were preincubated with different live LAB strains, the IL-4 and IL-5 production induced after specific *D pteronyssinus* allergen stimulation was largely reduced in an LAB/PBMC ratio–dependent manner. This effect was independent of the species-strains of LAB studied. For example, IL-5 inhibition was equal to 60% ± 2% for LAB1 and 62% ± 3% for LAB4 (*L rhamnosus* GG, 10:1 ratio; Fig 4, *A* and *B*). Similar effects were observed when PBMCs from allergic patients were stimulated with SEA. In this case although IL-4 and IL-5 production was very high compared with the production obtained with *D pteronyssinus*, LAB markedly reduced the IL-5 secretion by 73% ± 3% and 75% ± 3% for LAB1 and LAB4, respectively (data not shown). The coincubation with LAB strongly increased the production of IFN-γ (Fig 4, *C*), which, in the presence of the related allergen, was less than 1 ng/mL.

Thus by reducing T_H2 cytokine production and by enhancing the IFN-γ production by PBMCs from allergic patients, LAB appeared to exhibit an anti-T_H2 activity.

Involvement of CD14+ monocytes in cytokine production

Experiments were carried out with different purified cell subpopulations to investigate the mechanism by which LAB are able to modify cytokine production. When purified human monocytes from healthy donors were preincubated with LAB and further cultivated in the presence of autologous T cells, followed by stimulation with SEA, IL-4 production was reduced to a level similar to that observed when total PBMCs were incubated with LAB in the presence of SEA superantigen (Fig 5, *A*). This inhibition effect was also observed with purified B cells, but the percentage of inhibition was slightly less pronounced than with monocytes (Fig 5, *B*). These APCs do appear to be directly or indirectly involved in the inhibition of T_H2 cytokine release in response to LAB.

IL-12 and IFN-γ produced by LAB inhibit T_H2 cytokine production

LAB stimulation leads to the production of IL-12 and IFN-γ. Experiments were performed in the presence of neutralizing anti-IL-12 and anti-IFN-γ antibodies to appreciate the role of these 2 cytokines in the T_H2 cytokine inhibition effect. As shown in Fig 6, neutralization of these pro-T_H1 cytokines restored the IL-4 production inhibited by the LAB1 strain, suggesting that these 2 cytokines could be involved in the inhibition mechanism.

DISCUSSION

Allergic diseases are characterized by a type 2 cytokine profile expression. Indeed, IL-4, IL-5, IL-13, and IL-9 are involved in the initiation and maintenance of the allergic reaction. In this report we showed that different LAB strains belonging to various species can modulate the T_H1/T_H2 balance by reducing T_H2 cytokine release and enhancing T_H1 cytokine production (IFN-γ). Thus such LAB could exert a beneficial effect in patients

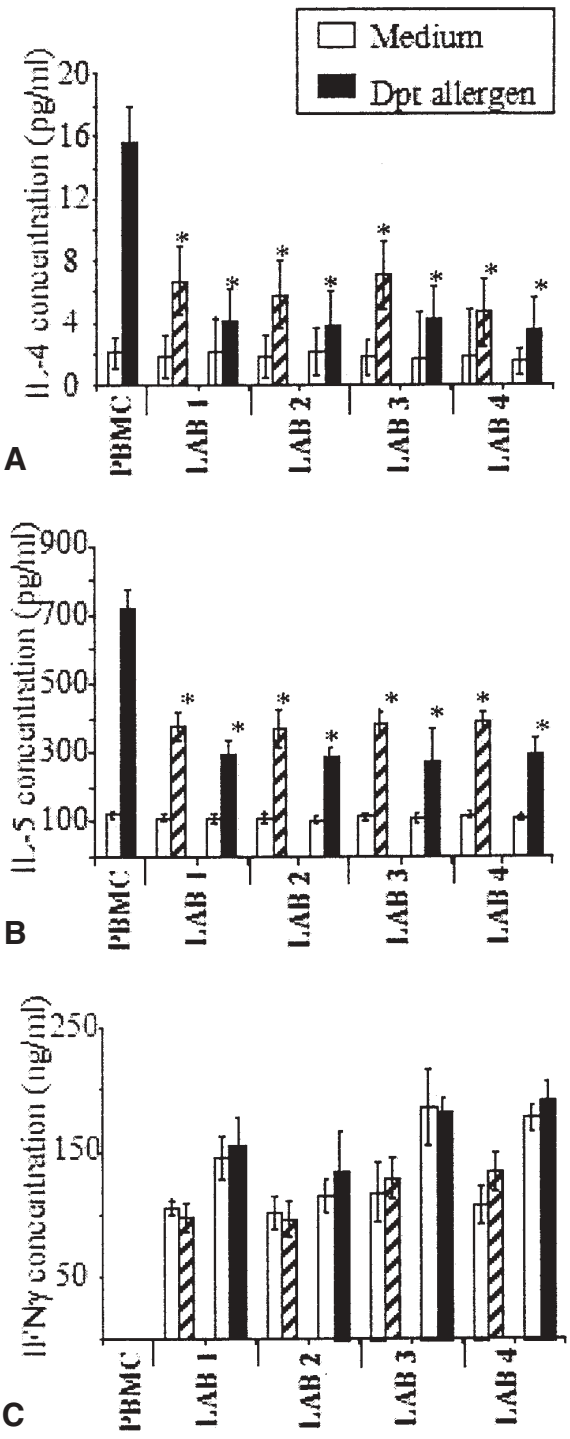

FIG 4. Inhibition of allergen-induced T_H2 cytokine profile. PBMCs from patients allergic to *D pteronyssinus* (*Dpt*; n = 5) were preincubated for 3 hours (at 2×10^6/mL) with 4 strains of LAB (LAB1, LAB2, LAB3, and LAB4) at 2 bacteria/cell ratios (1:1 *[striped bars]* or 10:1 *[solid bars]*) before related allergen-dependent stimulation (1 IR/mL) or not. IL-4 **(A)**, IL-5 **(B)**, and IFN-γ **(C)** production was determined by means of specific ELISA after 48 hours' stimulation. The *asterisk* indicates a significant ($P \leq .05$) inhibition of T_H2 cytokine production in comparison with the SEA-induced response.

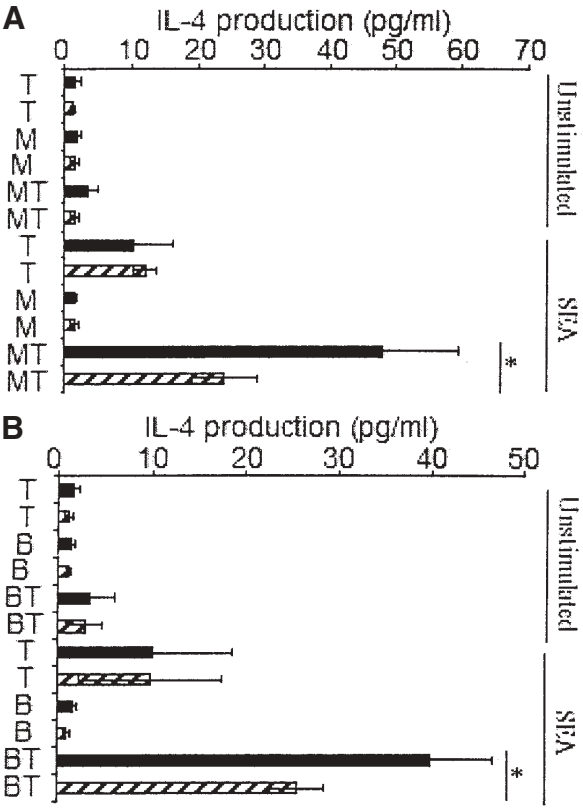

FIG 5. Involvement of monocytes in the inhibition of T_H2 cytokine production. **A,** Monocytes (*M*; 2×10^5/mL) of healthy donors were preincubated or not in the presence of autologous $CD4^+$ T cells (*T*; 2×10^6/mL) for 3 hours with LAB1 (10:1 ratio for LAB/monocytes) before stimulation with SEA (2 μg/mL). **B,** $CD19^+$ B cells *(B)* from healthy donors (2×10^5/mL) were preincubated or not in the presence of autologous $CD4^+$ T cells (*T*; 2×10^6/mL) for 3 hours with LAB1 (10:1 ratio) before stimulation with SEA (2 μg/mL). IL-4 concentration was determined by means of ELISA after 48 hours' culture. The *asterisk* indicates a significant ($P \leq .05$) inhibition of T_H2 cytokine production in comparison with SEA-induced production.

with allergic asthma by means of their inhibitory effect on T_H2 cytokine production.

To demonstrate this effect, we first used a T_H2 cytokine–producing cellular model (ie, PBMCs stimulated with the superantigen SEA). Several reports indicate that PBMCs secrete T_H2 cytokines after stimulation with superantigens by using similar signaling pathways as those involved with usual antigens.[23] In our study, when PBMCs were preincubated with LAB, T_H2 cytokine production was reduced. As reported previously,[22] this inhibitory effect was obtained with all the tested gram-positive LAB but not in the presence of the gram-negative bacterium *E coli* TG1. The inhibition was demonstrated to be dependent on the dose of LAB used, with higher bacterial doses leading to more pronounced reduction of IL-4 and IL-5 production. Such a dose-response effect was reported in vitro for IL-10 and IL-12 release by monocytes from healthy donors on incubation with different strains of gram-positive bacteria.[20] We also demonstrated that the viability of bacteria was not

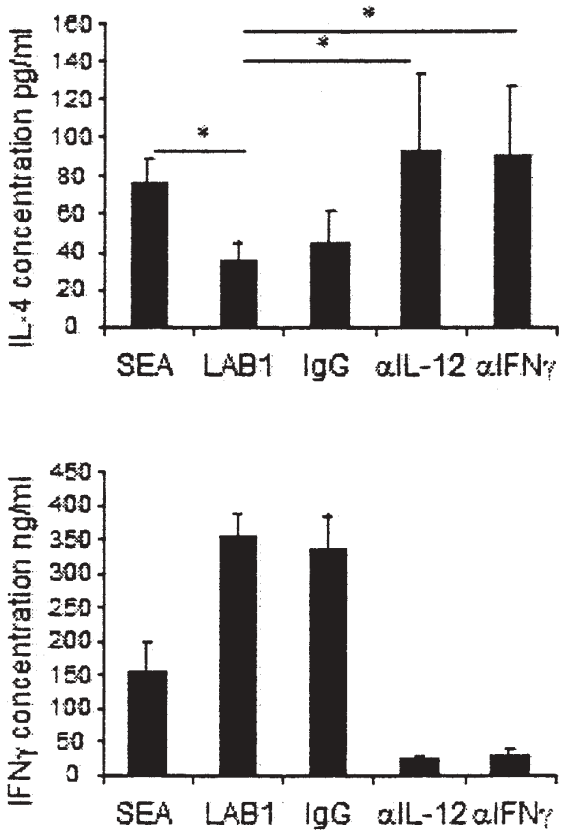

FIG 6. Involvement of IL-12 and IFN-γ in the LAB anti-T_H2 effect. PBMCs from healthy donors (n = 5) were preincubated for 3 hours (at 2×10^6/mL) with LAB1 strain at a bacteria/cell ratio of 10:1 in the presence of neutralizing anti-IL-12 *(αIL-12)* or anti-IFN-γ *(αIFN-γ)* antibodies (10 μg/mL) before SEA stimulation (2 μg/mL). In the negative control, IgG2a antibodies were used at the same concentration. IL-4 and IFN-γ concentrations were determined by means of specific ELISA after 48 hours' stimulation. The *asterisk* indicates a significant ($P \leq .05$) difference between cytokine concentrations.

absolutely required for the inhibitory effect because heat-killed or paraformaldehyde-treated bacteria performed equally well as exponentially growing strains. Interestingly, the production of the T_H2 cytokines IL-4, IL-5, and IL-13 by PBMCs from allergic patients stimulated with the related allergen was found to be highly reduced when PBMCs were incubated in vitro with LAB. To the best of our knowledge, this is the first report of a beneficial effect of LAB on cells from patients sensitized to an aeroallergen. Indeed, previous reports are all related to allergic symptoms caused by food allergens. For example, Kalliomaki et al[18] described that after *L rhamnosus* GG oral supplementation for 8 weeks, children with atopic dermatitis and cow's milk allergy exhibit a transient early upregulation in IFN-γ production and a later decrease in IL-4 production. These observations correlate well with studies performed in mice in which specific LAB strains were shown to modify the T_H2 profile induced in response to ovalbumin or casein. In these experiments LAB reduced the IgE response, as well as the allergen-dependent inflammatory response.[15,16,24-26]

Because the inhibitory effect was observed even when the LAB were added after SEA or *D pteronyssinus* stimulus (data not shown), our observations suggest that LAB might act by regulating directly or indirectly the signaling pathway required for T_H2 cytokine production. Several factors can modulate IL-4 and IL-5 release. Indeed, IFN-γ was described as a potential regulator of IL-4 synthesis. In addition, IL-12 was reported as a key cytokine in the initiation of IFN-γ production by human PBMCs. LAB were capable of inducing IL-12 secretion by PBMCs, as well as by monocytes (data not shown). In our experimental setting, APCs (ie, monocytes) were absolutely required to generate the inhibitory effect on IL-4 production. This effect mediated by APCs could either be due to bacteria phagocytosis, which is mainly associated with a type 1 response, or to the involvement of toll-like receptors, the stimulation of which can also lead to a T_H1 profile. Indeed, the large amounts of IL-12 produced by LAB-stimulated APCs contribute to this mechanism by activating signal transducer and activator of transcription (STAT) 4, which is known to transactivate IFN-γ directly. Moreover, Miettinen et al[27] demonstrated that gram-positive bacteria, such as GG, were able to indirectly induce STAT (mainly STAT1 and STAT3) DNA-binding activity in primary macrophages through cytokines, and following binding to the IFN-γ activation site element leads to IFN-γ production. In this way, in our experimental model, the production of IL-4 and IL-5 could be modulated.

Allergy was recently described as being the result of a deficit of bacterial stimulation during childhood. Our results confirm and extend previous observations that LAB might have a beneficial effect against allergic diseases by reducing T_H2 cytokine production. Consequently, LAB could restore the host capacity to limit the response against aeroallergens and the development of allergy.

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