

The Effect of *Lactobacillus casei* on an Experimental Model of Atopy

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The immunomodulation of IgE synthesis in the allergic process mediated by the Th1/Th2 cytokine balance is now better understood. Lactic acid bacteria (LAB) are used as microorganisms to improve health. Also well demonstrated is the effect of these bacteria as an adjuvant on the mucosal and systemic immune response. These previous reports lead us to study if some LAB, such as Lactobacillus casei strain CRL 431 which has been determined its immunopotentiator capacity, is able to modulate IgE synthesis in a mouse experimental model of allergy induced with ovalbumin. Animals were sensitized with ovalbumin by injection and aerolization. A challenge with the antigen after four weeks of IgE induction was done. L.casei was administered for 2 days at a concentration of 10⁹ cells day⁻¹ before sensitization, before challenge, before sensitization, and before and after challenge. In the control animal, an increase in the IgE levels, with diminution in the IFN- γ production and high level of IL-4 release was observed. In the animal treated with L.casei a down regulation of IgE synthesis by increasing of IFN- γ and diminution of IL-4 cytokines was determined. This effect was only observed when L. casei was administered before sensitization, but not before challenge. These results would indicate that the L. casei would induce Th1 activation able to increase IFN- γ cytokine with diminution of IL-4 released by Th2 lymphocytes avoiding the switch toward IgE synthesis. However, L. casei does not have an effect when Th2 are triggered and IL-4 levels are increased. The strain L. casei CRL 431 would be able to modulate Th1/Th2 cytokine balance.

Keywords: *L. casei*, allergy, IgE, immunomodulation, IFN- γ , IL-4

INTRODUCTION

Over the past several years progress has been made in understanding the regulation of IgE production in different species (Benner *et al.*, 1991). Induction of IgE synthesis is under control of Th₂ cytokines. These T cells are characterized by the production among them of IL-3, IL-4, IL-5, IL-6 and IL-13. However, the profile of cytokines from T cells differs in children and adults (Lilic *et al.*, 1997). Some of these cytokines, such as IFN- γ , control the production of IgE by B cells and play an important role in the activation and differentiation of effector cells, eosinophils and mast cells in the allergic response (Maggi *et al.*, 1988). The critical role of IL-4 in the stimulation of IgE production has been demonstrated in different experimental models (Sadick *et al.*, 1990; Urban *et al.*, 1991). In contrast IFN- γ has been shown to have an inhibitory effect on 'in vitro' IgE production by murine and human lymphocytes (Chrétien *et al.*, 1990).

Although the mechanism of action is not entirely defined, IFN- γ is known to antagonize the effect of IL-4 on IgE synthesis in the presence of CD4 T cells (Shirakawa *et al.*, 2000).

IgE is important in the early and late phase of allergic responses (Ishizaka, 1981), thus the regulation of this immunoglobulin is important in the prevention of allergic diseases.

Much attention has been focused on the importance of the intestinal microflora as a mucosal barrier (Wells *et al.*, 1988; Gaboriau-Routhiau *et al.*, 1997) and on the probiotic immunomodulating capacity over the host immune response in experimental models. Probiotics such as lactic acid bacteria (LAB) have been reported to exhibit beneficial effects (Perdigon *et al.*, 1993; Matsuzaki *et al.*, 1997; Pelto *et al.*, 1998) and to induce several cytokines (Matsuzaki *et al.*, 1996).

In previous work we demonstrated that a strain of *Lactobacillus casei* could be used as an oral immunomodulator (Perdigón *et al.*, 1993; Petrino *et al.*, 1995; Petrino *et al.*, 1996). These reports suggest that *L. casei* has the potential to regulate the immune response of the host, without undesirable side effects.

This research was performed to examine the influence of oral administration of *L. casei* on the diminution of IgE levels in an experimental model of atopy, induced by ovalbumin, in mice.

MATERIAL AND METHODS

Animals

BALB/c mice, each weighing 25–30 g were obtained from a randomly bred closed colony kept in our Microbiology Department. The animals were housed in plastic cages at room temperature, each experimental group consisting of 40–50 mice and for each assay 5–6 mice were used.

Sensitization and Challenge Protocol

Animals were given two intradermic injections (0.05 ml) of 1% ovalbumin (OVA) (chicken OVA, grade V, Sigma, St Louis, MO, USA) 15 days apart. A week after the last inoculation, the immunization was continued using a daily exposure to OVA 1% for 20 min each day for a period of 10 days. The aerosol was generated with an ultrasonic nebulizer (RESPIREX, particle size 1–6 μ m) connected to a plastic exposure chamber (51). Aerosol was given in groups of maximal five animals.

Four weeks after the last nebulization, the mice were again challenged with OVA 1% for 20 min by aerosolization, for 3 consecutive days.

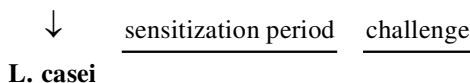
Administration of Lactic Acid Bacteria

The microorganism used was *Lactobacillus casei* CRL 431, from the CERELA culture collection. The *Lactobacillus* was cultured in Deman, Rogosa and Sharpe (MRS) broth

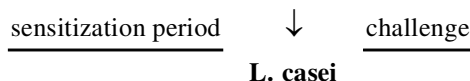
(Oxoid) for 8 h at 37°C. The viable culture was suspended in 5 ml of sterile non-fat milk (NFM, 10%) at a concentration of 10^9 cells/day/mouse. It was administered at 20% (v/v) in the drinking water for 2 consecutive days.

The experimental groups were as follows:

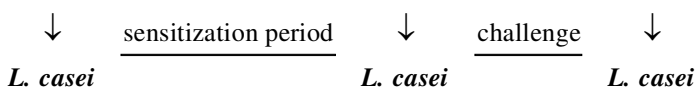
- (1) Groups of mice were given *L. casei* for two days before the sensitization period.



- (2) Animals where *L. casei* was administered for two days prior to the challenge, second series of exposure with OVA 1%.



- (3) Animals given *L. casei* several times (two days previous to the sensitization period, before and after challenge).



- (4) Control group without treatment with *L. casei*.

Determination of Specific Anti-OVA IgE Levels

Levels of OVA-specific mouse IgE were measured by ELISA test, using horseradish peroxidase (HRP)-conjugated rat anti-mouse IgE monoclonal antibody (MAb) (Pharmingen, San Diego, CA, USA). The samples were collected on days 2, 9, 15 and 23 (samples S1, S2, S3 and S4, respectively), after the sensitization period. After challenge, samples were collected on days 2 and 5 (S5 and S6)

Immunoplates (Maxisorb F96, Nunc) were incubated with 200 μ l per well of chicken OVA at a concentration of 10 μ g ml⁻¹ in 0.06 M-bicarbonate-carbonate buffer, pH 9.6, for 18 h at 4°C. The plates were washed three times with phosphate-buffered saline (PBS) and 0.1% Tween 20, and then blocked with 2% non-fat milk in PBS (200 μ l per well) for 1 h at room temperature. Then, 100 μ l aliquots of serum samples (diluted by one-half) were added to each well. The plates were incubated for 1 h at 37°C, washed three times with PBS and 200 μ l of HRP anti-mouse IgE MAb was added. They were incubated for 1 h at 37°C. 200 μ l of *o*-phenildiamine (8 mg) – 30% hydrogen peroxide (50 μ l) – citrate buffer (20 ml) pH 4.5 was added. The reaction was developed at room temperature for 20–30 min in darkness. It was stopped by addition of 20 μ l of 2 N-sulphuric acid and read with an ELISA reader at 493 nm. The results were expressed as nanograms of specific OVA-IgE per ml of serum.

Cytokine Levels

The cytokines examined were IL-4 and IFN- γ in serum samples from animals of group 1 and 4. These were measured at the same periods of time as the specific IgE anti-OVA.

For cytokine detection, mouse IL-4 and IFN- γ , the Immunoassay kits from R&D System were used. Both assays employed the quantitative sandwich enzyme immunoassay technique (ELISA test). The results were expressed as picograms per ml of serum.

Histological Preparation From Lung

Mice from group 3 and 4 were sacrificed and lungs were removed from test and control groups in order to quantify mast cells. Tissues were collected on the last day of sensitization

(sample 1), on day 23 post sensitization (sample 2), the day before the challenge (sample 3), and five days post challenge (sample 4).

These samples were washed with saline solution and fixed in ice cold alcohol 96° at 2–4 °C for 24h. Then they were dehydrated and embedded in paraffin using routine methods.

Serial paraffin sections (4 µm) of the lung of each mouse were obtained. In order to study mast cells associated with bronchial tissue, the histological samples were stained with Alcian Blue Safranin O at pH 1 (Tas, 1977).

The cells associated with the peribronchial area were observed with a video microscope (Olympus). This allowed us to count mast cells in the peribronchial area (three slices per mice). The results were expressed as number of cells mm⁻².

Statistical Analyses

Data were expressed as the mean of *n* independent experiments ± standard error of the mean (SEM). The Student's *t*-test was used to calculate the statistical significance of the results.

RESULTS

Effect of *L. casei* on Specific IgE Levels

The OVA specific IgE values in serum each group determined by the ELISA test, are shown in Figure 1 (a) and (b).

L. casei, administrated previous to the sensitization (group 1) decreased OVA specific IgE with significant differences ($P < 0.01$) in relation to control sensitized mice (Figure 1 (a)). Ig E was also inhibited after challenge. Levels of IgE in mice fed with *L. casei* for 2 days before challenge (group 2) were significantly decreased with regard to the control. These results are expressed in Figure 1(b). In group 3, the effect of the repetitive doses of *L. casei*, before

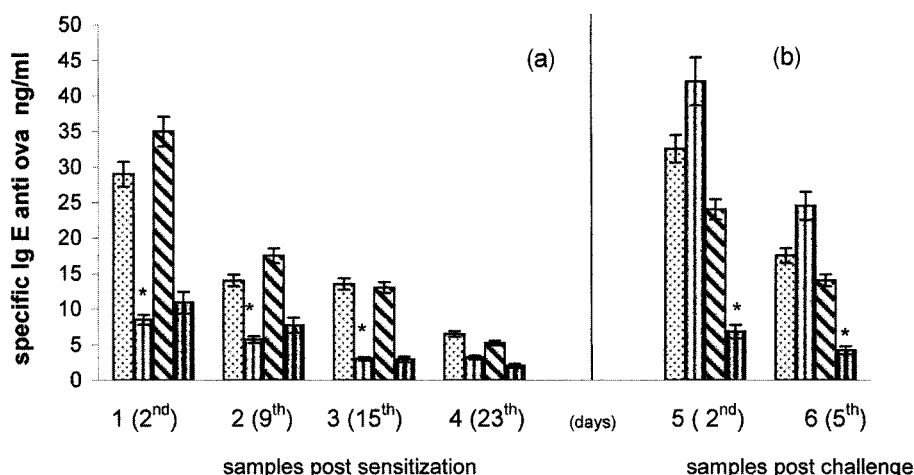


FIG. 1. Effect of *L. casei* on the levels of specific IgE anti-OVA in an allergic model. Groups of animals were given *L. casei* before sensitization, one group before challenge and another with a repeated dose: before sensitization and before and post challenge. Serum samples were taken on days 2, 9, 15 and 23 post sensitization (a) and on days 2 and 5 post-challenge with OVA (b). Control values IgE anti-OVA: 0–4 ng ml⁻¹. Bars represent mean values of $n = 5 \pm \text{SD}$. * $P < 0.01$.

■ Group (1) of mice treated with *L. casei* for two days before sensitization (a) and (b); ▨ Group (2) of animals sensitized with ova and feeding with *L. casei* during two days before challenge (a) and (b); ▩ Group (3) of mice were given *L. casei* prior to the sensitization period and before and after challenge, * $P < 0.01$; ▤ Group (4) of sensitized and challenged control animals, untreated with *L. casei* (a) and (b).

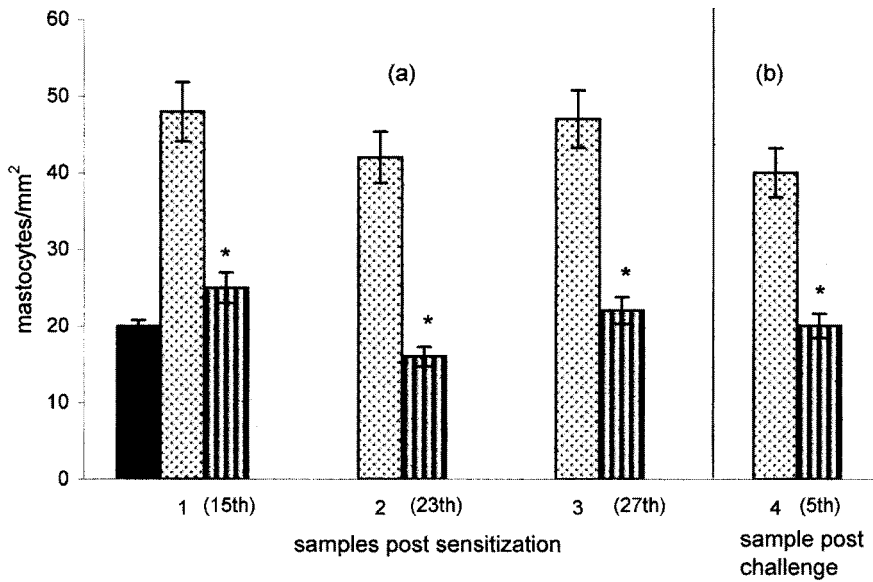


FIG. 2. Effect of *L. casei* on the mastocytes associated to bronchial tissue. ■ Group (3) of mice were given *L. casei* prior to the sensitization period and before and after challenge, * $P < 0.01$; ▨ Group (4) of sensitized and challenged control animals, untreated with *L. casei* (a) and (b); ■ Values from control animals untreated with OVA: 19 ± 4 mastocytes mm^{-2}

sensitization and previous- and post-challenge, showed a significative diminution of Ig E values in all the periods assayed in relation to the control group, reaching control values ($2\text{--}4 \text{ ng ml}^{-1}$).

Effect of *L. casei* on the Mast Cells Associated With the Peribronchial Area

Based on the results obtained in group 3, related to the levels of IgE, we determined the number of mast cells associated with the peribronchial area in this group. The counting of these cells showed the positive effect of *L. casei*. We obtained values similar to the control (Figure 2) from mice untreated with OVA.

Effect of *L. casei* on Cytokine Production

We analysed the cytokines IFN- γ and IL-4, measured only in group 4, the sensitized controls, and group 1, treated with *L. casei* prior to sensitization.

The samples were tested at the same time as specific IgE anti-OVA determination. Previously we determined that *L. casei* administered for two days to non-sensitized mice stimulated IFN- γ production (18 pg ml^{-1}), as compared to the non-exposed control (up to 2 pg ml^{-1}).

Our results show that *L. casei* stimulated IFN- γ production until day 9 post-sensitization (Figure 3(a)), but after OVA challenge the values were similar to the controls (Figure 3(b)).

As regards to IL-4, we observed that *L. casei* administered prior to sensitization diminished significantly ($P < 0.01$) IL-4 levels (Figure 4 (a)) in relation to the untreated animal. However, after challenge *L. casei* was not effective and the IL-4 values were similar to the sensitized controls (Figure 4(b)).

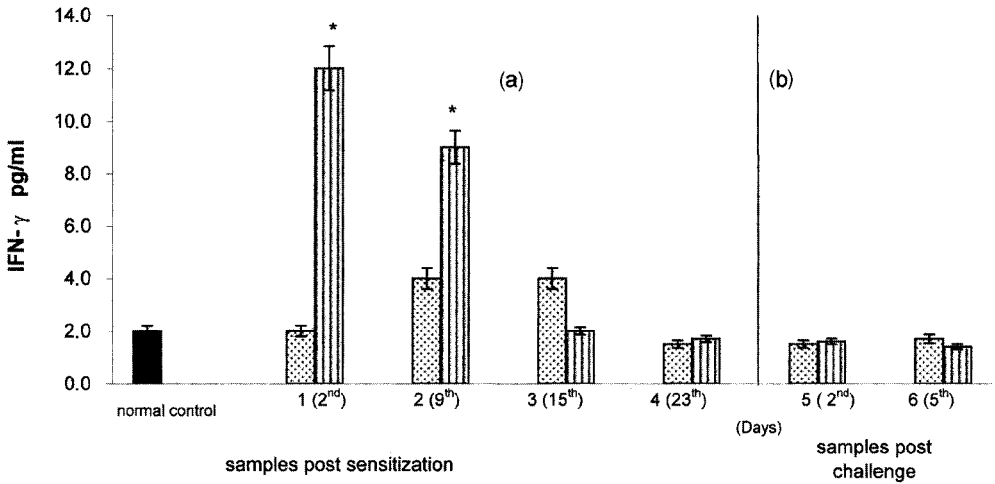


FIG. 3. Effect of *L. casei* on the levels of IFN- γ in host sensitized to OVA. Group of animals were given *L. casei* before sensitization. Serum samples were taken on the same days than those for specific IgE determination. (a) Levels of IFN- γ post sensitization; (b) IFN- γ levels after challenge. Bars represent mean values of $n = 5 \pm$ SD, $*P < 0.01$. ■ Group (1) of animals with previous feeding with *L. casei* for two days and then sensitized (a) and (b); □ Group (4) of sensitized and challenged control animals, untreated with *L. casei* (a) and (b); ▨ Values from control animals untreated with ovalbumin = up to 2 pg mL^{-1} .

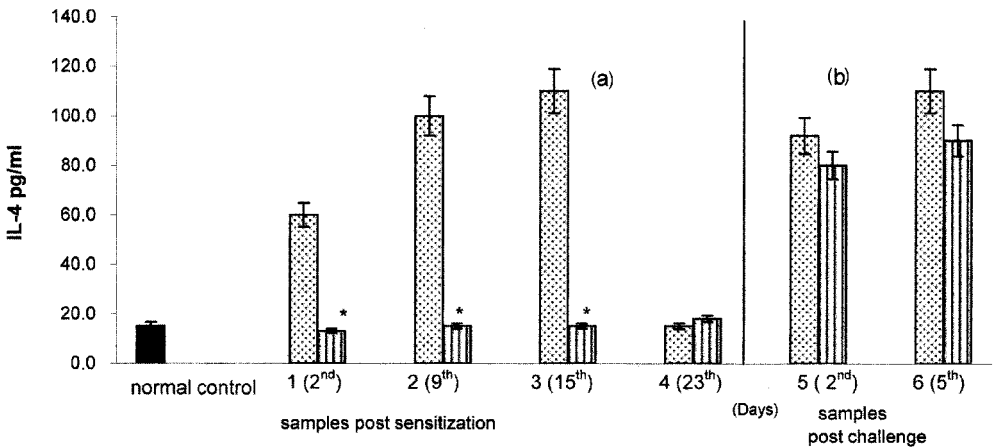


FIG. 4. Effect of *L. casei* on the IL-4 production in an allergic process. Groups of animals were given *L. casei* before sensitization. Serum samples were taken on the same days as those for specific IgE determination. (a) Levels of IL-4 post sensitization; (b) IL-4 levels after challenge. Bars represent mean values of $n = 5 \pm$ SD, $*P < 0.01$. ■ Group of animals with previous feeding for two days with *L. casei* and then sensitized (a) and (b); □ Group of sensitized and challenged control animals, untreated with *L. casei* (a) and (b); ▨ Control values for untreated mice with ovalbumin: up to 15 pg mL^{-1} .

DISCUSSION

The intestinal microflora is an important constituent of the gut mucosal barrier. In the absence of intestinal microflora, antigen transport is increased and the induction of oral tolerance may be abrogated (Moreau & Corthier, 1988).

The use of specific microorganisms, called probiotics, able to influence the intestinal microflora, may aid in host protection by stimulation of cytokines released from the immune cells associated to gut mucosa. These probiotics strains also could influence the down regulation of an allergic process, modulating the balance Th1/Th2, which is important in the regulation of IgE synthesis through cytokine release. Attention has recently been focused on the lactic acid bacteria microorganisms capable of regulating the host immune response in experimental systems, especially, the genus *Lactobacillus* (Matsuzaki *et al.*, 1998).

The primary objective of this study was to establish a model of atopy in BALB/c mice similar to that reported by Rentz *et al.* (1994), to determine the validation of use of *L. casei* as an immunomodulator in this process. This microorganism was selected due to its ability to survive passage through the gastrointestinal tract and to exhibit immunopotentiator capacity (Perdigón *et al.*, 1993; Petrino *et al.*, 1996). To produce an effective sensitization with OVA, aerosolization was necessary. Intradermic injections were not enough to reach a high level of IgE. After the sensitization period we detected in the serum from control animals, high levels of specific IgE anti-OVA and a significant increase in the levels of IL-4, with low levels of IFN- γ at various time points. After challenge, the profile of specific IgE, and IL-4 were increased even more in this group of animals (Figures 1,3 and 4).

We demonstrated the effect of *L. casei* in the regulation of IgE production in our model of atopy. This effect was also showed by Matsuzaki *et al.* (1998). We observed a marked diminution of IgE when the probiotic was administered prior to the antigen sensitization period (Figure 1). This regulatory effect may be due to the increase of IFN- γ released by Th1 cells (Figure 3), which were activated by *L. casei*, inducing down regulation in the IL-4 levels (Figure 4). This interleukin may also play a predominant role in the pathogenic of airway inflammation in atopic asthma, leading to bronchial hyperresponsiveness with mast cells and eosinophilic airway infiltration (Brusselle *et al.*, 1995). We found that *L. casei* was able to modulate the increase of mast cells in the peribronchial area after sensitization and before challenge with OVA (Figure 2). This effect, we think, was due to a diminution in the IL-4 levels. The diminution in the number of mast cells is an important fact considering the role for mast cells in the development of several chronic diseases such as asthma and in innate immunity (Wedemeyer *et al.*, 2000).

It has been shown that probiotics promote antigen specific immune responses, particularly in the IgA class and can reverse intestinal permeability (Isolauri *et al.*, 1993; Majamaa *et al.*, 1995). Moreover, lactobacilli and yogurt have been used to improve IFN- γ levels (Halpern *et al.*, 1991) and inhibit TNF- α release, reducing allergic inflammation. TNF- α plays an important role in inflammatory processes, depending on the Th1/Th2 cytokine balance (Hernandez-Pando & Rook Gaw, 1994).

The preservation in the balance of cytokine produced by Th₁ cells and Th₂ cells has been reported (Coffman & Carty, 1986) to be an important factor in the regulation of IgE production. Therefore the functional augmentation of Th1 cells and the inhibition of Th2 cells are critical in the regulation of Ig E synthesis.

The beneficial effect observed with *L. casei* was not a long-term response. It was not effective in increasing IFN- γ levels when the animals were challenged with aerosolized OVA, however *L. casei* was effective when it was administered at various times during the development of this model of atopy (Figure 1).

Our observations would indicate that repeated doses of *L. casei* could immunomodulate the allergic process via Th₁ cytokine production, and down regulate the hypersensitivity reaction.

Our finding would justify the use of probiotics as a preventive in food allergy, where the mechanisms of this process and Th1/Th2 paradigm are being closely examined (Helm & Burks, 2000). We demonstrated the immunomodulatory capacity of *L. casei* in an atopy model.

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