Study of the Possible Mechanisms Involved in the Mucosal Immune System Activation by Lactic Acid Bacteria

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ABSTRACT

The induction of a mucosal immune response is not easy due to the development of oral tolerance, but under some conditions, bacteria can activate this immune system. Antigens administered orally can interact with M cells of Peyer's patches or bind to the epithelial cells. We have demonstrated that certain lactic acid bacteria are able to induce specific secretory immunity, and others will enhance the gut inflammatory immune response. The aim of this work was to establish the reason for these different behaviors and to define possible mechanisms involved in the interaction of lactic acid bacteria at the intestinal level. We studied IgA+ and IgM+ B cells comparatively in bronchus and intestine and CD4+ T cells and IgA anti-lactic acid bacteria antibodies in the intestinal fluid, induced by oral administration of *Lactobacillus casei*, *Lb. delbrueckii* ssp. *bulgaricus*, *Lb. acidophilus*, *Lb. plantarum*, *Lb. rhamnosus*, *Lactococcus lactis*, and *Streptococcus salivarius* ssp. *thermophilus*. The increase in the IgA+ B cells in the bronchus means that these lactic acid bacteria were able to induce the IgA cycle by interaction with M cells from Peyer's patches or intestinal epithelial cells. The IgM+ cells increased when the stimulus did not induce the switch from IgM+ to IgA+. The increase in the CD4+ cells suggests interaction of Peyer's patches and enhancement of the B- and T-cell migration. The anti-lactic acid bacteria antibody is related to the processing and presentation of the microorganisms to the immune cells. We demonstrated that *Lb. casei* and *Lb. plantarum* were able to interact with Peyer's patch cells and showed an increase in IgA+, CD4+ cells, and antibodies specific for the stimulating strain. *Lactobacillus acidophilus* induced gut mucosal activation by interaction with the epithelial cells without increase in the immune cells associated with the bronchus. Although *Lb. rhamnosus* and *Strep. salivarius* ssp. *thermophilus* interact with epithelial cells, they also induced an immune response against their epitopes. *Lactococcus lactis* and *Lb. delbrueckii* ssp. *bulgaricus* induced an increase of IgA+ cells entering the IgA cycle but not CD4+ cells; thus, these bacteria would have been bound to epithelial cells that activated B lymphocytes without processing and presenting of their epitopes. We did not determine specific antibodies against *Lc. lactis* or *Lb. bulgaricus*.
(**Key words**: lactic acid bacteria, intestinal interactions, immunomodulation)

Abbreviation key: **FITC** = fluorescein isothiocyanate conjugated, **GALT** = gut associated lymphoid tissue, **LAB** = lactic acid bacteria.

INTRODUCTION

The intestinal mucosa is the first line in host defense, and it is exposed to a great number of antigens, many of which are innocuous, while others are potentially harmful. The induction of the immune response at the intestinal level is not always easy. The mechanisms governing immune responsiveness and unresponsiveness in the intestine are not well understood. Orally administered antigens interact with the gut associated lymphoid tissue (**GALT**), which is a well developed immune network that is not only involved in protection of the host from pathogens but also in preventing the host from reacting to ingested protein. Thus, orally administered proteins often induce systemic hyporesponsiveness to the fed proteins; this mechanism is called oral tolerance (6). The maintenance of this process depends on the amount of antigen ingested. Although most dietary antigens are degraded, some intact or partially degraded antigens are absorbed into the systemic circulation. Oral tolerance can be abrogated and an immune response induced (4). This immune response

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is mainly a humoral immune response mediated by IgA producing cells and secretory IgA, which constitute almost 80% of all antibodies produced in mucosal associated tissue (16, 18). These antibodies inhibit the microbial adherence and also prevent absorption of antigens from mucosal surfaces (14). Although all of the immune cells are present on the gut mucosa, the cytotoxic cellular immune response is limited by immunoregulatory mechanisms to avoid intestinal damage (24).

Antigen uptake occurs through the specialized system represented by the M cells overlying Peyer's patches or across normal epithelium overlying the lamina propria (5, 11).

Peyer's patches are the major inductive sites in the intestine in which the antigens have the potential to interact with all of the specialized cells that induce an active immune response between macrophage and dendritic cells (antigen-presenting cells) and T and B lymphocytes. The activation of the oral immune system is clearly associated with activation of T cells in the Peyer's patches. However, many T cells are present in the diffuse lymphoid system of the lamina propria. If the antigen crosses the normal epithelium, it has the potential to present and to activate lamina propria T cells and to induce an immune response.

When the mucosal immune response is induced, primed T and B cells migrate through the lymphatic system and then enter the peripheral blood circulation via the thoracic duct. Extravasation of the immune cells occurs not only in the gut lamina propria but also in other mucosal sites such as respiratory tissues, urogenital tissues, and mammary and salivary glands. This migration is known as the IgA cycle (23), and its distribution from the gut to other mucosal sites has been described as the common system of mucosa (17). This concept implies that oral immune stimulation can induce immunity in mucosal sites remote from the gut.

Much relevant literature (2, 7, 9) has demonstrated the importance both to human and animal health of the lactic acid bacteria (**LAB**) present in fermented foods and probiotic preparations. Some LAB can affect protective immunity against pathogens and tumors and have the ability to increase the mucosal immune response (9, 10, 13, 15).

In our laboratory, we have demonstrated the capacity of certain LAB to enhance both systemic and mucosal immunity. Furthermore, we have shown that dose can influence the effect achieved (1, 21, 22, 27).

Although much research has been done, the use of LAB for therapeutic purposes is still limited. The effect of LAB may be influenced by differences between strains, model systems, or poor viability. Thus, it is very difficult to generalize from results of one experiment.

Knowledge of the possible mechanisms by which LAB interact at the intestinal level and then stimulate the immune cells could improve the use of these microorganisms as immunomodulator substances.

The aim of this research was to study the effect of LAB on immune cells associated with the mucosa and other immune parameters to determine the possible site of interaction of these microorganisms with the small intestine. This knowledge could explain why not all of the LAB could stimulate the immune system in the same way.

MATERIAL AND METHODS

Microorganisms Used

The bacterial strains used for the experiments were *Lb. casei* CRL 431, *Lb. acidophilus* CRL 924, *Lb. plantarum* CRL 936, *Lb. delbrueckii* ssp. *bulgaricus* CRL 423, *Lb. rhamnosus* CRL 74, *Lc. lactis* CRL 526, and *Strep. salivarius* ssp. *thermophilus* CRL 412 from the Centro de Referencias para Lactobacilos (CERELA, Tucumán, Argentina) culture collection. *Lactobacillus casei*, *Lb. acidophilus*, *Lb. rhamnosus*, and *Lb. plantarum* were cultured for 8 h at 37°C in MRS broth (Oxoid Ltd., Hampshire, England); *Strep. thermophilus* and *Lc. lactis* were cultured 8 h at 37°C and 30°C, respectively, in LAPTg broth. All bacteria were harvested by centrifugation at $5000 \times g$ for 10 min and were washed three times with sterile saline solution.

Animals and Feeding Procedure

The BALB/c mice weighing 25 to 30 g were obtained from the random-bred colony kept in our department at the Institute of Microbiology. Each experimental group consisted of 5 to 6 mice.

Each culture of LAB was suspended in 5 ml of sterile nonfat milk and was administered at 20% (vol/vol) in the drinking water to different groups of mice for 2, 5, or 7 consecutive d at a dose of 10^9 cells/d per mouse. The control group received sterile milk in the drinking water given under the same conditions as for the test group.

All of the mice were fed ad libitum with a conventional balanced diet.

Tissue Sections

At the end of each feeding period with the different LAB, mice were anesthetized and slaughtered, and

the small intestine and the lower respiratory tract were removed. Tissues were placed in ethanol and were processed by the technique of Saint Marie (25). Tissues were fixed, dehydrated, and embedded in paraffin at 56°C. From each tissue, 4-μm serial paraffin sections were cut.

Immunofluorescence Test

The IgA, IgM-secreting cells, and CD4$^+$ lymphocytes were determined on histological slices from the gut and bronchus.

Direct immunofluorescence was performed using the respective monospecific antibodies (α- or μ-chain specific) conjugated with fluorescein isothiocyanate (**FITC**) (Sigma Chemical Co., St. Louis, MO) or FITC-conjugated monoclonal antibodies specific for CD4$^+$ lymphocytes (Gibco BRD Life Technologies, Neutroquimica, Argentina).

Negative controls were run using the respective unlabelled antibodies before incubation with FITC-conjugated antibodies.

Results were expressed as the mean of the number of positive cells per 10 fields of light microscope (100×).

Anti-LAB Antibodies and ELISA Test

The anti-LAB antibodies present in intestinal fluid were measured by ELISA. Assays were performed at the end of each feeding period and for each LAB that was assayed.

The procedures used for collection of intestinal fluid and ELISA were as described previously by Alvarez et al. (1) and used goat anti-mouse IgA (α-chain specific conjugated peroxidase; Sigma Chemical Co.). Absorbance was measured at 493 nm.

Control values were those obtained from intestinal fluid of untreated mice for each LAB under study.

Statistical Analysis

The number of different immune cells measured were determined as arithmetic means of the values obtained from (n = 5 to 6) determinations expressed for each surface marker. Statistical comparisons were conducted using Student's test.

RESULTS

Determination of the Number of IgM$^+$ Cells in GALT

The number of IgM$^+$ B cells was measured because their importance is second only to that of IgA in protection of mucosal surfaces.

We observed that only *Lc. lactis*, *Lb. acidophilus*, and *Lb. plantarum* were able to increase the number of IgM-secreting cells in the lamina propria of the small intestine for different periods of administration: 2, 5, and 7 d, respectively (see Figure 1).

Study of IgA Producing Cells on GALT

Oral administration of all LAB strains studied increased the number of IgA cells in the lamina propria. The effect was dose dependent. The most remarkable effect was obtained with *Lb. casei*, *Lb. plantarum*, *Lb. delbrueckii* ssp. *bulgaricus*, and *Strep. salivarius* ssp. *thermophilus* (see Figure 2). We also observed a significant decrease ($P < 0.01$) when *Lb. plantarum* was administered for 5 d.

Effect of LAB on CD4$^+$T Cells

The CD4$^+$ T cells are important in the induction of specific immune responses, and they participate in the switch from IgM to IgA.

We demonstrated that only *Lb. casei* and *Lb. plantarum* were able to increase CD4$^+$ T cells in the lamina propria of the small intestine. The effect was dose dependent.

The values obtained with other LAB were similar to those of controls with exceptions for *Lb. rhamnosus* at 2, 5, and 7 d and for *Lc. lactis* at 7 d, for which we observed a significant decrease ($P < 0.01$) in the number of CD4$^+$ cells. These results are in Figure 3.

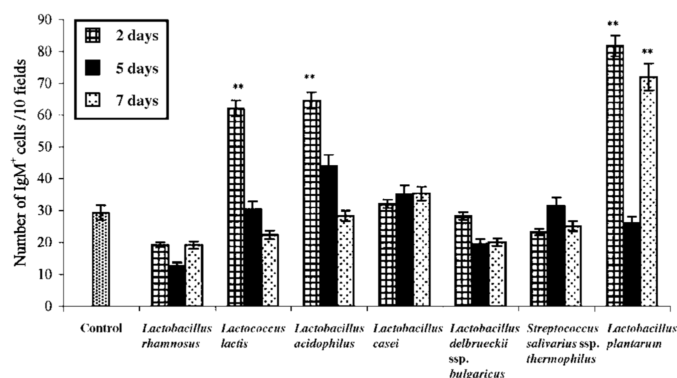

Figure 1. Effect of oral administration of different LAB on the number of IgM cells present in lamina propria of the small intestine tissue. The IgM B cells were measured by an immunofluorescence test using μ-chain monospecific antibody after 2, 5, or 7 d of feeding with each lactic acid bacteria assayed. The bars indicate arithmetic means (±SD) of the number of cells in 10 fields (100×) expressing the marker. $^{**}P < 0.01$.

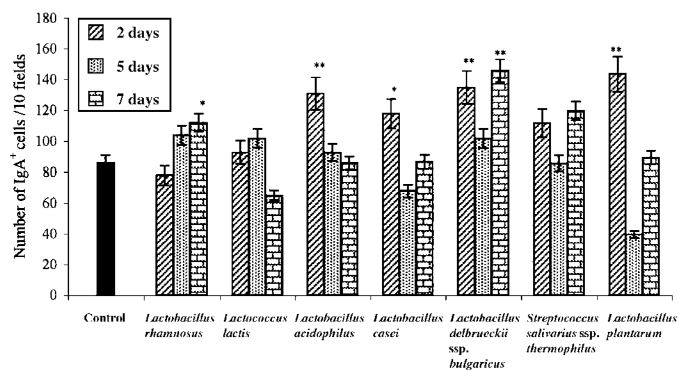

Figure 2. Effect of oral administration of different lactic acid bacteria on the number of IgA+ B cells. They were measured by an immunofluorescence test using monospecific antibody after 2, 5, or 7 d of feeding with each lactic acid bacteria assayed. The bars indicate arithmetic means (±SD) of the number of cells in 10 fields (100×) expressing the marker. *$P < 0.05$. **$P < 0.01$.

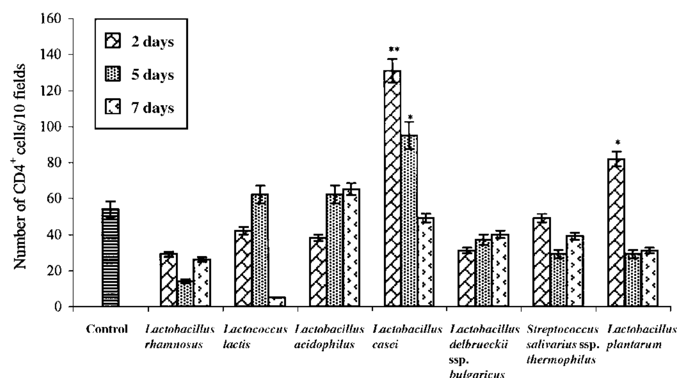

Figure 3. Effect of oral administration of different lactic acid bacteria on the number of CD4+ T cells. They were measured by an immunofluorescence test using CD4 monoclonal antibody after 2, 5, or 7 d of feeding with each lactic acid bacteria assayed. The bars indicate arithmetic means (±SD) of the number of cells in 10 fields (100×) expressing the marker. *$P < 0.05$. **$P < 0.01$.

Determination of the IgA B Cells Associated with Bronchus Tissue

We studied the IgA-secreting cells associated with bronchus associated lymphoid tissue as a measure of cellular migration. Oral antigen stimulation can induce IgA B cells present in the gut to enter to the IgA cycle and to increase the number of these cells in sites distant from the intestine (such as bronchus) under the concept of common system of mucosa.

We observed that all of the LAB assayed, with the exception of *Lb. acidophilus*, were able to increase the number of IgA+ cells in bronchus. This effect was dose dependent. These results are shown in Figure 4.

Study of the Anti-LAB Antibodies

This assay was performed to determine whether the LAB as antigens were degraded and presented to the immune cells to induce antibody production against their epitopes. Antigen degradation may be in the Peyer's patches or in the epithelial cell. We determined that only *Lb. casei*, *Lb. rhamnosus*, *Strep. salivarius* ssp. *thermophilus*, and *Lb. plantarum* were able to induce antibody production (see Table 1).

DISCUSSION

In the induction of the gut immune response, the increase in the number of IgA producing cells is due to the switch of IgM+ B cells present in Peyer's patches to IgA+ B cells under the influence of cytokines released by CD4+ T-helper lymphocytes type 2 (Th2) (12, 19). An increase in the number of IgM+ cells in lamina propria would mean that the stimulus was only able to mobilize these B lymphocytes without activation of Th2 lymphocytes. Only stimulation by *Lc. lactis* (2 d), *Lb. acidophilus* (2 and 5 d), and *Lb. plantarum* (2 and 7 d) (Figure 1) led to an increase in the number of IgM+ cells in the lamina propria.

To have good stimulation of intestinal mucosa, the number of IgA+ B lymphocytes must be only slightly higher than in the normal control (3). High numbers of IgA+ cells could have harmful effects on the host and favor an increase in intestinal permeability, which occurs with coeliac disease (26). When we determined the number of IgA+ cells present in the small intestine, we saw different patterns between the LAB assayed for the different periods of adminis-

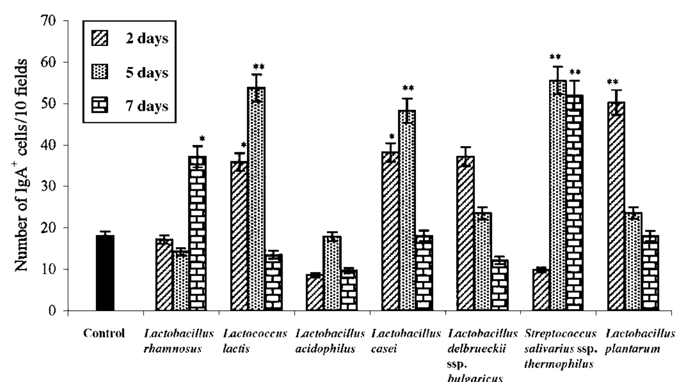

Figure 4. Effect of oral administration of different lactic acid bacteria on the number of IgA cells associated the bronchus associated lymphoid tissue. IgA+ B cells were measured by an immunofluorescence test using monospecific antibody after 2, 5, or 7 d of feeding with each lactic acid bacteria assayed. The bars indicate arithmetic means (±SD) of the number of cells in 10 fields (100×) expressing the marker. *$P < 0.05$. **$P < 0.01$.

TABLE 1. Determination of antibodies for anti-lactic acid bacteria (LAB) by ELISA.[1]

Microorganisms	Control		LAB administration (absorbance at 493 nm) 2 d		5 d		7 d	
	$\overline{X}$	SD	$\overline{X}$	SD	$\overline{X}$	SD	$\overline{X}$	SD
Lactobacillus casei	0.083	0.042	0.274**	0.022	0.342**	0.053	0.352**	0.028
Lactobacillus rhamnosus	0.167	0.031	0.226	0.057	0.476**	0.019	0.362**	0.025
Streptococcus salivarius ssp. *thermophilus*	0.163	0.027	0.285*	0.035	0.252*	0.010	0.132	0.051
Lactobacillus delbrueckii ssp. *bulgaricus*	0.124	0.018	0.143	0.030	0.147	0.051	0.151	0.042
Lactobacillus acidophilus	0.382	0.048	0.369	0.063	0.402	0.055	0.394	0.065
Lactobacillus plantarum	0.148	0.010	0.280**	0.032	0.193	0.060	0.207*	0.028
Lactococcus lactis	0.095	0.018	0.156	0.033	0.150	0.040	0.086	0.037

[1]The mice (n = 5) were fed with different LAB for 2, 5, or 7 consecutive d. At the end of each feeding period, they were slaughtered, and the intestinal fluid was recovered to perform an ELISA test. Control mice were without treatment.

*$P < 0.05$.

**$P < 0.01$.

tration (Figure 2), which suggested that the dose administered could affect the results. The great decrease in the IgA+ cells observed for d 5 of *Lb. plantarum* may be due to a down regulation process to avoid an increase in the inflammatory immune response. When we analyzed the number of IgA+ cells on bronchus tissue, we observed that *Lb. casei*, *Lb. plantarum*, *Strep. salivarius* ssp. *thermophilus*, *Lb. rhamnosus*, *Lb. delbrueckii* ssp. *bulgaricus*, and *Lc. lactis* were able to increase the number of IgA+ B lymphocytes in bronchus (Figure 4). This effect was also dose dependent. The increase in IgA+ cells in GALT and bronchus associated lymphoid tissue suggest that cellular migration was taking place.

Antigen-presenting cells from the Peyer's patches or lamina propria of the small intestine may induce an antigen specific proliferative response of T cells (20). However, T cells and IgA+ B cells can leave the Peyer's patches following antigen stimulation and increase the number of T cells in the lamina propria. If the antigen interaction is with epithelial cells, CD4+ T cells can be activated to stimulate other immune cells through the release of cytokines, but they are not able to migrate to sites distant from the intestine (20). Clear differences were noted in the profile of cytokines when T cells were stimulated in lamina propria. The presentation of antigen by cells from the lamina propria would lead to a lack of conventional activation and differentiation (29).

When we studied the number of CD4+ T cells, we observed that most of the LAB assayed were not able to increase this population with exception of *Lb. casei* and *Lb. plantarum* for some periods of administration. On the contrary, the most frequent effect observed was a decrease in the number of these cells especially for *Lb. rhamnosus* and *Lc. lactis* (Figure 3). We do not think that these LAB are able to induce selectively a suppression of immune response. We believe that the decrease observed could be related to the interaction of these LAB with the intestinal epithelial cells and to the levels of cytokine released, which would not be enough to induce clonal expansion of the T cells present in lamina propria. The CD4+ increase in the lamina propria means not only cellular migration but also interaction of LAB with M cells of the Peyer's patches. This fact would mean that only *Lb. casei* and *Lb. plantarum* could induce an effective stimulation from Peyer's patches of the immune cells associated with the gut. The diminution of these cells might also be due to the mechanisms of oral tolerance, which can be mediated by deletion, anergy, or active cellular suppression for which the determining factor is the dose of antigen fed. Low doses favor active suppression, whereas high doses favor deletion and anergy (28).

When we determined whether the LAB were processed as antigens and whether they induced an immune response against their own epitopes, we detected antibodies against LAB for *Lb. casei*, *Lb. plantarum*, *Lb. rhamnosus*, and *Strep. salivarius* ssp. *thermophilus*, which indicated that these LAB were degraded and presented to the immune cells (Table 1).

We postulated the following hypothesis, taking into account results obtained for number of IgA+ and CD4+ cells and antibodies against LAB, about the way in which the different LAB can interact with the small intestine. If LAB induce an increase in the number of IgA+ cells in gut and bronchus, on the CD4+ T cells, and in the immune response against their epitopes, the interaction could have been through M cells at the Peyer's patches such as was

observed using *Lb. casei* and *Lb. plantarum*. When we observed increases in IgA^+ cells in the gut and bronchus, but no increase in the $CD4^+$ T cells, the interaction would have been on the intestinal epithelium cells independent of whether or not the LAB were processed as antigen. The IgA cycle may also be increased by mobilization of the IgA^+ cells present in the mesenteric lymph node (28), which we observed with *Lb. rhamnosus* and *Strep. salivarius* ssp. *thermophilus*, which were processed and presented as antigen. *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Lc. lactis* could interact at epithelial level without processing these microorganisms but increasing the IgA cycle.

We believe that *Lb. acidophilus* has only a local effect on the gut when interacting with the epithelial cells. It was unable to induce the IgA^+ cycle or to increase the number of $CD4^+$ T cells. The effect induced would be exerted only by the cytokines released by the stimulation of the epithelial cells.

In this report we demonstrated that LAB can interact at different levels in the small intestine. These interactions could explain why the mucosal immunostimulation by LAB is not the same for all bacterial LAB species.

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