Immunomodulation of Human Blood Cells Following the Ingestion of Lactic Acid Bacteria

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ABSTRACT

Because of the lack of data that convincingly show immunomodulatory properties of lactic acid bacteria in humans, a study was performed in which healthy volunteers were divided into two groups and given a fermented milk product supplemented with *Lactobacillus acidophilus* strain La1 or *Bifidobacterium bifidum* strain Bb 12 for 3 wk. Blood was sampled throughout the study to assess changes in lymphocyte subsets or leukocyte phagocytic activity following consumption of the fermented products. No modifications of lymphocyte subpopulations were detected. In contrast, phagocytosis of *Escherichia coli* sp. in vitro was enhanced after the administration of both fermented products. The increment in phagocytosis was coincident with fecal colonization by the lactic acid bacteria and persisted for 6 wk after ingestion of the fermented products. By this time, the fecal lactobacilli and bifidobacteria had returned to concentrations prior to consumption. Nonspecific, anti-infective mechanisms of defense can be enhanced by the ingestion of specific lactic acid bacteria strains. These strains can be used as nutritional supplements to improve the immune function of particular age groups, i.e., the neonate or the elderly, for which these functions are diminished. (**Key words**: lactic acid bacteria, immunomodulation, phagocytosis, leukocytes)

Abbreviation key: **BBRM** = bacterial biological response modifier, **LAB** = lactic acid bacteria, **LPS** = endotoxic lipopolysaccharide.

INTRODUCTION

Immunomodulation has been observed after administration of lactic acid bacteria (LAB) in several animal studies (17), but few human studies (13, 22), and LAB may enhance the nonspecific resistance of the host to infections and tumors (7) or act as adjuvants of specific immune responses (8, 10, 17, 18, 24). These activities have been observed after LAB intake by oral and parenteral administration (21), but neither the cell populations initially reacting to the bacterial signals nor the endogenous mediators or cytokines produced have been identified.

Different LAB strains have different biological activities in the host, but the association between particular bacterial features and biological activity remains obscure. Nevertheless, relevant bacterial characteristics are the species specificity, the survival of the inoculum in the gastrointestinal tract, its persistence above a critical level of colonization, and its capacity to attach to mucosal surfaces. With respect to the active components, many bacterial biological response modifiers (BBRM), present on the surface of Gram-positive and Gram-negative bacteria, come in contact with different host microenvironments (12, 16). Some of the well-characterized moieties include cell-wall peptidoglycans, lipoteichoic acids, and endotoxic lipopolysaccharides (LPS) (23). Interestingly, peptidoglycans and LPS may share cellular receptors on lymphoid and myeloid cells (4). Although certain BBRM are only active through the parenteral route, others are also active orally, but it is not clear whether the latter generate a signal locally at the intestinal mucosal surface or whether they need to translocate through the mucosal barrier to another

host microenvironment in order to trigger a biological response.

No direct evidence exists that LAB given orally modulates the immune response of the human host. A study using human volunteers was therefore initiated. Two LAB strains were selected on the basis of their biological characteristics and added to a fermented milk: *Lactobacillus acidophilus* strain LA 1, which binds to human enterocytes in vitro (1), and *Bifidobacterium bifudum* strain Bb 12, which has a high colonization capacity in humans (F. Rochat, 1991, personal communication). Both fermented products were given orally, and the subsequent effects on blood cell subsets and phagocyte activity were examined.

MATERIALS AND METHODS

Volunteers and Experimental Design

Healthy adult human volunteers (12 females and 16 males) from 23 to 62 yr of age (mean 36.9) were randomly distributed into two groups that received two different types of fermented products in addition to their normal diet. For the first 3 wk of the study, both groups received milk but, for the second 3 wk, received a fermented milk supplemented with a *B. bifidum* strain Bb 12 (group 1, daily intake 1×10^{10} cfu) or *L. acidophilus* strain LA1 (group 2, daily intake 7×10^{10} cfu). In the final 6 wk of the study, both groups again received milk, but no fermented products. Volunteers consumed 120 ml of milk or fermented milk three times daily (morning, midday, and evening). This amount represented an acceptable volume of fermented products and corresponded to a dose of bacteria that has been shown to change the level of fecal colonization (F. Rochat, 1991, unpublished data). A total of four blood samples were taken at the beginning of the study before volunteers received milk (time 0) and at the end of each of the three feeding periods.

Lymphocyte Subsets in Peripheral Blood

Blood samples were collected in sterile EDTA-blood collection tubes, and flow cytometric analysis of human leukocyte subsets was performed using the protocol of the Simultest™ IMK Plus kit (Becton-Dickinson,

Basel, Switzerland), which contains monoclonal antibodies to T cells (CD3+), activated T cells (CD3+, HLA-DR+), B lymphocytes (CD19+), helper-inducer (CD3+, CD4+), suppressor-cytotoxic (CD3+, CD8+) lymphocytes, and natural killer cells (CD3-, CD16+, CD56+).

Phagocyte Activity of Peripheral Blood Cells

Leukocyte phagocyte activity in peripheral blood was quantitatively determined using flow cytometry and fluorescein isothiocyanate-labeled opsonized *Escherichia coli* sp. (PHAGOTEST™; Becton-Dickinson). Briefly, 100 μl of fresh heparinized blood were mixed with 20 μl of an *E. coli* bacterial suspension (1 × 10⁹/ml) such that the ratio of bacteria to leukocytes was approximately 20:1 (vol/vol). This mixture was then incubated for 10 min at 37°C in a water bath with controlled shaking. After quenching to remove free floating or bacterial cells attached to, but not ingested by, the leukocytes, the blood was lysed and fixed, and DNA staining with a propidium iodide solution was subsequently performed. Measurements were with a FACScan™ flow cytometer using a blue-green excitation light (488 nm). During data acquisition with LYSIS II™ software (Becton-Dickinson), a live gate was set in the red fluorescence histogram such that the only events considered were those positive for propidium iodide staining, thus corresponding to human diploid cells. In analysis of the data, the overall phagocyte activity of the blood cells and that of individual cell populations (neutrophils and monocytes) were examined.

Fecal Colonization by LAB

Fecal specimens were taken before the period of fermented milk intake, on d 21 of the study period, and 12 d after product consumption. Freshly passed fecal samples were analyzed for the total bifidobacteria, the total lactobacilli, and for *L. acidophilus* La1.

The lactobacilli were counted on MRS agar (Difco, Detroit, MI) with antibiotics (.8 mg/ml of phosphomycine, .93 mg/ml of sulfamethoxazole, and 50 μg/ml of trimethoprim), and the bifidobacteria were counted on Eugon agar (Becton Dickinson) with tomato juice. Plates were incubated anaerobically (Anaerocult A,

Merck, Darmstadt, Germany) for 48 h at 37°C, and the different types of colonies were observed and isolated. The lactobacilli were identified by cell morphology, and the carbohydrate profile was determined by API 50CHL (Bio Mérieux, Marcy L'Etoile, France). Thereby, the total lactobacilli and, more specifically, the Lal colonies were assessed. Bifidobacteria were counted after identification by cell morphology and API 32A testing (Bio Mérieux).

Statistical Analysis

The data were analyzed using ANOVA for repeated measures. Ratios and differences in phagocytosis rates of the different phagocytic populations in blood were compared using a covariance analysis (ANCOVA) with repeated measures and Scheffé contrasts (20).

RESULTS

Reference ranges were provided by Becton-Dickinson for total T and B cells, activated T cells, T helper to T suppressor ratios, and natural killer cells for hematologically normal Causasian adults. Because 1 volunteer in each group showed ratios of T helper to T suppressor cells of <.5 and 1 volunteer in group 1 showed a high percentage of T cell activation (>26%), those volunteers were excluded from the study. In blood samples from the remaining volunteers, no significant modifications were detected in the proportions of the blood lymphocyte subsets or in the degree of T-cell activation at the time points examined (data not shown).

Although volunteers were randomly distributed into the two groups, the overall activity of blood phagocytes (granulocytes and monocytes) at the beginning of the study was slightly greater for the group that received Lal (P = .051; Figure 1). For the individual groups, this activity did not change after 3 wk of abstention from fermented products. However, the increase in overall activity was significant for both groups after LAB ingestion (P < .0001), which was still more evident in the group receiving bifidobacteria. Six weeks after consumption of fermented products, overall phagocytic activity decreased for both groups (group 1, P = .001; group 2, P = .05); the
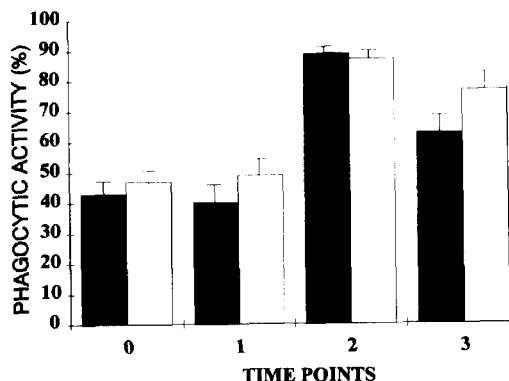

Figure 1. Percentage of phagocytes (granulocytes and monocytes) showing phagocytic activity in the peripheral blood of volunteers receiving fermented milk supplemented with *Bifidobacterium bifidum* strain Bb12 (■) or *Lactobacillus acidophilus* strain Lal (□). Mean (± SEM) phagocytic activity is shown at time point 0, the beginning of the study; time point 1, after receiving milk; time point 2, after 3 wk of fermented milk; and time point 3, 6 wk after the end of fermented milk consumption.

change was more evident for the group consuming bifidobacteria, but final values were still considerably higher than those at time point 0.

Because granulocytes represent approximately 60% of the leukocytes in group 1 (Bb12) and about 53% of those in group 2 (Lal) (data not shown), the greater increment between time points 1 and 2 for group 1 than for group 2 (P < .05) may be attributable to the greater proportion of granulocytes present, especially because the granulocyte population was affected more by LAB ingestion (Figures 2 and 3). Therefore, whether this difference is due to a different effect between diets is not known; indeed, covariance analysis showed that granulocytes have a higher increase in phagocytic activity after fermented milk consumption than do monocytes (P < .004). Nevertheless, the phagocytic activity of granulocytes (P < .0001 for both groups between time points 1 and 2) mimicked that of blood phagocytes as a whole (Figure 2). Monocytes represented approximately 4 to 8% of the leukocytes for both groups. The proportion was a little greater at time 0 for group 2 (Lal) (group 1 = 6.71, SEM ± .38; group 2 = 7.96, SEM ± .54; P = .07), as was the percentage of these cells showing phagocytic activity (P =
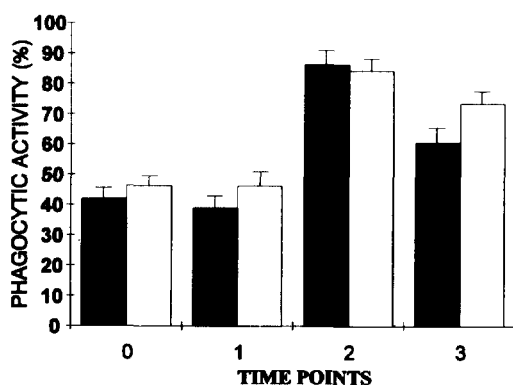
Figure 2. Percentage of granulocytes with phagocytic activity in the peripheral blood of volunteers receiving fermented milk supplemented with *Bifidobacterium bifidum* strain Bb12 (■) or *Lactobacillus acidophilus* strain La1 (□). Mean (± SEM) phagocytic activity is shown at time point 0, the beginning of the study; time point 1, after receiving milk; time point 2, after 3 wk of fermented milk; and time point 3, 6 wk after the end of fermented milk consumption.
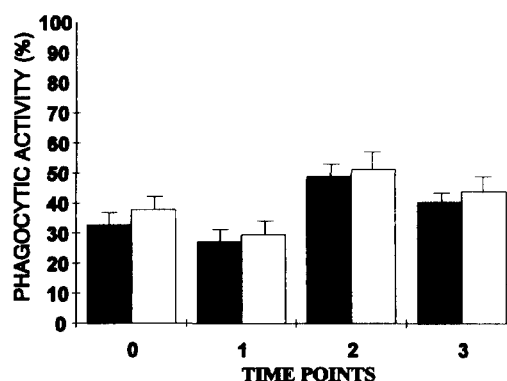
Figure 3. Percentage of monocytes showing phagocytic activity with respect to the total monocyte population in peripheral blood of volunteers receiving fermented milk supplemented with *Bifidobacterium bifidum* strain Bb12 (■) or *Lactobacillus acidophilus* strain La1 (□). Mean (± SEM) phagocytic activity is shown at time point 0, the beginning of the study; time point 1, after receiving milk; time point 2, after 3 wk of fermented milk; and time point 3, 6 wk after the end of fermented milk consumption.

.051). However, this latter difference was less significant by time point 3 ($P = .09$).

Ingestion of bifidobacteria and lactobacilli caused significantly increased phagocytic activity by monocytes (for both groups, $P < .001$) at time point 2 (Figure 3); however, the contribution of this effect to the overall increase in phagocytic activity was not great because the proportion of monocytes in the blood leukocyte population was small. Of interest, the phagocytic activity increased at time point 2, even though the proportion of monocytes present in the blood decreased [Group 1, 6.47 ± .47% (SEM) at time point 1, vs. 4.11 ± .38% at time point 2; group 2, 6.75 ± .75% at time point 1, vs. 4.54 ± .60 at time point 2]. By the final time point, the phagocytic capacity of the monocytes decreased for both groups.

Clearly, ingestion of LAB increased the overall phagocytic activity of blood leukocytes, particularly granulocytes. Furthermore, the changes in phagocytic activity for the granulocytes followed a pattern similar to that exemplified by the blood cells as a whole in that a significantly enhanced uptake of opsonized fluorescein isothiocyanate-labeled *E. coli*, observed immediately after the period of yogurt ingestion, was still evident, although at lower amounts, 6 wk after yogurt consumption

had ended. However, because of the initial disparity between the two experimental groups, no comparison between groups could be made except that, 6 wk after yogurt consumption had ended, the group receiving lactobacilli showed phagocytic activity more comparable with that observed during ingestion. Whether these changes were the result of cellular activation or the effect of a seric factor (opsonin) that increased the phagocytic activity is not known.

Viable counts of lactobacilli and bifidobacteria in the feces of volunteers were recorded. Fecal counts of the relevant bacteria were detected in each of the two volunteer groups. Fecal counts of bifidobacteria increased significantly (9.00 vs. 7.82 log cfu/g of feces; $P < .05$) in group 1 after the consumption of fermented milk. Lactobacilli also increased in group 2 (6.46 vs. 5.24 log cfu/g of feces; $P < .05$; Table 1). However, 12 d after the end of the fermented milk consumption, bacterial counts returned to initial counts. The strain La1 was detected in all of the volunteers consuming it.

According to the API characteristics, most of the lactobacilli and bifidobacteria present in the feces of the volunteers during the feeding period corresponded to the strains present in the respective fermented milk products and

TABLE 1. Fecal counts of bifidobacteria and lactobacilli in groups 1 and 2.

Treatment	Bifidobacteria[1]				Lactobacilli[1]			
	Group 1[2]		Group 2		Group 1		Group 2[3]	
	$\overline{X}$	SEM	$\overline{X}$	SEM	$\overline{X}$	SEM	$\overline{X}$	SEM
Before	7.82[a]	.56	8.29	.26	5.52	.49	5.24[a]	.39
During	9.00[b]	.07	8.41	.20	5.53	.41	6.46[b]	.26
After	8.21[a]	.32	8.13	.35	5.74	.50	4.98[a]	.38

[a,b]Means in the same column with different superscripts differ $(P < .05)$.

[1]Log_{10} colony-forming units per gram of feces; n = 13.

[2]Group 1 received 1.10^{10} cfu of bifidobacteria/d.

[3]Group 2 received 7.10^{10} cfu of lactobacilli/d.

were not detected in feces prior to consumption of fermented milk.

DISCUSSION

This study reports that at least one parameter of nonspecific immune activity–phagocytic activity in peripheral blood–was enhanced following the ingestion of fermented milk products. These products, supplemented with *L. acidophilus* or *B. bifidum*, promoted uptake of opsonized *E. coli*. The increment in overall phagocytic activity immediately after the 3-wk ingestion period was statistically higher for the group receiving *B. bifidum*. However, this difference might have been due to the greater number of granulocytes present. At the last time point, phagocytic activity started to decrease, especially for the group receiving *B. bifidum*, but, for both groups, the final activity was nevertheless higher than that at time 0; fecal colonization had returned to the original values by that time.

Both granulocyte and monocyte populations were affected by LAB ingestion. Because the granulocytes showed a greater increment in phagocytic activity than monocytes (AN-COVA; $P < .004$) and because the latter were present in substantially lower numbers in whole blood, the global enhancement of phagocytic activity was considered to be due to an increased contribution by the granulocyte population. To our knowledge, this study is the first with humans to report an alteration in granulocyte function following oral administration of LAB.

The mechanisms by which LAB consumption mediates this effect are not known.

Specific LAB strains may act as BBRM for the immune function of the host. To act as BBRM, the lactic acid microflora need to attain a given degree of colonization or at least attain a minimal amount during transit. In this study, oral administration of LAB to healthy volunteers modified a marker of the nonspecific immune activity, which was coincident with an augmented colonization of the feces. Moreover, the increased phagocytic activity persisted for some time after colonization returned to the original values.

The host-bacteria interactions involved are not fully understood. How can a normally noninvasive bacteria, given in quantities far inferior to that of the normal microflora, influence systemic immunity? The possibility exists that an unusually large inoculum of LAB targets a compartment of the host's mucosal immune system, which, under normal circumstances, is not highly colonized. Obvious bacterial targets in the host's intestine are the Peyer's patches of the gut-associated lymphoid tissue. The epithelium covering this lymphoid tissue has a proportion of M cells, which are thought to be more permeable to luminal components and, as such, to permit a better interaction with underlying immune cells. Certainly, increased antibacterial activity of Peyer's patch lymphocytes has been described in mice following administration of yogurt containing live bacteria (3). However, the Peyer's patches may not be the exclusive site of action. A bacterial signal that does not necessarily require contact between bacteria cells and intestinal cells could act directly on the epithelial cells, the intraepithelial lymphoid

compartment of the proximal small bowel, or both.

In support of this hypothesis, we have previously found (E. J. Schiffrin, 1992, unpublished data) that colonization of germ-free mice with live LAB cells, *L. acidophilus* strain LA1, *L. casei* strain GG, or a hydrolysate of an *Lactobacillus delbruekii* ssp. *bulgaricus* culture, increased the intraepithelial cell population in the proximal small bowel; however, no changes in the intraepithelial cell population were observed in the distal ileum or the colon. Upon activation, this lymphoid population is able to produce cytokines, such as interferon-γ and interleukin-2 (5, 6). Failure to detect an up-regulation of major histocompatibility complex class II molecules on the enterocytes does not exclude the possibility that these mediators are nevertheless produced and act locally in the intraepithelial microenvironment to alter the systemic immunoreactivity of the host. In some reductionist in vitro models, LAB induced interferon-γ production by human lymphocytes (22) and interferon-α production by peritoneal macrophages (14). Some LAB products not only have the capacity to induce endogenous production of tumor necrosis factor-α when given orally in the mouse, (2), but they also enhance other macrophage and neutrophil functions. Moreover, LAB products can alter macrophage functions in vitro with a strain-dependent pattern (9). Hatcher and Lambrecht (9) have reported that the phagocytosis of viable *Salmonella typhimurium* by murine macrophages was increased by the pretreatment of the phagocytes with cell-free extracts of *L. acidophilus* and *Bifidobacterium longum*. However, treatment with *B. longum* induced higher macrophage phagocytic capacity. We report for the first time that oral administration of LAB to human volunteers alters the function of the granulocyte population in the blood.

Because of the disparity between groups at the beginning of the study, differences, if any, cannot be described between the two bacterial strains used in this study. Both strains evidently mediated a similar effect on the activity, and the granulocytes were the main target. This effect may be mediated by the modulation of cell surface molecules that are involved in bacterial uptake by leucocytes: for example, the induction of β-2 integrin molecules (C3b receptor/Mac-1, LFA-1) or of the Fcγ receptors

(11), which are involved in phagocytosis of opsonized particles or bacteria and other cellular events (19) in which contact between cells is required. Alternatively, a seric factor, such as an opsonin, may also play a role in the biological effect. Both possibilities need further investigation.

For newborns or the elderly, immunocompetent blood cells show defective functions. At birth, the number of polymorphonuclear leukocytes in peripheral blood, and their phagocytic capacity, are lower than those of adult cells (15). Administration of specially selected LAB strains could potentially correct these defects.

CONCLUSIONS

Although the killing capacity of leukocytes was not examined in these studies, signals generated at the surface of the intestine or very close to it probably enhance phagocytic activity of blood granulocytes. The lack of changes in the lymphoid cell population in peripheral blood or the proportion of leukocytes present in peripheral blood suggests that this subtle physiological change can be achieved without major alterations of the host homeostasis. Thus, nonspecific, anti-infective mechanisms of defense are improved without causing severe systemic responses, such as those seen in the acute phase response.

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