Immune modulation of blood leukocytes in humans by lactic acid bacteria: criteria for strain selection¹⁻³

Eduardo J Schiffrin, Dominique Brassart, Alain L Servin, Florence Rochat, and Anne Donnet-Hughes

ABSTRACT Lactic acid bacteria in food can transiently colonize the intestine and exert beneficial effects (probiotic). Survival during intestinal transit or adhesion to epithelium or both seem to be important for modifying the host’s immune reactivity. Because Lactobacillus acidophilus strain La1 is adherent to enterocytes in vitro, we hypothesize that contact with immune cells may occur in vivo. However, Bifidobacterium bifidum strain Bb12, which shows high fecal colonization, is another potential immunomodulator. Twenty-eight volunteers were divided into two groups and given a fermented product containing one of the two strains. Lymphocyte subsets and leukocyte phagocytosis activity were studied in blood. No modifications were detected in lymphocyte subsets. In contrast, phagocytosis of Escherichia coli ssp. was enhanced in both groups (P < 0.001 for both). Bacterial adhesion to enterocytes, fecal colonization, or both seem to be valuable selection criteria for immunomodulation. Antifungal mechanisms of defense can be enhanced after ingestion of specific lactic acid bacteria strains. Am J Clin Nutr 1997;66:515S–20S.

KEY WORDS Lactic acid bacteria, immune modulation, phagocytosis, leukocytes, yogurt, fermented milk

INTRODUCTION Immune modulation has been observed after administration of lactic acid bacteria (LAB) in animal studies (1) and a few human studies (2, 3). Oral or parenteral administration of LAB may strengthen nonspecific mechanisms of defense against infection and tumors (4, 5); or, LAB may act as adjuvants in antigen-specific immune responses (6–9). Cell populations initially reacting to such bacterial signals could include blood leukocytes, B and T lymphocytes, accessory cells of the immune system, and mucosal epithelial cells such as those in the intestine. However, bacterial signaling to the host’s cell populations (membrane receptors and perhaps bacterial metabolic products) and the mediators of such responses are not well defined.

Bacterial strains have different biological activities in the host, but the association between particular bacterial features and biological activities remains obscure. Bacterial characteristics that seem relevant include species specificity, inoculum survival in the gastrointestinal tract, resistance to gastric acidity and bile toxicity, capacity to attach to mucosal surfaces, and transient colonization of the gut above a "critical" level. Bacterial components that represent biological response modifiers of the host are present on the surface of microorganisms and interact with different host microenvironments (10, 11). Bacterial biological response modifiers can be active parenterally, orally, or both, but whether the oral route can generate a signal locally at the intestinal mucosal surface or whether the modifiers need to translocate through the mucosal barrier to another host compartment is not clear. Some of the bacterial moteties with immunomodulatory activities are peptidoglycans, lipoteichoic acids, and endotoxic lipopolysaccharide (12). Interestingly, peptidoglycans and lipopolysaccharide may share cellular receptors on lymphoid and myeloid cells (13), although such receptors have not been identified on epithelial cells. Other bacterial products such as staphylococcal enterotoxin superantigens bind major histocompatibility complex class II molecules and stimulate T cells bearing particular T cell receptor VB chain, causing a massive T cell stimulation (14).

Variation in biochemical composition could explain the diverse biological activities obtained with different bacterial strains, but this is difficult to prove. An interesting alternative approach is to delineate the interactions between prokaryotes and eukaryotes. Maintenance of immune system homeostasis depends to some extent on cell-cell contacts. One can speculate that interactions of the eukaryotic cellular network with bacterial cells could also be relevant for the homeostasis of the immune system. An in vitro screening system for LAB strains adherent to human enterocytes seems pertinent especially because these cells are the first host intestinal cells with which ingested bacteria come into contact.

To examine whether oral administration of LAB does indeed modulate the immune status of the host, a human volunteer study was organized. Lactobacillus acidophilus strain La1, which binds to human enterocytes in vitro (15), and Bifidobacterium bifidum strain Bb12, which has a high colonization capacity of human volunteers (R Rochat, personal communication, 1991), were added to fermented milks. Both fermented milks were given orally and the subsequent effects on blood cell subsets and phagocytic activity were examined.

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SUBJECTS AND METHODS

In vitro screening studies

*L. acidophilus* La1, La3, La10, and La18 strains from the Nestec collection (Lausanne, Switzerland) were grown in De Man, Rogosa, Sharpe (MRS) broth in anaerobic conditions. Enterocyte-like Caco-2 cells were obtained from Jorgen Fogh (Sloan Kettering Memorial Cancer Center, Rye, NY) and grown in Dulbecco modified Eagle’s minimal medium (25 mmol glucose/L) (Eurobio, Paris) supplemented with 20% fetal calf serum (Boehringer Mannheim, Mannheim, Germany) and 1% nonessential amino acids.

Monolayers of Caco-2 cells, seeded at a concentration of 1.4 × 10⁴ cells/cm², were prepared on glass coverslips placed in six-well tissue culture plates (Corning Glass Works, Corning, NY) at 37 °C in a 10% carbon dioxide, 90% air atmosphere. The culture medium was changed daily.

Confluent cellular monolayers were washed twice with phosphate-buffered saline (PBS) before reincubation with *L. acidophilus* (1 mL, 4 × 10⁸ bacteria/mL) for 1 h in spent supernate, treated supernate, or fresh MRS broth diluted 1:1 in culture medium. The monolayers were then washed with sterile PBS, fixed in methanol, Gram stained, and examined under light microscopy. Each adherence assay was performed in triplicate. For each monolayer the number of attached bacteria was assessed in 20 different microscopic fields. The participation of the spent supernate in adherence was determined before and after trypsin treatment (2.5 g/L for 60 min at 37 °C). To determine the influence of Ca²⁺ on adherence to lactobacilli, monolayers were washed with EGTA (ethyleneglycoltaeacetic acid; 20 mmol/L) in PBS after the incubation period with bacteria.

After the bacterial adhesion assay, monolayers were fixed with 2.5% glutaraldehyde in 0.1 mol PBS/L, postfixed with OsO₄, dehydrated in a graded series of ethanol, and passed through a graded series of amyl acetate. Cells were dried in a critical point dryer, coated with gold, and examined in a scanning electron microscope (JSM 25S; Jeol, Paris).

In vivo human studies

Healthy adult human volunteers (n = 12 females and 16 males) aged from 23 to 62 y (mean: 36.9 y) were randomly distributed into two groups. For the first 3 wk of the study, both groups received milk; in the following 3 wk, the volunteers received a fermented milk supplemented with either *B. bifidum* strain Bb12 [group 1, daily intake: 1 × 10¹⁰ colony forming units (cfu)] or *L. acidophilus* strain La1 (group 2, daily intake: 7 × 10¹⁰ cfu). In the final 6 wk of the study, both groups received milk again, but no fermented products. Volunteers consumed 120 mL milk or fermented milk three times a day. This amount corresponds to a quantity of bacteria shown previously to change the level of fecal colonization (F Rochat, unpublished data, 1991). Four blood samples were taken: at the beginning of the study before the subjects received milk (time point 0), after the first 3-wk period (time point 1), after the second 3-wk period (time point 2), and after the final 6-wk period (time point 3).

EDTA-treated blood samples were taken 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 cells (CD³⁺, CD4⁺), suppressor-cytotoxic lymphocytes (CD³⁺, CD8⁺), and natural killer cells (CD³⁺CD16⁺, CD56⁺).

Flow cytometry determination of leukocyte phagocytic activity in peripheral blood was performed using fluorescein isothiocyanate–labeled opsonized *Escherichia coli* ssp. (Phagotest; Becton Dickinson). Briefly, 100 μL fresh heparinized blood was mixed with 20 μL of an *E. coli* bacterial suspension (1 × 10¹²/L) such that the ratio of bacteria to leukocytes was ~20:1 (by vol). This mixture was then incubated for 10 min at 37 °C in a waterbath with controlled shaking. After quenching, the blood was lysed and fixed and DNA staining with a propidium iodide solution was performed. Samples were analyzed with a FACScan flow cytometer (Becton Dickinson) using a blue-green excitation light wavelength (488 nm). During data acquisition with LYSYS II software (Becton Dickinson), only events positive for propidium iodide staining, and thus corresponding to human diploid cells, were considered. In analysis of the data, the overall phagocytic activity of the blood cells and that of individual cell populations (neutrophils and monocytes) was examined.

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

The lactobacilli were counted on MRS agar (Difco, Detroit) with antibiotics (phosphomycine, 0.8 g/L; sulfamethoxazole, 0.93 g/L; and trimethoprim, 50 g/kg) 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. Lactobacillus colonies were identified by cell morphology and the carbohydrate profile was determined by using API 50CHL (Bio Mérieux, Marcy L’Étoile, France). Thereby, total lactobacilli and, more specifically, the La1 colonies were assessed. Bifidobacteria were counted after identification by cell morphology and API 32A testing (Bio Mérieux).

Statistics

Data were analyzed by using analysis of variance for repeated measures. Ratios and differences in rates of phagocytosis of the different phagocyte populations in blood were compared by using analysis of covariance with repeated measures and Scheffé contrasts (16).

RESULTS

In vitro screening studies

The four human *L. acidophilus* strains examined differed in their ability to adhere to differentiated monolayers of Caco-2 cells (Table 1). *L. acidophilus* La1 strongly adhered to epithelial cells whereas La3 was weakly adherent. For both strains adhesion was mainly calcium independent. La10 and La18 strains were not adherent. The results clearly show the strain variation for adhesion capacity in this in vitro system. By scanning electron microscopy it was possible to observe a diffuse pattern of adhesion of La1 to Caco-2 monolayers (Figure 1).
TABLE 1
Adhesion of *Lactobacillus acidophilus* strains to human intestinal epithelial cells

<table>
<thead>
<tr>
<th>Lactobacillus strain</th>
<th>Number of lactobacilli adhering to 100 Caco-2 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without EGTA</td>
</tr>
<tr>
<td><em>L. acidophilus</em> L1</td>
<td>155 ± 26</td>
</tr>
<tr>
<td><em>L. acidophilus</em> L3</td>
<td>66 ± 21</td>
</tr>
<tr>
<td><em>L. acidophilus</em> L10</td>
<td>18 ± 3</td>
</tr>
<tr>
<td><em>L. acidophilus</em> L18</td>
<td>23 ± 7</td>
</tr>
</tbody>
</table>

"* x ± SE. EGTA, ethyleneglycotetraacetic acid.

The reduced adherence of L1 seen in fresh media or trypsin-treated supernate (data not shown) suggests that the spent supernate contains an extracellular proteinaceous component produced by the bacteria that plays a crucial role in the association of L1 to the enterocyte apical membrane.

In vivo human studies

No major differences were observed in proportions of blood lymphocyte subsets or degree of T cell activation in the two groups throughout the study. However, a significant increase in global phagocytic activity of blood phagocytes (granulocytes and monocytes) was observed in both groups after LAB ingestion: 38.9 ± 3.7% before LAB ingestion compared with 86.5 ± 1.4% after for group 1 and 46.3 ± 3.4% before compared with 84.4 ± 0.8% after for group 2 (*P < 0.0001*) (Table 2 and Figure 2). Six weeks after the end of consumption of fermented products, overall phagocytic activity decreased in both groups (group 1, *P = 0.001*; group 2, *P = 0.05*); the diminution was more evident in group 1, but final values were still considerably higher than those at time point 0. The granulocyte population was affected more by LAB ingestion than were monocytes; covariance analysis showed that phagocytic activity increased more in granulocytes after fermented milk consumption than in monocytes (*P < 0.004*). Nevertheless, the phagocytic activity of granulocytes (*P < 0.0001* for both groups between time points 1 and 2) mimicked that of blood phagocytes as a whole (data not shown).

Monocytes represented ~4–8% of the leukocytes in both groups. Ingestion of bifidobacteria and lactobacilli caused significantly increased phagocytic activity by monocytes (for both groups, *P < 0.001*) at time point 2 (data not shown); 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 is small.

FIGURE 1. Scanning electron micrograph of differentiated Caco-2 monolayers with adherent *Lactobacillus acidophilus* strain L1. Diffuse pattern of adhesion is shown.
**DISCUSSION**

The polarized human intestinal epithelial cell line Caco-2, which is a valuable model for the selection of adherent LAB strains (17), identified La1 as a highly adherent strain and showed the importance of the spent supernate in the adhesion process. Consequently, we chose the La1 strain to study immunomodulation by LAB in humans. Likewise, *B. bifidum* Bb12 was chosen on the basis of its high colonization capacity of human feces, even though a weak adhesion was seen in the in vitro system (D. Brassart, personal communication, 1992).

The in vivo human study supports the use of the two bacterial strains as probiotics because nonspecific phagocytic activity of peripheral blood cells was enhanced after ingestion of fermented milk products supplemented with either of the two selected LAB. The increment in overall phagocytic activity immediately after the 3-wk ingestion period started to decrease by the last time point, when fecal colonization returned to original values. But for both strains, the final activity was nevertheless higher than that at time point 0. Although both granulocytes and monocytes are the targets for this effect, the global enhancement in phagocytic activity was considered to be mainly due to the granulocyte population, which constitutes a greater proportion of the blood cells and exhibited a greater increment in phagocytic activity.

The host-bacteria interactions that mediate this immunomodulatory effect are not known. Presumably, LAB would first need to attain a minimal level of transient colonization in the gut. The correlation between fecal colonization and immunomodulatory capacity observed in this study certainly supports this assumption. However, how can a normally noninvasive bacteria given in quantities far inferior to that of the normal microflora influence systemic immunity? Perhaps the inoculum of LAB targets host cells at a level of the intestine that is not highly colonized. Possible targets are the Peyer’s patches of the gut-associated lymphoid tissue, the epithelium of which has a

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**B. bifidum** strain Bb12

**L. acidophilus** strain La1
proportion of specialized cells called M cells, which are more permeable to luminal components and as such encourage interaction with underlying immune cells. Indeed, increased antibacterial activity of Peyer’s patch lymphocytes has been described in mice after administration of yogurt containing live bacteria (18).

However, a bacterial signal could also act directly on epithelial cells or the intraepithelial lymphoid compartment of the proximal small intestine. In support of this concept, we observed previously (EJ Schiffin, unpublished data, 1992) that colonization of germ-free mice with live LAB cells, L. acidophilus strain La1, L. casei strain GG, or a hydrolysate of an L. bulgaricus culture, increased the proportion of intraepithelial cells in the proximal but not the distal small bowel. This lymphoid population, on activation, can produce cytokines, such as interferon γ and interleukin 2 (19, 20). Cytokines released from these lymphoid populations may then act locally or alter the systemic reactivity of the host. It is tempting to speculate that for some probiotic activities such as immunomodulation or competition with some pathogens the host target of LAB is localized in the proximal gastrointestinal tract, whereas for other pathogens, i.e., Shigella ssp., the target is localized in the colon.

LAB and their products can induce in vitro interferon γ production by human lymphocytes (3) and interferon α production by peritoneal macrophages (21). In addition, LAB products given orally can induce endogenous tumor necrosis factor α production in mice (22) and also enhance other macrophage and neutrophil functions. Moreover, LAB products can alter macrophage functions in vitro with a species-dependent pattern as shown by Hatcher and Lambrecht (23).

We provide direct evidence that oral administration of LAB to human volunteers alters the function of blood phagocytes. However, we could not detect differences between the two bacterial treatments. Both LAB strains were selected based on two different positive criteria for immunomodulation and the general pattern was similar in both experimental groups. Therefore, the results, although positive, suggest that the cellular events are more complex than originally thought. This effect may be mediated by the modulation of cell surface molecules involved in bacterial uptake by leukocytes such as β-2 integrin molecules (C3b receptor-Mac-1, LFA-1) or the Fcγ receptors (24, 25). Alternatively, a serum factor, such as an opsonin, may also explain the observed biological effect. Both possibilities need further investigation.

Administration of specially selected LAB strains could potentially correct immune defects seen in the elderly and neonates (26), whose lower numbers of polymorphonuclear cells have decreased phagocytic activity. Although the killing capacity of leukocytes was not examined in these studies, signals generated at the surface of the intestine or close to it probably enhance phagocytic activity of blood granulocytes without causing major alterations in host homeostasis. Thus, non-specific, antiinfective mechanisms of defense are improved without causing severe systemic responses such as those seen in the acute-phase response.

REFERENCES
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