Gut Mucosa Morphology and Microflora Changes in Malnourished Mice after Renutrition with Milk and Administration of *Lactobacillus casei*

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MS 98-332: Received 11 December 1998/Accepted 25 May 1999

**ABSTRACT**

Nutrition plays a key role in maintaining the balance of the intestinal microflora. Malnutrition disturbs the ecological barrier and induces histological damage. We evaluated modifications induced by renutrition with nonfat milk (NFM) and *Lactobacillus casei* administration (for 2 days) on the bacterial gut population and structural and ultrastructural gut modifications in malnourished mice. Balb/c mice suffering from a malnutrition process immediately after weaning (for 21 days) were divided into four groups and were given NFM for 0, 7, 14, and 21 days. Another group was treated in a similar way, but after different periods of NFM administration, mice in this group received *L. casei* for two consecutive days. All experimental animals were sacrificed by cervical dislocation, and both the microflora and the histological structure of the intestine were studied. In malnourished animals, a decrease in the numbers of *Lactobacillus* and anaerobic microorganisms was observed, whereas there was an increase in the number of *Enterobacteriaceae*. In animals treated with NFM and NFM plus *L. casei*, we could observe an important improvement on the microflora in the small and large intestines but no differences between both treatments. Structural and ultrastructural studies showed a slight improvement 7 days after treatment with NFM, and for 14 and 21 days after renutrition, the mice showed normal intestinal villi, whereas the additional feeding with *L. casei* for two consecutive days, after different periods of renutrition, yielded an earlier improvement (7 days).

One of the key functions of the intestine is to prevent lumen bacteria and endotoxins from reaching systemic organs and tissues. Failure of this intestinal barrier function results in the systemic spread of bacteria from the gut to systemic organs, a process that is called bacterial translocation. Protein malnutrition disrupts the normal ecology of the microflora (30), thus particularly affecting strict anaerobes (21), and it may disrupt the normal microflora, thereby producing overgrowth of certain members of the indigenous flora (1). Protein malnutrition also impairs host immune response and antibacterial defenses (7, 24), enhances susceptibility to infection (10), and leads to mucosal atrophy (25). These changes can alter the gut barrier function and induce bacterial translocation (32). It has previously been described that malnutrition is also associated with villous atrophy, abnormal mucin formation, and impairment in the secretion of secretory immunoglobulin A (28, 29).

During the renutrition process, both the composition of the diet and the administration route have a profound influence on intestinal morphology and function. Enteral nutrition seems to be superior to parenteral nutrition, and a high-protein enteral diet improves systemic immunity and reduces the incidence of infection (13, 16).

It has been described that the addition of bacterial supplements, such as selected lactic acid bacteria or fermented milk, to an enteral feeding formula would improve not only the nutritional state but also the intestinal microflora and immune system (4, 11, 19, 20), and such supplements can eliminate toxins (26) and assist in the regulation of mucus production.

In previous works (18), we demonstrated that yogurt or *Lactobacillus casei*, administered in a case involving malnutrition caused by severe protein deficiency, improved

**TABLE 1. Body weight in malnourished mice after different periods with NFM and NFM plus *L. casei***

<table>
<thead>
<tr>
<th>Experimental groupsb</th>
<th>Body weightc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>24.9 ± 1.9</td>
</tr>
<tr>
<td>Malnourished</td>
<td>14.5 ± 2.0</td>
</tr>
<tr>
<td>Malnourished + Lc</td>
<td>13.2 ± 1.9</td>
</tr>
<tr>
<td>Malnourished + 7NFM</td>
<td>17.2 ± 1.2</td>
</tr>
<tr>
<td>Malnourished + 7NFM + Lc</td>
<td>17.4 ± 1.6</td>
</tr>
<tr>
<td>Malnourished + 14NFM</td>
<td>20.8 ± 1.5</td>
</tr>
<tr>
<td>Malnourished + 14NFM + Lc</td>
<td>21.3 ± 1.3</td>
</tr>
<tr>
<td>Malnourished + 21NFM</td>
<td>22.5 ± 1.1</td>
</tr>
<tr>
<td>Malnourished + 21NFM + Lc</td>
<td>23.5 ± 1.3</td>
</tr>
</tbody>
</table>

a Animals were treated as was explained in the text.
b NFM, nonfat milk; Lc, *L. casei.*
c Results are expressed as the mean ± SD of determinations. *n = 15* mice (per group).

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several immunological parameters, such as peritoneal macrophage activity and the number of immunoglobulin A-secreting cells on lamina propria of the small intestine. However, we did not observe any improvement in the normal microflora, and the translocation process could not be avoided.

The aim of the present work was to study the effects of the addition of L. casei, as an oral adjuvant to a renutrition diet, on the microbacterial translocation and the ultrastructural modification of the epithelial cells in malnourished mice.

MATERIALS AND METHODS

Animal model. BALB/c mice from a closed colony were malnourished, using a protein-free diet supplemented with vitamins (2.2%), minerals (4%), and essential fatty acids (5%), for 21 days. This process was begun immediately after weaning. The animals that had lost about 40% of their body weight (with respect to normal controls) were selected for the experiment. The normal control received a commercial diet. Each experimental group consisted of five to six mice per assay.

Renutrition diet. At the end of the malnutrition period, a renutrition process was begun by administering nonfat milk (NFM; 10%) for 0, 7, 14, and 21 days.

L. casei administration. After each renutrition period, the mice were fed with L. casei subsp. casei, which was obtained from the Centro de Referencia para Lactobacilos culture collection. The supplement was administered for two consecutive days at a concentration of 10^8 cells/day/mouse; the concentration was suspended in 5 ml of sterile NFM and administered in 150 ml of drinking water. During this period, the mice continued on a protein-free diet without NFM administration. L. casei was identically administered to malnourished and well-nourished controls.

Intestinal microflora and translocation studies. These studies were carried out on all experimental groups. At the end of the different feeding periods, the animals were sacrificed by cervical dislocation, and the small intestine, the large intestine,

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Lactobacillus</th>
<th>Gram-negative bacilli</th>
<th>Gram-positive cocci</th>
<th>Anaerobic microorganisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>8.8 ± 0.8</td>
<td>4.4 ± 0.6</td>
<td>1.1 ± 0.7</td>
<td>4.0 ± 0.4</td>
</tr>
<tr>
<td>Malnourished</td>
<td>5.5 ± 0.7</td>
<td>6.1 ± 0.5</td>
<td>3.4 ± 0.5</td>
<td>1.4 ± 0.4</td>
</tr>
<tr>
<td>Malnourished + Lc</td>
<td>5.2 ± 0.9</td>
<td>5.4 ± 0.7</td>
<td>0.2 ± 0.1^d</td>
<td>2.7 ± 0.4^c</td>
</tr>
<tr>
<td>Malnourished + 7NFM</td>
<td>8.2 ± 0.4^e</td>
<td>6.3 ± 0.5</td>
<td>3.3 ± 0.4</td>
<td>3.5 ± 0.7^c</td>
</tr>
<tr>
<td>Malnourished + 7NFM + Lc</td>
<td>5.3 ± 0.5</td>
<td>3.3 ± 0.4</td>
<td>0.7 ± 0.1^d</td>
<td>3.0 ± 0.5^c</td>
</tr>
<tr>
<td>Malnourished + 14NFM</td>
<td>8.3 ± 0.6^e</td>
<td>4.8 ± 0.5</td>
<td>2.6 ± 0.2^d</td>
<td>3.4 ± 0.4^c</td>
</tr>
<tr>
<td>Malnourished + 14NFM + Lc</td>
<td>8.6 ± 0.5^e</td>
<td>4.7 ± 0.6</td>
<td>2.2 ± 0.2^d</td>
<td>3.6 ± 0.3^c</td>
</tr>
<tr>
<td>Malnourished + 21NFM</td>
<td>7.5 ± 0.3^e</td>
<td>4.6 ± 0.3</td>
<td>2.0 ± 0.3^d</td>
<td>3.2 ± 0.3^c</td>
</tr>
<tr>
<td>Malnourished + 21NFM + Lc</td>
<td>8.7 ± 0.5^e</td>
<td>4.4 ± 0.4</td>
<td>1.2 ± 0.1^d</td>
<td>3.8 ± 0.4^c</td>
</tr>
</tbody>
</table>

^a Animals were sacrificed at 0, 7, 14, and 21 days postrenutrition. The samples were plated onto selective media and incubated under aerobic and anaerobic conditions. NFM, nonfat milk; Lc, L. casei.

^b Values measure log_{10} CFU viable bacteria of mice; n = 5 (per group). Values given as mean ± SD of determinations.

^c Significant increase (P < 0.01) compared with malnourished control.

^d Significant decrease (P < 0.01) compared with malnourished control.

TABLE 3. Microbial population from large intestine^a

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Lactobacillus</th>
<th>Gram-negative bacilli</th>
<th>Gram-positive cocci</th>
<th>Anaerobic microorganisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>8.4 ± 0.6</td>
<td>5.7 ± 0.8</td>
<td>2.3 ± 0.3</td>
<td>9.5 ± 0.6</td>
</tr>
<tr>
<td>Malnourished</td>
<td>6.7 ± 0.5</td>
<td>6.1 ± 0.7</td>
<td>5.0 ± 0.7</td>
<td>4.8 ± 0.3</td>
</tr>
<tr>
<td>Malnourished + Lc</td>
<td>6.2 ± 0.7</td>
<td>6.8 ± 0.5</td>
<td>0.6 ± 0.1^d</td>
<td>8.4 ± 0.6^c</td>
</tr>
<tr>
<td>Malnourished + 7NFM</td>
<td>8.6 ± 0.6</td>
<td>7.1 ± 0.6</td>
<td>1.0 ± 0.1^d</td>
<td>4.3 ± 0.2</td>
</tr>
<tr>
<td>Malnourished + 7NFM + Lc</td>
<td>7.7 ± 0.6</td>
<td>7.1 ± 0.5</td>
<td>0.8 ± 0.0^d</td>
<td>8.2 ± 0.7^c</td>
</tr>
<tr>
<td>Malnourished + 14NFM</td>
<td>8.5 ± 0.7^e</td>
<td>6.6 ± 0.5</td>
<td>2.9 ± 0.3^d</td>
<td>5.9 ± 0.3^c</td>
</tr>
<tr>
<td>Malnourished + 14NFM + Lc</td>
<td>8.2 ± 0.6^e</td>
<td>6.5 ± 0.6</td>
<td>1.6 ± 0.1^d</td>
<td>8.7 ± 0.6^c</td>
</tr>
<tr>
<td>Malnourished + 21NFM</td>
<td>8.0 ± 0.7^e</td>
<td>7.6 ± 0.5</td>
<td>1.6 ± 0.1^d</td>
<td>7.3 ± 0.3^c</td>
</tr>
<tr>
<td>Malnourished + 21NFM + Lc</td>
<td>8.3 ± 0.7^e</td>
<td>5.7 ± 0.6</td>
<td>2.0 ± 0.1^d</td>
<td>8.4 ± 0.4^c</td>
</tr>
</tbody>
</table>

^a Animals were sacrificed at 0, 7, 14, and 21 days postrenutrition. The samples were plated onto selective media and incubated under aerobic and anaerobic conditions. NFM, nonfat milk; Lc, L. casei.

^b Values measure log_{10} CFU viable bacteria of mice; n = 5 (per group). Values given as mean ± SD of determinations.

^c Significant increase (P < 0.01) compared with malnourished control.

^d Significant decrease (P < 0.01) compared with malnourished control.
the spleen, and the liver were removed aseptically. The intestinal contents from both the small and large intestines were homogenized in 5 ml of peptone water, and serial dilutions from 1/10 to 10\(^{10}\) were made. The spleen and liver were treated similarly for translocation studies. All dilutions were plated onto brain–heart infusion supplemented with blood, cysteine, hemin, and kanamycin for analysis of anaerobic microorganisms (23); LAPTg (Bacto tryptone, Bacto yeast extract, glucose, peptone, and salts) (Difco, Buenos Aires, Argentina) and brain–heart infusion for analysis of aerobic microorganisms; Lactobacilli selective for analysis of lactobacilli; mannitol (Difco) for analysis of staphylococci; and MacConkey (Difco) for analysis of enterobacteria. Plates were incubated under aerobic and anaerobic conditions (Gas Pack System [Ersvne, Buenos Aires, Argentina]) at 37°C for 48 h (23). At the end of the incubation period, the bacteria were counted, and the results were expressed as the logarithm of the number of bacteria per gram of content. Translocation was determined to be either positive or negative.

**Histological preparation.** Samples from the ileum near Pey er’s patches were taken from animals of different experimental groups for histological studies. The samples were stained with Mallory dye (15). This staining technique gives collagen a dark blue color, and slime, amyloid, and other hyaline tissues are colored light blue. The nucleus, cytoplasm, elastic fibers, and fibrin stain red.

**Electronic microscopy.** After different feeding periods, mice from experimental and control groups were sacrificed. The small intestine of each mouse was carefully removed for ultrastructural studies by transmission and scanning electron microscopy, described previously by Aguero et al. (1).

**Statistical analysis.** Results were expressed as the mean of five or six samples plus or minus standard deviation (Student’s t test). The data were analyzed by analysis of variance of repeated measurements followed by a multiple-comparison test (17, 33).

**RESULTS**

**Determination of the weight increase during administration of renutrition diet.** The results (Table 1) show a significant increase \((P < .005)\) in body weight in malnourished animals that were fed with NFM compared with malnourished controls. After 21 days, the body weight of malnourished animals was near that of the normal controls. Supplementation with *L. casei* following different renutri-
tion periods did not increase body weight in malnourished animals treated only with NFM.

**Quantification and identification of the microbial population in the intestine and determination of bacterial translocation.** A modification in the microflora from the small and large intestines could be observed after malnutrition (Tables 2 and 3). The process induced a significant decrease in the number of lactobacillus and anaerobic microorganisms and an increase in the number of gram-negative bacilli, with an enhancement in the *Escherichia coli* and *Klebsiella* populations in both the small and the large intestine. Fourteen days of treatment with NFM induced an increase in anaerobic microorganisms and lactobacilli and more diversity of genera among gram-negative bacilli. The experimental groups supplemented with *L. casei* showed values that were near those of normal controls.

Milk renutrition and *L. casei* addition induced a significant decrease in the number of gram-positive cocci compared with malnourished controls in almost all experimental groups. Translocation was observed in malnourished mice but was not observed following refeeding.

**Histological studies of the small intestine.** After malnutrition, an important reduction in the size of microvilli, an increase in the number of microvilli per area of villi, and an alteration of the microvilli were detected by scanning (Figs. 1b and 2b) and transmission (Figs. 1a and 2a) electron microscopy. Using transmission electron microscopy, a slight recovery of intestinal microvilli was found in animals treated with NFM for 7 days. However, these animals showed cytoplasmic edema, which induces dispersion of the cisternum from the endoplasmic reticulum and mitochondria (Fig. 3a). Animals whose diet had been supplemented with *L. casei* did not show these effects at that period of treatment (Fig. 4a). The coexistence of morphologically normal villi and hypertrophic villi was observed with scanning electron microscopy (Figs. 3b and 4b).

After 14 and 21 days of treatment with NFM, microvilli were more uniform, and a prominent rugose endoplas-
mic reticulum was found. It showed extended cisternum, which suggested an important synthesis of protein (Figs. 5a and 7a). No differences in the number and morphology of villi were found using scanning electron microscopy (Figs. 5b and 7b). Animals whose diet had been supplemented with *L. casei* after 14 and 21 days of treatment with NFM showed an earlier improvement (Figs. 6a, 6b, 8a, and 8b).

The increased activity of goblet cells that was attributable to protein stimulus was detected by light microscopy, using Mallory stain, in all periods assayed (Figs. 9 through 12).

**DISCUSSION**

Malnutrition induces a prolonged alteration of the small intestine microflora. The loss of nutrients that are essential for epithelial integrity may affect the function of the gastrointestinal mucosa (12, 14). Other authors have observed a decrease in mucous secretion and an induction of bacterial translocation (2, 6). It has been demonstrated that enteral nutrition has several advantages over parenteral nutrition in terms of the recovery of the gastrointestinal mucosa functions, and therefore, enteral nutrition is now widely used (1, 16).

Milk and milk products represent important sources of dietary proteins for humans, and these proteins are known to serve as a source of biologically active peptides. The interaction of the peptides with the intestinal mucosa is important both in terms of the peptides’ potential effects on the physiology of the mucosa and in terms of mucosa transport functions. The targets for milk-derivative peptides in the intestinal mucosa are mainly located on epithelial cells (8). Nutritional rehabilitation with a milk formula supplemented with proteins, minerals, and multivitamins speeds up weight gain (31).

In our malnourished experimental model, renutrition was effected using bovine milk at different periods in time. An enhancement in body weight, which was not modified by *L. casei* addition, could be observed (Table 1). However,
it has been described that lactic acid bacteria are able to increase the weight up to that of a well-nourished host when they are added to the diet (27).

Establishment of the intestinal flora occurs in a sequential way and is characteristic for each animal species. Under healthy conditions, this microflora is stable and prevents exogenous bacterial colonization with newly ingested bacterial species (22). Under conditions of malnutrition, this flora is severely affected, and bacterial translocation can be induced (9). Bacterial translocation observed in malnourished mice was stopped with milk feeding, and *L. casei* did not impair the effect that milk had on bacterial translocation. However, supplementation of the diet with probiotic bacteria is not always beneficial to the host if these substances are administered before adequate renutrition occurs (5, 7–9).

In our study, we demonstrated that milk renutrition can restore the intestinal flora and that *L. casei* supplementation enhances these effects (Tables 2 and 3). Malnutrition also produces villous atrophy and damage of the gut barrier function and induces an increase in intestinal permeability (3). When analyzing the effects of milk renutrition on the histological structures of the small intestine, an improvement in the length of the villi—during the different periods of milk renutrition—was observed. There was also an enhancement in goblet cell activity, and this effect was even more remarkable when *L. casei* was added (Figs. 5 through 8). The effects in our study were similar to those demonstrated with dietary fibers, which reduce the deleterious effects of malnutrition and bacterial translocation (3).

Ultrastructural studies through transmission and scanning electron microscopy showed that milk renutrition for 7 days improved the disorganized brush border and enterocyte activity, whereas *L. casei* induced a faster recovery of microvilli and epithelium cells.

We demonstrated the importance of the addition of lactic acid bacteria, such as *L. casei*, in terms of the gastrointestinal function in malnutrition. We also believe that *L. casei* could be a good mucosal adjuvant in this process, taking into account the impaired gut barrier functions that
are closely associated with abnormal gastrointestinal mucosal immunology. Bearing in mind that malnutrition produces great alterations in the mucosal immune responses and that enterocytes play an important role in the cytokine production (to evoke an immune response), the quick recovery of these cells could contribute to the enhancement of the immunity and improvement of intestinal permeability.

ACKNOWLEDGMENTS

This work was supported by a grant from Consejo Nacional de Investigaciones Científicas y Técnicas (Argentina, grant no. PIP 5011) and a grant from Consejo de Investigaciones—Universidad Nacional de Tucumán, Tucumán University (Argentina, grant no. DO 64/96).

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