Increased Enterocyte Production in Gnotobiotic Rats
Mono-Associated with Lactobacillus rhamnosus GG

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There is increasing scientific and commercial interest in using beneficial microorganisms (i.e., probiotics) to enhance intestinal health. Of the numerous microbial strains examined, Lactobacillus rhamnosus GG has been most extensively studied. Daily intake of L. rhamnosus GG shortens the course of rotavirus infection by mechanisms that have not been fully elucidated. Comparative studies with germfree and conventional rats have shown that the microbial status of an animal influences the intestinal cell kinetics and morphology. The present study was undertaken to study whether establishment of L. rhamnosus GG as a mono-associate in germfree rats influences intestinal cell kinetics and morphology. L. rhamnosus GG was easily established as a mono-associate in germfree rats. After 3 days of mono-association, the rate of mitoses in the upper part of the small intestine (jejenum 1) increased as much as 14 and 22% compared to the rates in germfree and conventional counterparts, respectively. The most striking alteration in morphology was an increase in the number of cells in the villi. We hypothesis that the compartmentalized effects of L. rhamnosus GG may represent a reparative event for the mucosa.

It is generally accepted that use of a probiotic, defined by Fuller as “a live microbial feed supplement which beneficially effects the host animal by improving its microbial balance” (6), is an interesting approach for prevention and treatment of some infectious intestinal diseases. One of the numerous strains on the market, Lactobacillus rhamnosus GG, has been extensively studied, especially for treatment of rotavirus diarrhea (9, 14, 22).

Acute gastroenteritis caused by rotavirus is a type of diarrhea that occurs worldwide and is primarily found in children 6 to 24 months old. Rotavirus diarrhea is the main cause of childhood death in developing countries, resulting in to close to 1 million deaths per year (7). In developed countries, a fatal outcome is rarely seen. However, many children have to be hospitalized; in the United States as many as 55,000 to 70,000 children per year have to be hospitalized (13).

Although much is known about rotavirus replication and growth conditions, the pathophysiological mechanism(s) behind rotavirus infection remains unclear. This infection is primarily an infection of the small intestine, especially the jejunum (3, 21). Most recently, a protein (NSP4) of rotavirus has been described as the first viral enterotoxin (23). In children, viral excretion in feces is maximal during the first 4 days of illness (3). Virus can be detected in mucosal homogenates from day 2 through day 8 in germfree (GF) mice exposed to rotavirus but persists for up to 13 days in conventional (Conv) mice (12). In humans as well as in animals, the virus disappears from the small bowel by a mechanism(s) not known yet.

Several mechanisms have been proposed to explain the therapeutic effect(s) of oral ingestion of L. rhamnosus GG during rotavirus infection. The most attention has been paid to stimulation of the immune response by L. rhamnosus GG, especially during production of immunoglobulin A (15, 17). Whatever the mechanism(s) is, administration of L. rhamnosus GG is associated

### Table 1. Effect of L. rhamnosus GG on the mitotic index in the intestines of young male rats

<table>
<thead>
<tr>
<th>Rats</th>
<th>Mitotic index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Duodenum</td>
</tr>
<tr>
<td>Control GF</td>
<td>27.3 ± 2.4</td>
</tr>
<tr>
<td>L. rhamnosus GG, 3 days</td>
<td>29.3 ± 2.7</td>
</tr>
<tr>
<td>L. rhamnosus GG, 21 days</td>
<td>28.0 ± 2.9</td>
</tr>
<tr>
<td>Control Conv</td>
<td>27.5 ± 2.4</td>
</tr>
</tbody>
</table>

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ab Value significantly different from the value for GF rats.
ab Value significantly different from the value for Conv rats.
with a significant reduction in fecal viral excretion (8, 9). These findings might have some relevance in light of the major role of rotavirus in diarrhea in day care centers (11). Another possible mechanism, not previously suggested, is that oral ingestion of *L. rhamnosus* GG could have a stimulatory effect on production of enterocytes, thereby causing wash-out of virus.

Recently, by performing comparative studies with GF and Conv rats and mice, we found that age, gender, and microbial status influence intestinal cell kinetics and morphology in a compartmentalized manner (1, 2). Reviewing previous investigations, we realized that little attention had been paid to standardization of these variables. Additionally, we are not aware of any previous publication describing intestinal cell kinetics in rats or mice mono-associated with probiotic microbes. The aim of the present study was to investigate the influence of *L. rhamnosus* GG on mucosal cell kinetics and morphology in different regions of the intestine of young male rats mono-associated with bacteria.

### MATERIALS AND METHODS

**Animals.** Ten young GF AGUS male rats (5), reared and maintained in a stainless steel isolators (10, 18), were divided into two groups of five. The rats were mono-associated in one isolator with *L. rhamnosus* GG for 3 or 21 days. Two groups of rats of the same age (35 days) (six GF rats and five Conv rats) were used as controls. The AGUS strain is our main rat strain, and it has been kept GF and inbred for close to 90 generations. In order to avoid genetic drift, the rats were mono-associated in one isolator with *L. rhamnosus* GG, 21 days 30.3 Control

**Mono-association.** The animals were mono-associated by using procedures established in the laboratory. Briefly, *L. rhamnosus* GG (ATCC 53503; Valio Ltd., Helsinki, Finland) was grown in MRS (de Man-Rogosa-Sharpe) medium (Merck, Darmstadt, Germany) anaerobically at 37°C for 24 h. Then aliquots (10 ml) were dispensed into sterile ampoules, which were heat sealed. The external surfaces were sterilized with chromosulfuric acid, and then the ampoules were transferred into the isolator. Inside the isolator, the ampoules were broken, and 0.5 to 1 ml (10⁹ CFU/ml) of the contents was given orally to each animal; the remaining contents were spread on the fur and bedding material.

**Protocol.** The rats were kept mono-associated for 3 or 21 days. On the day of the experiment, food was withdrawn in the morning; 2.5 h later, the cells were blocked in the metaphase by a vincristine injection (1 mg/kg; Oncovin; Lilly S.A., Fegersheim, France) given intraperitoneally (24, 25). All rats were subjected to laparotomy following intraperitoneal injection of mebumal (25 mg/kg) exactly 4 h after the vincristine injection. To verify that bacteria became established, samples of the contents of the small intestine, cecum, and colon were diluted in MRS broth, incubated anaerobically at 37°C, and inspected daily for growth for 3 days.

**Preparation of specimens.** Specimens (2 cm) were taken from the duodenum, from the jejunum 15 and 40 cm distal to the pyloric region (Jej-land Jej-2, respectively), and from ileum 5 cm proximal to the ileo-cecal junction. Samples (1 cm) were also taken from the cecum at its base and from the colon 2 to 3, 5, 6, and 8 to 9 cm from the cecum (C-1, C-2, and C-3, respectively). Each specimen was placed on a microprobe filter (pore size, 0.2 µm; Schleicher & Schuell, Dassel, Germany) and cut open along its longitudinal axis to obtain access to crypts and villi. The specimens were fixed for 3 h in Carnoy’s solution (60% methanol, 30% chloroform, 10% acetic acid) and for 20 h in 70% ethanol and embedded in paraffin. A number of six 3-µm-thick sections were cut 100 µm from each other and stained with hematoxylin and eosin. All specimens were coded and examined in a blind fashion with a light microscope (magnification, ×200; Leica DM I.S. Wetzlar, Germany).

**Mitotic index.** The total numbers of mitotic cells and cell nuclei were counted in the left columns of 30 well-oriented crypts in all sections, and the mitotic index was calculated with the following formula: MI = N_m/N_c × 100, where N_m is the number of mitotic cells and N_c is the total number of cells in the left column of the crypt.

**Number of epithelial cells.** The number of cells in the left columns of 30 well-oriented small intestine and colonic crypts and in the villi of the small intestine were counted.

**Depth of crypts and height of villi.** In the small intestine the depths of 20 well-oriented crypts were measured from the base of the crypt to the crypt-villus junction, and the heights of 20 villi were measured from the crypt-villus junction to the tip of the villus by using a micrometer in the ocular eyepiece (magnification, ×100). In a similar way, the depths of colonic crypts were measured from the base of the crypt to the flat margin of the colonic mucosa.

**Statistical analysis.** Results are expressed as means ± standard deviations. The one-way analysis of variance test was used to evaluate the differences between groups. The significance level used was *P* < 0.05.

### RESULTS

The animals remained healthy throughout the study. In all rats mono-associated with bacteria, *L. rhamnosus* GG was present in the luminal contents at a concentration of at least 10⁷ CFU/g of contents (data not shown).

The microscopic evaluations created a vast amount of data; therefore, only significant results are commented upon below.

**Mitotic index for rats mono-associated with *L. rhamnosus* GG compared with mitotic indices for GF and Conv rats.** After 3 days of mono-association, the mitotic activity in the jejunum was increased compared with the activity in GF rats, and after 21 days, the mitotic activity in Jej-1 and the cecum was increased compared with the activity in GF rats (Table 1).

After 3 days of mono-association, the mitotic activity in the jejunum and ileum was increased compared with the activity in Conv rats, and after 21 days, the mitotic activity in Jej-1 and the cecum was increased compared with the activity in Conv rats.

**Number of cells in the crypts of rats mono-associated with *L. rhamnosus* GG compared with the numbers of cells in the crypts of GF and Conv rats.** After 3 days of mono-association, the number of crypt cells in the large intestine was increased com-

### TABLE 2. Effect of *L. rhamnosus* GG on the total number of crypt cells and the depth of the crypt in young male rats

<table>
<thead>
<tr>
<th>Rats</th>
<th>Duodenum</th>
<th>Jejunum (15 cm)</th>
<th>Jejunum (40 cm)</th>
<th>Ileum</th>
<th>Cecum</th>
<th>Colon (2-3 cm)</th>
<th>Colon (5-6 cm)</th>
<th>Colon (8-9 cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control GF</td>
<td>25.6 ± 2.5</td>
<td>21.0 ± 0.8</td>
<td>20.9 ± 1.6</td>
<td>20.1 ± 0.9</td>
<td>24.2 ± 3.5</td>
<td>22.2 ± 3.6</td>
<td>25.0 ± 2.6</td>
<td>23.3 ± 2.3</td>
</tr>
<tr>
<td><em>L. rhamnosus</em> GG, 3 days</td>
<td>28.9 ± 1.9</td>
<td>22.6 ± 2.1</td>
<td>23.7 ± 3.1</td>
<td>22.3 ± 1.6</td>
<td>33.5 ± 3.1ab</td>
<td>26.9 ± 2.2a</td>
<td>29.4 ± 2.4a</td>
<td>29.3 ± 3.6a</td>
</tr>
<tr>
<td><em>L. rhamnosus</em> GG, 21 days</td>
<td>30.3 ± 2.2ab</td>
<td>23.3 ± 1.7ab</td>
<td>23.0 ± 0.8ab</td>
<td>22.1 ± 1.0ab</td>
<td>36.7 ± 2.0ab</td>
<td>25.3 ± 1.3a</td>
<td>30.5 ± 3.8ab</td>
<td>29.3 ± 2.1ab</td>
</tr>
<tr>
<td>Control Conv</td>
<td>26.5 ± 1.0</td>
<td>21.7 ± 1.3</td>
<td>22.0 ± 1.8</td>
<td>20.3 ± 1.1</td>
<td>28.7 ± 2.4</td>
<td>24.5 ± 2.6</td>
<td>27.4 ± 3.3</td>
<td>28.5 ± 1.5</td>
</tr>
</tbody>
</table>

*Value significantly different from the value for GF rats.*

*Value significantly different from the value for Conv rats.*

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**TABLE 2.** Effect of *L. rhamnosus* GG on the total number of crypt cells and the depth of the crypt in young male rats

**GF on the total number of crypt cells and the depth of the crypt in young male rats**
pared with the number in GF rats, and after 21 days, the numbers of crypt cells in all intestinal compartments (except C-1) were increased compared with the numbers in GF rats (Table 2).

After 3 days of mono-association, the number of crypt cells in the cecum was increased compared with the number in Conv rats, and after 21 days, the numbers of crypt cells in all intestinal compartments (except C-1) were increased compared with the number in GF rats, and after 21 days, the crypts in the duodenum, jejunum, ileum, and cecum were increased compared with the numbers in Conv rats.

**Depth of the crypts in rats mono-associated with L. rhamnosus GG compared with the depths of the crypts in GF and Conv rats.** After 3 days of mono-association, the crypts in the small intestine and cecum were shorter in the rats mono-associated with *L. rhamnosus* than in GF rats, and after 21 days, the crypts in the jejunum were shorter in the rats mono-associated with *L. rhamnosus* than in GF rats. However, after 21 days, the crypts in the cecum and C-2 were deeper in the rats mono-associated with *L. rhamnosus* GG than in the GF rats (Table 2).

After 3 days of mono-association, the crypts in the duodenum, ileum, cecum, C-1, and C-2 were shorter in the rats mono-associated with *L. rhamnosus* than in Conv rats, and after 21 days, the crypts in the duodenum, Jej-1, and C-1 were shorter in the rats mono-associated with *L. rhamnosus* than in Conv rats.

**Number of villus cells in rats mono-associated with L. rhamnosus GG compared with the numbers of villus cells in GF and Conv rats.** After 3 and 21 days of mono-association, the number of villus cells in the small intestine was increased compared with the number of villus cells in GF rats (Table 3).

After 3 and 21 days of mono-association, the number of villus cells in the small intestine was also increased compared with the number of villus cells in Conv rats.

**Height of villus in rats mono-associated with L. rhamnosus GG compared with height of villus in GF and Conv rats.** After 3 days of mono-association, the villi were taller in the duodenum and jejenum but shorter in the ileum (Table 3). After 21 days of mono-association, the villi in the duodenum and jejunum were taller in rats mono-associated with *L. rhamnosus* GG than in Conv rats.

**DISCUSSION**

Our results demonstrate that *L. rhamnosus* GG is easily established as a mono-associate in GF rats. Establishment did not cause any discomfort in the animals. The results indicate that *L. rhamnosus* GG had a compartmentalized effect upon rat intestinal mucosa. After 3 days of mono-association, the mitotic index for the upper part of the jejunum increased 14 and 22% compared to data obtained for the GF and Conv rats, respectively. A similar increase in cell production has been observed in GF rats exposed to a Conv flora for 3 days (26). As is shown in Table 1, young male GF rats express higher jejunal mitotic activity than Conv rats in that region of the intestine, for reasons thought to be due to increased dietary intake (1). It is tempting to speculate that differences in the mitotic indices between GF and Conv animals may partially account for the differences in the intestinal persistence of rotavirus in such animals (12). The increased number of crypt cells was not followed by deeper crypts or taller villi, indicating that there was a reduction in individual cell size. In fact, both the villi and the crypts tended to be shorter, especially after 3 days of mono-association. Most probably, the reduction in cell size is a cophenomenon of increased cell production.

Several mechanisms may contribute to these effects. Utilizing a human colonic adenocarcinoma cell line (IEC-6), Olaya et al. showed that components of the cell wall of *Escherichia coli* and the cytosol of *Lactobacillus acidophilus* are sources of epithelial cell mitogens (19). Members of the same group presented data linking this mitogenic effect(s) to the presence of lipopolysaccharides in gram-negative bacteria and lipotheoid acid/peptidoglycan in a gram-positive organism (20). In a recent study, Kitazawa

### TABLE 2—Continued

<table>
<thead>
<tr>
<th>Rats</th>
<th>Duodenum</th>
<th>Jejunum (15 cm)</th>
<th>Jejunum (40 cm)</th>
<th>Ileum</th>
<th>Cecum</th>
<th>Colon (2-3 cm)</th>
<th>Colon (5-6 cm)</th>
<th>Colon (8-9 cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crypt depth (µm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>126.9 ± 8.2</td>
<td>95.4 ± 9.6</td>
<td>96.0 ± 11.6</td>
<td>85.7 ± 9.7</td>
<td>135.3 ± 4.1</td>
<td>124.8 ± 12.4</td>
<td>134.8 ± 10.5</td>
<td>134.9 ± 8.9</td>
<td></td>
</tr>
<tr>
<td>105.0 ± 13.3a</td>
<td>79.3 ± 10.7b</td>
<td>76.7 ± 11.0b</td>
<td>71.1 ± 11.0b</td>
<td>121.3 ± 10.7b</td>
<td>115.4 ± 12.3b</td>
<td>132.7 ± 18.6b</td>
<td>131.0 ± 28.4</td>
<td></td>
</tr>
<tr>
<td>123.0 ± 16.1b</td>
<td>81.0 ± 4.8c</td>
<td>80.6 ± 8.6c</td>
<td>76.5 ± 9.4</td>
<td>145.8 ± 7.7c</td>
<td>110.2 ± 19.5b</td>
<td>163.1 ± 22.4c</td>
<td>134.8 ± 24.1</td>
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</tr>
<tr>
<td>143.2 ± 8.1</td>
<td>89.9 ± 5.7d</td>
<td>84.4 ± 7.4</td>
<td>86.4 ± 4.7</td>
<td>151.6 ± 21.8</td>
<td>148.8 ± 19.0</td>
<td>167.2 ± 20.6</td>
<td>157.4 ± 24.9</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Rats</th>
<th>No. of villus cells</th>
<th>Height of villus (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Duodenum</td>
<td>Jejunum (15 cm)</td>
</tr>
<tr>
<td>Control GF</td>
<td>75.4 ± 0.6</td>
<td>63.0 ± 3.0</td>
</tr>
<tr>
<td><em>L. rhamnosus</em> GG, 3 days</td>
<td>100.1 ± 7.0ab</td>
<td>79.7 ± 4.7a</td>
</tr>
<tr>
<td><em>L. rhamnosus</em> GG, 21 days</td>
<td>101.4 ± 6.7ab</td>
<td>84.2 ± 6.8ab</td>
</tr>
<tr>
<td>Control Conv</td>
<td>68.0 ± 4.8</td>
<td>56.7 ± 4.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Rats</th>
<th>Duodenum</th>
<th>Jejunum (15 cm)</th>
<th>Jejunum (40 cm)</th>
<th>Ileum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control GF</td>
<td>75.4 ± 0.6</td>
<td>63.0 ± 3.0</td>
<td>55.2 ± 2.8</td>
<td>38.4 ± 2.4</td>
</tr>
<tr>
<td><em>L. rhamnosus</em> GG, 3 days</td>
<td>100.1 ± 7.0ab</td>
<td>79.7 ± 4.7a</td>
<td>68.7 ± 8.0ab</td>
<td>47.3 ± 3.3ab</td>
</tr>
<tr>
<td><em>L. rhamnosus</em> GG, 21 days</td>
<td>101.4 ± 6.7ab</td>
<td>84.2 ± 6.8ab</td>
<td>74.8 ± 1.2ab</td>
<td>50.5 ± 2.1ab</td>
</tr>
<tr>
<td>Control Conv</td>
<td>68.0 ± 4.8</td>
<td>56.7 ± 4.4</td>
<td>50.4 ± 3.3</td>
<td>41.3 ± 4.6</td>
</tr>
</tbody>
</table>

* Value significantly different from the value for GF rats.

ab Value significantly different from the value for Conv rats.
et al. (16) showed that chromosomal DNA, purified from 12 of 16 strains of *L. acidophilus*, induced proliferation of splenic lymphocytes. The nucleotide sequences, consisting of only A and T nucleotides, were characterized as B-lymphocyte-specific mitogen (16). However, no experiments were performed on enterocytes. It seems reasonable to assume that the enterocytes in our rats mono-associated with bacteria were exposed to a large amount of lipothiocoid acid/peptidoglycan and probably also to oligonucleotides similar to those present in *L. acidophilus*. Whether and to what extent these three substances may act alone or in concert as a driving force(s) for increased mitogenic activity in enterocytes are still not known.

Whatever the mechanism is, the mitogenic effect of *L. rhamnosus* GG found in the present study may have a beneficial effect(s) in some clinical situations. Increased cell production contributes to enhanced mucosal regeneration. In addition to this reparative effect, the increased cell production could act as a wash-out mechanism for pathogenic microbial agents. Thus, use of *L. rhamnosus* GG in the treatment of a rotavirus diarrhea may increase cell production in the jejunum, leading to wash-out of infected cells, thereby shortening the period of fecal excretion of the virus. Previous investigations have also shown that *L. rhamnosus* GG reduces the severity of rotavirus infections (8, 9). The wash-out function related to the presence of *L. rhamnosus* GG may also be involved in this reduction of diarrhea symptoms.

In the present study, the observation period was limited to 3 weeks. Consequently, extrapolation of our results to more long-lasting or chronic inflammatory bowel conditions has to be made with the greatest care. If the mitogenic effect(s) of *L. rhamnosus* GG lasts for extended periods of time, it seems reasonable to assume that there is a reparative effect on more chronic conditions as well. The chronic inflammatory factors might be microbial pathogens or chemical agents. Experimentally, ex-GF rats challenged with acute or chronic proinflammatory agents provide excellent models for testing possible prophylactic and/or therapeutic effects of probiotics.

This study represents a first attempt to study the mitogenic effect(s) of a probiotic strain in ex-GF animals. It would be reasonable to assume that a similar mitogenic mechanism(s) may be found when other probiotics are used. It can be hypothesized that various probiotics may act on different compartments within the gastrointestinal tract. A strategic goal could be to orchestrate a group of probiotics that act on specific compartments, which could be used in specific pathophysiological conditions in patients.

ACKNOWLEDGMENTS

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