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Orally Administered Bovine Lactoferrin Inhibits Bacterial Translocation in Mice Fed Bovine Milk

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Feeding of bovine milk to mice induced a high incidence of bacterial translocation from the intestines to the mesenteric lymph nodes, and the bacteria involved were mainly members of the family *Enterobacteriaceae*. Supplementation of the milk diet with bovine lactoferrin or a pepsin-generated hydrolysate of bovine lactoferrin resulted in significant suppression of bacterial translocation. Our findings suggest that this ability of lactoferrin to inhibit bacterial translocation may be due to its suppression of bacterial overgrowth in the guts of milk-fed mice.

It is well recognized that breast feeding helps protect human infants from intestinal infections (11–13). Lactoferrin is an iron-binding glycoprotein found prominently in mammalian colostrum and mature milk (18). Because it displays broad-spectrum antimicrobial activity in vitro, lactoferrin is thought to have a role in protection of breast-fed infants against bacterial infection (4, 5). The antimicrobial role of lactoferrin in vivo remains unclear, however, since few studies have been done to examine its role in the host defense in practice (20). We have recently reported that orally administered bovine lactoferrin (bLF) or a pepsin-generated hydrolysate of bLF, bLFH, inhibits the proliferation of endogenous members of the family *Enterobacteriaceae* and orally inoculated clostridia in the guts of mice fed bovine milk (22, 23). These results prompted us to study how ingested lactoferrin may contribute to the host defense through its bacteriostatic action against intestinal bacteria. During this study, we found that milk feeding induced a high incidence of translocation of endogenous bacteria from the intestines of mice. By using this experimental model, we examined whether addition of bLF or bLFH to milk exerts a suppressive effect on bacterial translocation. Bacterial translocation is defined as the passage of viable bacteria from the gastrointestinal tract through the mucosal epithelium to other sites, such as the mesenteric lymph nodes (MLN), spleen, liver, and bloodstream (3). To investigate the interaction between bacterial translocation and intestinal bacteria, the influence of a milk diet and the effect of bLF or bLFH on the fecal microflora of mice were examined. The influence of bLF or bLFH on the intestinal mucosal barrier and the immune defense, which are known to be impaired in other animal models of bacterial translocation (7, 9, 21), was also examined.

Influence of bLF and bLFH on fecal microflora. Four-week-old female BALB/c specific-pathogen-free mice were obtained from Nihon SLC (Shizuoka, Japan). The mice were kept in a specific-pathogen-free room. They were initially fed a commercial pelleted diet with the same lot number (F-2; Funabashi Farms Co., Chiba, Japan) and tap water ad libitum for 7 days to allow them to become accustomed to the new environment. The mice (at 5 weeks of age) were then used in the animal experiments. The influence of diet on the intestinal microflora was examined by using six groups of five mice each. Each group was fed pellets; milk containing bLF at a concentration of 0, 0.5, 1, or 2%; or milk containing 2% bLFH for 7 days. Native bLF and bLFH were prepared as described previously (22). Other conditions of the animal experiments were the same as those described previously (22). At appropriate intervals, fresh feces were collected separately from each mouse by temporarily transferring the mouse to another aluminum cage for about 5 min, and the number of enterobacteria in the feces was monitored. Enterobacteria were identified with the Enterotube II system (Becton Dickinson Overseas Inc., Tokyo, Japan) as described previously (22). Bacterial numbers were expressed as CFU per gram of feces. Data were expressed as the mean value ± the standard deviation of samples. The data were analyzed statistically by one-way analysis of variance and the multiple-range test of Tukey-honest significant difference. The time course of changes in the numbers of fecal enterobacteria in the groups fed different diets is shown in Fig. 1. When mice were fed milk only, the number of enterobacteria in the feces increased greatly, to a level about 100 times higher within 7 days than that observed before milk feeding. Addition of 2% bLF or 2% bLFH to the milk diet suppressed the proliferation of fecal enterobacteria during the period of feeding. The bacterial numbers in mice fed milk containing 2% bLF or 2% bLFH did not increase after day 2 and were significantly lower at day 7 than in the group fed milk only ($P < 0.05$). After feeding of each diet for 7 days, the fecal microflora of each mouse was assayed. Bacteriological analysis of the fecal microflora was performed by the methods of Mitsuoka et al. (16, 17) as described previously (23). Total bacteria and strict obligate anaerobic bacteria in the feces of mice were assayed by the methods of Mitsuoka et al. by using anaerobic prereduced medium 10 (6, 15). Each assay was run in duplicate. Medium 10 was inoculated under flowing oxygen-free CO_2 gas, while the other media were inoculated in air. Bacteria isolated from feces were identified by colony morphology and cellular features, Gram stain, spore formation, catalase activity, and aerobic and anaerobic growth characteristics. Table 1 shows the fecal microflora of mice fed the different diets for 7 days. The

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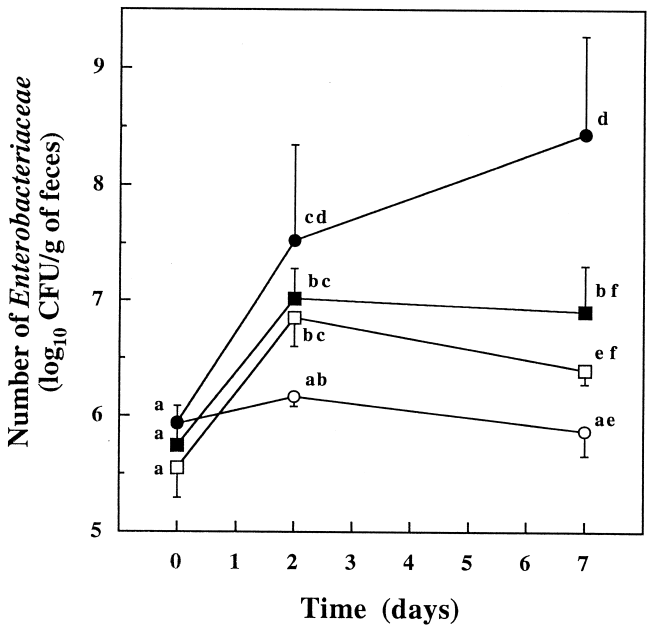

FIG. 1. Effect of administered bLF or bLFH on the proliferation of fecal enterobacteria in mice fed bovine milk. Each group of mice was fed pellets (○), bovine milk (●), milk containing 2% bLF (□), or milk containing 2% bLFH (■). The values are expressed as mean $\log_{10}$ CFU per gram ± the standard deviation ($n = 5$). Values within each group on different days and among the four groups on the same day with different letters are significantly different ($P < 0.05$) by the multiple-range test of Tukey-honest significant difference.

most prevalent bacteria in the fecal microflora of mice fed pellets were members of the family *Bacteroidaceae*. Compared with the group fed pellets, substantially higher numbers of streptococci, lactobacilli, bifidobacteria, and clostridia, as well as enterobacteria, were observed in the group fed milk only ($P < 0.05$). Such a tendency for bacterial overgrowth in the guts of mice fed milk has been reported previously (14). On the other hand, there was a tendency for the numbers of these bacteria, except bifidobacteria, to decrease in the groups fed milk containing bLF at different concentrations compared with the group fed milk only. The bacteriostatic effect of bLF against fecal enterobacteria was dependent on the concentration of bLF. The incidence of clostridia was four of five in the group fed milk only but one of five in the groups fed pellets or milk containing 2% bLF or bLFH. No significant difference in the numbers or incidence of fecal bacteria, except the numbers of total bacteria and anaerobic gram-positive cocci, was observed among the groups fed pellets, milk containing 2% bLF, or milk containing 2% bLFH ($P < 0.05$). On the basis of these results, the influence of diet on bacterial translocation was examined after 7 days of feeding.

Effect of bLF and bLFH on bacterial translocation. The second experiment was performed to determine whether milk feeding induces bacterial translocation in mice and whether addition of bLF to milk influences bacterial translocation. Mice were randomly divided into five groups of 10 mice each. Each group was fed pellets, milk, or milk supplemented with one of the following proteins at a concentration of 2%: bLF, bLFH, or casein sodium (ALANATE 180; New Zealand Dairy Board, Wellington) as a control protein. After receiving these diets for 7 days, the mice were anesthetized by exposure to chloroform and their organs were removed by a sterile technique under aerobic conditions. The MLN (the middle MLN), spleen, and liver were weighed and then immediately homogenized with a glass tissue grinder in an anaerobic prereduced diluent (16) with oxygen-free CO_2 flowing. Each half of the MLN or spleen homogenate and a 200-μl aliquot (in duplicate) of the liver homogenate were spread onto Trypticase soy agar (BBL, Becton Dickinson, Cockeysville, Md.) and BL agar (17) (Eiken Chemical Co., Tokyo, Japan), a nonselective agar medium. Both of these media were supplemented with 5% sterile defibrinated horse blood (NIPPON BIO-SUPP. Center, Tokyo, Japan). After aerobic incubation (Trypticase soy agar) for 24 h or anaerobic incubation (BL agar) in steel jars (Hirayama Manufacturing Corp., Tokyo, Japan) with steel wool covered by reduced copper under an atmosphere of CO_2 gas for 48 h at 37°C, bacterial CFU in the MLN, liver, and spleen were assayed. Table 2 shows the incidence of bacterial translocation to the MLN and the numbers of translocating bacteria in the groups of mice fed different diets. The incidence of bacterial translocation was expressed as the percentage of the number of mice with viable bacteria in the MLN (the middle MLN) per total number of mice ($n = 10$) tested. Incidences among the groups were analyzed statistically by chi-square analysis and the Fisher exact test. Translocating bacteria were isolated from the MLN of all of the groups at various rates of incidence but never from the spleen or liver. The pellet-fed group showed the lowest incidence (20%) of bacte-

TABLE 1. Influence of diet on fecal microflora of mice[a]

Fecal microflora	Mean $\log_{10}$ CFU/g of feces ± SD_{n-1} (frequency of occurrence [no. of mice yielding bacteria/5 examined])					
	Milk	Milk + 0.5% bLF	Milk + 1% bLF	Milk + 2% bLF	Milk + 2% bLFH	Pellets
Total bacteria	10.9 ± 0.2^{bc}	11.0 ± 0.2^{b}	10.9 ± 0.1^{bc}	11.0 ± 0.2^{b}	10.6 ± 0.1^{c}	10.6 ± 0.1^{c}
Enterobacteria	8.4 ± 0.8^{b} (5)	7.5 ± 0.9^{bd} (5)	6.8 ± 0.2^{cd} (5)	6.4 ± 0.1^{cd}(5)	6.9 ± 0.4^{cd} (5)	5.9 ± 0.2^{c} (5)
Staphylococci	4.5 ± 1.1 (5)	4.0 ± 1.0 (3)	4.6 ± 0.9 (5)	4.1 ± 1.0 (5)	5.1 ± 0.8 (4)	4.6 ± 0.6 (5)
Streptococci	8.1 ± 0.6^{b} (5)	5.9 ± 0.7^{c} (5)	6.8 ± 0.3^{c} (5)	6.9 ± 0.9^{bc} (5)	7.0 ± 0.6^{bc} (5)	6.3 ± 0.1^{c} (5)
Lactobacilli	10.2 ± 0.2^{b} (5)	10.2 ± 0.4^{bd} (5)	9.6 ± 0.4^{bc} (5)	9.4 ± 0.5^{cd} (5)	9.6 ± 0.4^{bc} (5)	9.1 ± 0.3^{c} (5)
Bacteroides	10.2 ± 0.2^{b} (5)	10.3 ± 0.2^{bc} (5)	10.3 ± 0.1^{bc} (5)	10.7 ± 0.1^{c} (5)	10.4 ± 0.1^{bc} (5)	10.5 ± 0.1^{bc} (5)
Bifidobacteria	10.4 ± 0.2^{bc} (5)	10.4 ± 0.1^{b} (5)	10.0 ± 0.2^{bcd} (5)	10.1 ± 0.5^{bcd} (5)	9.7 ± 0.4^{cd} (4)	9.5 ± 0.3^{d} (5)
Clostridia	9.7 ± 0.2 (4)	9.0 ± 0.3 (4)	9.2 ± 0.2 (3)	9.4 (1)	7.6 (1)	8.6 (1)
Fusiform bacteria	9.1 ± 0.3 (3)	9.4 ± 0.4 (4)	9.4 ± 0.1 (3)	9.4 ± 0.2 (3)	9.2 ± 0.3 (4)	9.3 ± 0.3 (3)
Other anaerobic gram-positive rods	8.9 ± 0.4 (4)	8.4 ± 0.1 (3)	8.3 ± 0.3 (3)	8.6 ± 0.7 (5)	8.3 ± 0.5 (5)	8.4 ± 0.7 (5)
Anaerobic gram-positive cocci	10.3 ± 0.5^{bc} (5)	10.6 ± 0.4^{b} (5)	10.6 ± 0.2^{bd} (5)	10.6 ± 0.1^{bd} (5)	9.9 ± 0.2^{cd} (5)	9.8 ± 0.3^{c} (5)

[a] Each group of five mice was fed pellets; bovine milk containing bLF at a concentration of 0, 0.5, 1, or 2%; or milk containing 2% bLFH for 7 days. Values with different superscript letters for each type of bacteria except clostridia are significantly different ($P < 0.05$) by the multiple-range test of Tukey-honest significant difference. SD_{n-1}, standard deviation.

TABLE 2. Influence of diet on bacterial translocation to the MLN of mice[a]

Group	Incidence of bacterial translocation (%)	No. of translocating bacteria (mean CFU/MLN ± SD_{n-1})					
		Total bacteria	Enterobacteria	Staphylococci	Streptococci	Lactobacilli	Anaerobic bacteria
Milk	100	57.6 ± 54.1^{b}	45.5 ± 53.5^{b}	0.2 ± 0.6	4.3 ± 6.6	6.2 ± 5.3^{b}	1.4 ± 1.9
Milk + 2% casein sodium	90	16.7 ± 16.6^{bc}	10.4 ± 17.1^{bc}	0	0	5.1 ± 2.7^{bc}	1.2 ± 1.5
Milk + 2% bLF	50*	0.7 ± 0.9^{c}	0.4 ± 0.9^{c}	0.2 ± 0.4	0	0	0.1 ± 0.3
Milk + 2% bLFH	40*	1.3 ± 2.1^{c}	0.9 ± 1.8^{c}	0	0	0.3 ± 0.6^{d}	0.1 ± 0.3
Pellets	20*	1.5 ± 3.0^{c}	0	0.8 ± 2.4	0	0.7 ± 2.1^{cd}	0

[a] Each group of 10 mice was fed pellets, bovine milk, or milk containing casein sodium, bLF, or bLFH at a concentration of 2.0% for 7 days. *, Significantly different ($P < 0.05$) versus the incidence of the group fed milk only by the Fisher exact test. Values with different superscript letters for each type of bacteria are significantly different ($P < 0.01$) by the multiple-range test of Tukey-honest significant difference. SD_{n-1}, standard deviation.

rial translocation to the MLN. In the group fed milk only, bacterial translocation was induced with a high incidence of 100%. Addition of 2% bLF or bLFH to milk resulted in a significant decrease in the incidence of bacterial translocation ($P < 0.05$). On the other hand, addition of 2% casein to milk did not show this effect. Among the translocating bacteria, mainly aerobic bacteria were recovered from the MLN of mice. Enterobacteria were the most prominent bacteria, followed by lactobacilli and streptococci. The numbers of translocating bacteria (total bacteria and enterobacteria) were significantly lower in the groups fed pellets or milk containing 2% bLF or bLFH than in the group fed milk only ($P < 0.01$). The group fed milk containing 2% casein showed intermediate numbers of translocating bacteria. Translocation of streptococci to the MLN was observed only in the group fed milk only. These findings, together with the changes observed in the fecal microflora, suggest that the ability of bLF to inhibit the translocation of intestinal bacteria may be due to its suppression of bacterial overgrowth in the guts of milk-fed mice. It has been reported previously that bLF exhibits a specific ability to suppress the proliferation of intestinal enterobacteria in the guts of milk-fed mice, unlike other proteins, including casein (22). The influence of diet on the gut mucosa of the small intestines of mice was also examined in the present study. No substantial effect of bLF on the small intestine was observed in relation to length, tissue weight, mucosal weight, mucosal protein, or DNA content (data not shown). The epithelial barrier appeared to be intact and to have normal architecture in all of the groups.

Mitogen response and histology. In the third experiment, the influence of diet on intestinal morphology and the systemic host immune response was examined by using four groups of five mice each. Each group was fed pellets, milk, milk containing 2% bLF, or milk containing 2% bLFH. After receiving each diet for 7 days, the mice were killed and the small intestines, ceca, and spleens were removed. Duodenum, jejunum, ileum, and cecum samples were examined histologically. Splenic mononuclear cells were prepared from the spleen for use in the mitogen assay, since the highest blastogenic response to mitogens has been observed for splenic lymphocytes (9). There was no significant difference in the blastogenic response to any mitogen tested, including concanavalin A, phytohemagglutinin, and lipopolysaccharide from *Escherichia coli* O111:B4 (Sigma Chemical Co., St. Louis, Mo.) between the groups fed pellets or milk (data not shown). Addition of bLF or bLFH to milk had no influence on the blastogenic response of splenic lymphocytes (data not shown). In the histological analysis of the intestinal mucosa, no apparent difference was observed in villous height, crypt depth, wall thickness, or number of goblet cells at any site among the groups tested (data not shown).

Bacterial translocation has been reported to be induced in various animal models by the following three basic pathophysiologic conditions: (i) disruption of the ecological balance of the indigenous microflora resulting in bacterial overgrowth with gram-negative enteric bacilli, (ii) impaired host immune defenses, and (iii) physical injury to the mucosal barrier (8). These three variables usually affect each other in animal models of bacterial translocation, including diet-induced bacterial translocation. The milk-fed mice used in this study represent a new animal model of diet-induced bacterial translocation. In this model, no apparent mucosal atrophy and no significant decrease of mitogen response or mucosal protein or DNA content were observed, at least after 7 days of milk feeding. Thus, the extent of bacterial translocation in this model seems to be correlated only with the numbers of fecal bacteria, mainly enterobacteria. These results suggest that bacterial translocation may be induced by overgrowth of intestinal bacteria alone without severe damage of mucosal function and without an impaired immune defense.

The in vivo mechanism by which bLF suppresses bacterial overgrowth and translocation is not clear, although its antibacterial properties have been demonstrated in many in vitro studies (1, 2, 4, 10, 18). Iron-free lactoferrin is known to inhibit bacterial growth in vitro by sequestering iron with its strong iron-chelating activity, producing an iron-deficient environment that does not support the growth of iron-requiring bacteria (4, 5). In addition, lactoferrin exerts bactericidal activity through direct interaction with the bacterial cell surface (1, 2, 10, 19). bLFH showed the same suppressive effect on bacterial translocation as undigested bLF in vivo. This result suggests that bLF can exert this activity in the gut of an animal after the bLF has been digested to some extent.

Considering the effects of bLF observed in this study, lactoferrin contained in mammalian milk may have the potential to protect infantile animals against gastrointestinal infections.

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