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Glutamine on the Luminal Microbial Environment After Massive Small Bowel Resection

To evaluate the oral glutamine (GLN) on the luminal microbes and bacterial translocation (BT) in short bowel, 45 Wistar rats were utilized in three groups; A (control), and B and C (short bowel, 85% of small bowel resected). The group A was fed with elemental diet (EmD), B with EmD+2% glycine, and C with EmD+2% GLN. The groups B and C were isocaloric and isonitrogenous. Wet weight, DNA, protein, and histomorphometry of the mucosa and parallel microbial culture from cecal contents, caval blood, and tissue blocks of the liver, spleen, and mesenteric lymph nodes were performed on the 5th, 10th, and 15th day. Mucosal growth was higher in group C than B. Colony forming units (CFU) from cecal contents increased more in group B than in C. BTs in A, B, and C were 7/15, 8/15, and 2/15, respectively. Total CFUs in blood and tissues were 5.8×10^4/g, 5.5×10^6/g, and 1.8×10^4/g, respectively. As for BT, the most frequent organism was Klebsiella in A (79.3%), but *E. coli* in B and C (94.2% and 55.6%). GLN seems to suppress luminal microbes, and reduces BT in short bowel due to enforced barrier function and proliferation of the mucosa.

Key Words : *Short Bowel Syndrome; Bacterial Translocation; Glutamine; Adaptation; Intestinal Mucosa*

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INTRODUCTION

In cases of massive small bowel resection, limitation of the oral feeding as well as intestinal motility changes have been speculated to induce bacterial overgrowth, translocation, and sepsis. Many factors that may promote the adaptation of the remaining bowel functions have been reported, such as pancreatico-biliary secretions, hormones, luminal nutrients (1-4) such as dietary fiber, short-chain fatty acid, and glutamine (GLN) (5, 6). However, there have been limited reports concerned about the relations between nutrients and floral changes in the gut (7, 8).

Discontinuation of the oral nutrient changes normal bacterial flora (7) and weakens the barrier function of the mucosa (8). GLN is also known to maintain the structure and function of the intestine (9), especially the intestinal permeability (10). Considering that IgA and secretory component excreted by the mucosal cells affect bacteria in the intestine, enterocytes may play the initial defense role against luminal microbes and toxins (11). Bacterial translocation (BT) represents penetration of the organisms through the mucosa (12), and it was presumed that the proliferation of luminal bacterial flora and BT might be suppressed by the supplementation of the GLN, which activates the structure and function of the mucosa. However, the relationship between GLN and luminal bacterial flora or translocation particularly in short bowel has not been thoroughly investigated.

The aim of the present study was to evaluate the promoting effect on the intestinal adaptation and the changes of the luminal microbial environment with subsequent blocking of the BT by administering GLN in rats after massive small bowel resection.

MATERIALS AND METHODS

Materials

Young male Wistar rats, weighing 200 to 250 g, were adopted, and fed EmD (elemental diet Energen, Samil Pharmaceuticals, Seoul, Korea) liberally through a drinking bottle from 5 days prior to the experimental period. The animals were divided into three groups: group A (n=15) as the control with sham operation, group B (n=15) and C as short bowel groups. In group A, EmD was fed. In group B, EmD with 2 g glycine (referred to as 'Gly' hereafter, GIBCO BRL, Grand Island, NY, U.S.A.)/100 mL was supplied, and in group C (n=15) with EmD with 2 g GLN (GIBCO BRL, Grand Island, NY, U.S.A.)/100 mL. In each animal, the amount of food taken was checked everyday.

Methods

For operation, rats were anesthetized by intramuscular injection of ketamine (0.01 mg/kg) and xylazine (0.001 mg/kg). Abdominal wall was sterilized with betadine solution, draped with sterile sheet to prevent contamination, and opened through a mid-line incision. The length of the small intestine from Treitz ligament to ileocecal valve was measured, and 85% of the measured intestine was resected, remaining 2 cm of ileum above the ileocecal valve and proximal jejunum. Intestinal tract was restored by one layer end-to-end jejunoileal anastomosis. From the proximal portion of the resected bowel, 2, 1, and 2 cm segments were taken as specimen for the study of mucosal wet weight, DNA and protein contents of mucosa, and histomorphometric changes. EmD feeding started when the animals were free to move after operation.

Observation of Results

Five rats from each group were sacrificed on the 5th, 10th, and 15th postoperative day. On opening the abdomen in sterile condition, tissue blocks of the mesenteric lymph nodes, liver and spleen, and 0.5 mL of blood from inferior vena cava were obtained for the quantitative microbial studies. MacConkey II agar plate (BBL, Cockeysville, Maryland, U.S.A.) and TSA II agar plate (BBL, Cockeysville, Maryland, U.S.A.) were used as bacterial culture media. After incubation at 37°C for 48 hr, the colony forming unit (CFU) was counted and the organisms were identified with the BBL Crystal ID System (BBL, Cockeysville, Maryland, U.S.A.).

For bacterial culture, the blood drawn in a heparinized syringe was diluted 100 folds with Hanks' Balanced Salt Solution (HBSS, 61200, pH 7.2, GIBCO BRL, Grand Island, NY, U.S.A.). Fifty microliters of the diluted blood was spread onto a culture media. Tissue blocks were put into empty disc to measure their weights and were grinded with dilution of 100 folds with HBSS. Fifty microliters of the tissue ground solution was spread onto the culture media and incubated.

The cecal content was put into an empty sterile disc, and was diluted by 100 folds with dilution solution, and then 50 μL of the diluted material was incubated on the media. In some cases, more dilution was implicated for the accurate count of CFUs.

The total length of the remaining small intestine after each experimental period was measured along the opposite side of the mesentery from the Treitz ligament to the ileocecal valve after resection of the whole intestine.

Wet weight, protein and DNA contents of the mucosa were examined as follows. The lumen of the resected intestine was washed out with ice-chilled saline solution. After eliminating the attached mesentery, intestine was fixed on a cork board with a tack around the proximal end. A 5 g weight was hanged at the distal end around ileocecal valve to give a universal expansion factor. Starting from 4 cm distal to the Trietz ligament, 2, 1, and 2 cm intestinal segments were obtained for the next studies. The lumen was opened longitudinally along the mesentery side. Mucosa was scraped out using a glass slide and weighed to measure the wet weight of the mucosa per unit length. Then, the obtained mucosa was ground, and the DNA content per unit length was measured with a WizardTM Genomic DNA Purification Kit (Promega, WI, U.S.A.) as the method by Sambrook et al. (13), and protein was assayed as by Lowry et al. (14).

The next 2 cm intestinal segment was spread and fixed with bamboo pins, placing the mucosa to the top. The specimen was fixed in 10% buffered formalin, embedded in paraffin, cut in 10 μm thickness, and stained with H&E. Microscopical photographs were taken at 40× magnification, and printed in 4×6 folds. Fifteen consecutive villi on the photographs were measured for the width and height of villus and crypt depth. Villus size ratio was obtained as villus height per villus width (15).

Statistical analysis

Each measurement was expressed as mean ±SD. The difference among the groups and the changes according to the experimental days were verified with two-way ANOVA test. $p<0.05$ was considered to represent statistical significance. The degree of BT was verified with Fisher's exact test using the number of animals showing positive bacterial culture in blood or tissue samples.

RESULTS

Amount of daily food intake

The daily food intake per 100 g of body weight was calculated at three time-points (day 5, 10, and 15). The mean amount was 49.3±6.3, 41.6±4.5, and 36.2±0.9 mL per day in group A, 15.1±3.0, 23.5±4.0, and 33.0±4.8 in group B, and 15.9±4.8, 23.2±2.4, and 25.3±3.4l in group C, respectively. However, no significant difference was noticed between groups B and C ($p=0.447$, Fig. 1).

Body weight

The percent weight change based on the weight at operation was 112.6±3.0, 125.4±5.3, and 135.1±2.7% in group A at day 5, 10, and 15, respectively. There was a significant increase compared to those of group B and C (Fig. 2, $p<0.001$). The percent weight changes in group B were 92.0±4.3, 87.3±6.3, and 78.8±10.6, and those in group C were 90.4 ±3.1, 89.9±6.8, and 91.8±10.2%, respectively. Both groups showed decreasing tendencies of body weight, but no significant difference was noted between group B and C ($p=0.184$).

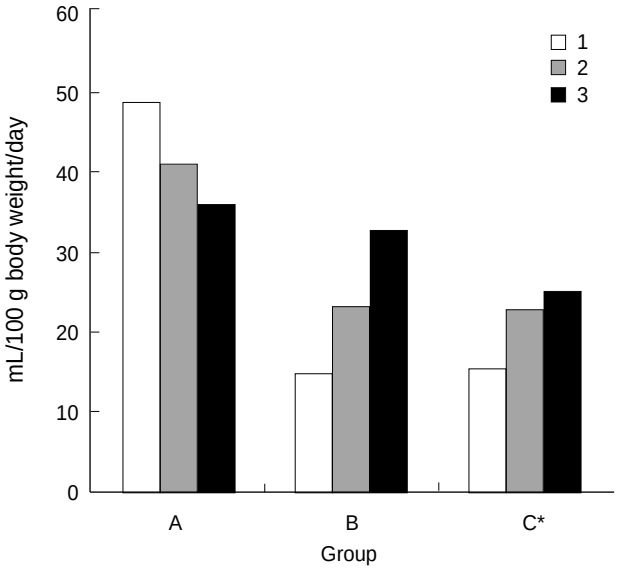

Fig. 1. Daily intake of elemental diet per body weight (100 g). No difference between B and C. p=0.447 between group B and C (two-way ANOVA test). Group A, elemental diet, normal small bowel; B, elemental diet added with 2% glycine, short bowel; C, elemental diet added with 2% glutamine, short bowel. 1, during the first 5-day period after small bowel resection; 2, during the second 5-day period after small resection; 3, during the third 5-day period after small bowel resection.

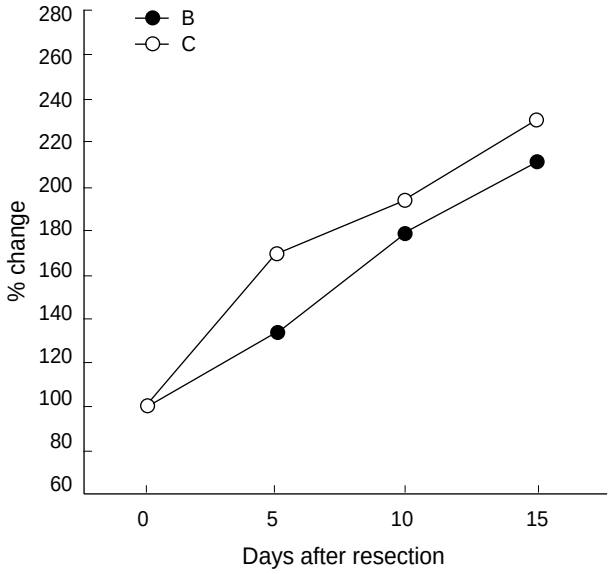

Fig. 3. Percent increase of the length of the remaining intestine after resection. p=0.071 between group B and C (two-way ANOVA test).

Length of intestine

The change of the intestinal length was expressed as percent change by the remaining bowel length at the time of operation. Bowel lengths in group B were 133.8±18.7, 178.2±11.1, and 211.1±32.3% and those in group C were 169.2±10.6, 194.2±14.2, and 230.7±6.3% at day 5, 10, and 15, respectively. There were gradual increases of intestinal length in both groups, but no significant difference was noted between the two groups (p=0.071, Fig. 3).

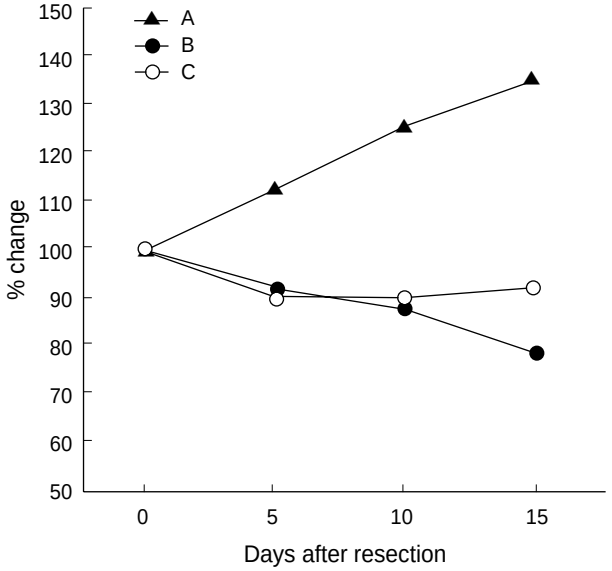

Fig. 2. Percent body weight change to the beginning. p=0.184 between group B and C (two-way ANOVA test).

Table 1. Measurements of wet weight, DNA and protein contents of the intestinal mucosa

	ED	Group		
		A	B	C
Weight	0		42.8±1.4	42.4±2.7
(mg/cm)	5	38.6±5.7	71.7±10.4	89.0±11.1
	10	24.7±4.3	75.6±13.5	86.3±11.2
	15	31.5±5.4	74.1±11.4	94.6±10.7
DNA	0		245.3±24.7	243.6±41.8
(μg/cm)	5	197.8±23.7	315.2±38.6	372.8±50.6
	10	200.6±25.2	313.8±32.3	378.0±47.0
	15	209.2±24.1	333.2±74.6	405.2±70.0
Protein	0		2,634±293.8	2,321±286.0
(μg/cm)	5	1,708±276.4	2,320±188.2	3,350±356.9
	10	1,698±73.3	2,932±324.8	3,056±323.5
	15	1,680±242.2	2,893±335.6	3,246±391.5

Values are expressed as mean±SD.
Group A, elemental diet with normal bowel; B, elemental diet added with 2% glycine after small bowel resection; C, elemental diet added with 2% glutamine after small bowel resection.
ED, experimental day; ED 0 is the day of small bowel resection.
The values of group B and C significantly more increased than those of group A (p<0.001, weight , DNA & protein; two-way ANOVA test). The values of group C increased more than those of group B (p=0.0009, mucosal weight; p=0.0035, DNA; p=0.0006, protein: two-way ANOVA test).

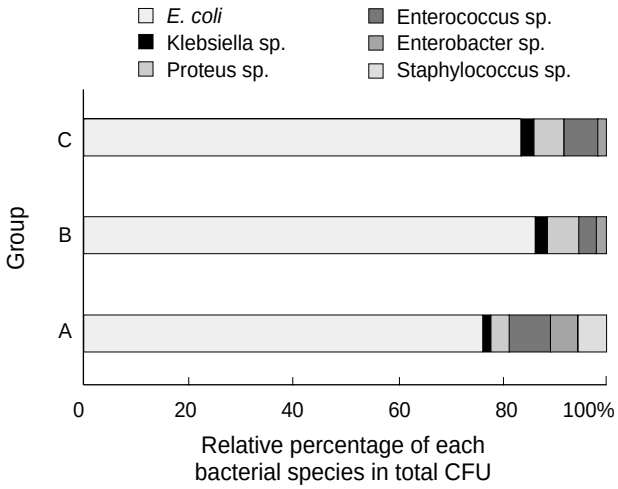

Fig. 4. Composition of the bacterial species isolated from cecal contents. CFU, colony-forming unit.

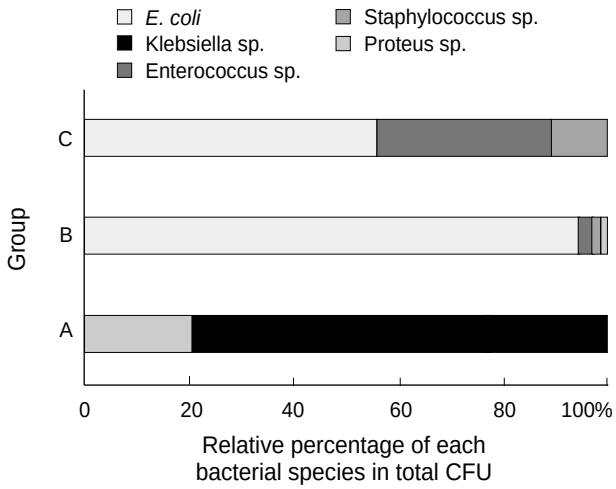

Fig. 5. Composition of the bacterial species translocated to the organs (liver, spleen, mesenteric lymph nodes, and blood from vena cava).

Table 2. Histomorphometric measurements of the mucosa

	ED	Group		
		A	B	C
Crypt depth	0		1.09±0.03	1.08±0.02
	5	1.23±0.13	1.56±0.16	1.60±0.13
	10	1.10±0.11	1.32±0.17	1.71±0.11
	15	1.12±0.17	1.42±0.06	1.73±0.11
Villus size ratio	0		3.89±0.13	3.89±0.15
	5	3.36±0.28	4.46±0.69	6.01±0.61
	10	3.18±0.47	3.87±0.86	4.62±0.68
	15	2.41±0.28	4.88±0.64	5.11±0.42

Values are expressed as mean±SD.
Definition of experimental groups and abbreviations are same as in Table 1.
Villus size ratio: height of villus divided by width of villus.
The values of group B and C are higher than those of group A (p< 0.001, crypt depth and villus size ratio: two-way ANOVA test). The values of group C are higher than those of group B (p=0.0001, crypt depth; p=0.0301, villus size ratio: two-way ANOVA test).

Table 3. Total CFU per gram of cecal contents

ED	Group		
	A	B	C
5	8.64±8.51	9.55±8.50	9.05±9.28
10	8.70±8.65	9.10±8.69	9.02±9.33
15	8.68±8.27	9.40±9.36	9.01±9.14

Values are expressed as mean log CFU±SD.
Definition of experimental groups and abbreviations are same as in Table 1.
The CFUs are higher in group B and C than in group A (p<0.05: two-way ANOVA test), and higher in group B than in group C (p=0.0279: two-way ANOVA test).

Wet weight, DNA and protein contents of mucosa

Wet weight and DNA and protein contents of the mucosa were significantly increased in group B and C compared to group A (Table 1, p<0.001). Mucosal growth was more remarkable in group C than in group B (p=0.0009; mucosal weight, p=0.0035; DNA content, p=0.0006; protein content).

Histomorphometric changes of mucosa

As shown in Table 2, the villus size ratio and crypt depth increased in group B and C compared to group A (p<0.001; villus size ratio and crypt depth). The ratio and depth increased more in group C than in group B (p=0.0001; crypt depth, p=0.0301; villus size ratio).

Bacterial translocation

Bacteria in the cecal contents; Log CFU per gram of cecal content in group B and C increased compared to in group A, and the increase was less significant in group C than in group B (Table 3). *E. coli,* Klebsiella sp., Proteus sp., Enterococcus sp., Enterobacter sp. and Staphylococcus sp. were identified. *E. coli* accounted for 76.5, 86.7, and 83.9% of the total microbial population in each group, respectively (Fig. 4).

A positive culture of bacteria from blood, lymph node, liver, and spleen was considered to represent bacterial translocation. The number of animals with BT is shown in Table 4. The rate of BT was significantly lower in group C than in group B (p<0.05, Fisher's exact test). Total CFU translocated was 5.8×10^4, 5.5×10^6, and 1.8×10^4/g in group A, B, and C, respectively. The translocation rates by each species are presented in Table 5. As shown in Fig. 5, *Klebsiella sp.* accounted for 79.3% of BT and *E. coli* for 21.7% in group A. *E. coli*

Table 4. Total number of animals with bacterial translocation to each organ by the microbial species

	Group	A			B			C		
	ED	5	10	15	5	10	15	5	10	15
Blood	*E. coli*	-	-	1	-	-	1	-	-	1
	Klebsiella sp.	-	-	5	-	-	-	-	-	-
	Enterococ. sp.	-	-	-	-	-	1	-	-	-
	Staphy. sp.	-	-	-	-	-	-	-	-	-
	Total	-	-	5	-	-	1	-	-	1
Liver	*E. coli*	-	-	1	1	-	2	-	-	1
	Kelbsiella sp.	-	-	3	-	-	1	-	-	-
	Proteus sp.	-	-	-	-	-	-	-	-	-
	Total	-	-	4	1	-	2	-	-	1
Spleen	*E. coli*	-	-	-	1	-	2	-	-	-
	Kelbsiella sp.	-	-	5	-	-	-	-	-	-
	Proteus sp.	-	-	-	-	-	1	-	-	-
	Enterococ. sp.	-	-	-	-	-	1	-	-	-
	Staphy. sp.	-	-	-	-	-	1	-	-	-
	Total	-	-	5	1	-	2	-	-	-
Peritoneum										
	E. coli	-	-	1	1	-	1	-	-	-
	Kelbsiella sp.	-	-	4	-	-	-	-	-	-
	Enterococ. sp.	-	-	-	-	-	2	-	-	-
	Total	-	-	5	1	-	2	-	-	-
MLN	*E. coli*	-	2	-	3	-	4	-	-	-
	Proteus sp.	-	-	-	1	-	2	-	-	1
	Enterococ. sp.	-	-	-	3	-	2	-	-	1
	Enterobac. sp.	-	-	-	-	-	2	-	-	-
	Staphy. sp.	-	-	-	1	-	2	-	-	-
	Total	-	2	-	3	-	5	-	-	2
		0/52	2/5 7/15	5/5	3/5	0/5 8/15	5/5	0/5	0/5 2/15*	2/5

Definition of experimental groups and abbreviations are same as in Table 1.
*p=0.05 between group B and C (Fisher's exact test).

was the predominant agent translocated in group B and C (94.2% and 55.6%, respectively).

DISCUSSION

Short bowel syndrome is a malabsorption state following massive resection of the small intestine for various conditions. It shows malnutrition, weight loss, diarrhea, dehydration, and electrolyte imbalance because of decreased absorptive surface and rapid transit time of the bowel (16, 17). The minimum length of intestine in humans to avoid short bowel syndrome is various depending on the patient age and the bowel status, but is considered to be 70 cm to 100 cm in case of preserved ileocecal valve (18, 19). In rats, short bowel syndrome occurred when 80% of the small bowel was resected (20). However, in the present pilot study, 80% resection was followed by weight gain, whereas 85% resection by diarrhea and weight loss. The remaining small intestine after massive resection undergoes adaptive responses of increased DNA synthesis within 12 to 24 hr (1, 2), followed by increments of villus height and crypt depth. Hyperplasia of mucosal epithelium, elongation and dilatation of the remaining intestine, and increased enzyme activity was also documented (2). These adaptive responses continue for 1 to 2 yr (17). During the adaptive period, luminal nutrients through oral administration are essential for the structural and functional integrity of the enterocytes (4, 21).

Table 5. Bacterial species and CFUs translocated in organs*

	Group		
	A	B	C
E.coli	1.2×10^4	5.2×10^6	1.0×10^4
Klebsiella sp.	4.6×10^4	-	-
Proteus sp.	-	14×10^5	6.0×10^4
Enterococcus sp.	-	1.1×10^5	-
Staphylococcus sp.	-	7.5×10^4	2.0×10^3

Values are expressed as CFU/g of tissue.
Definition of experimental groups and abbreviations are same as in Table 1.
*organs; liver, spleen, mesenteric lymph nodes, and blood from vena cava.

GLN is the most abundant amino acid in blood and free amino acid pool of the body. It acts as a nitrogen transfer vehicle, regulator of protein synthesis, precursor for nucleic acid biosynthesis, and a specific fuel for enterocytes (9) and other rapidly dividing cells such as renal tubular cells, malignant cells, fibroblasts, vascular endothelial cells, and lymphocytes. Therefore, GLN is speculated to affect the activities of gut associated lymphoid tissue (GALT). EmD, currently utilized in this study, contains 0.5% GLN in the formula. As GLN is known to be effective at the concentration of around 2% (22, 23), it was added more to reach 2.5% as final concentration.

BT, first described in 1968, is a term to describe any passage of microorganisms from one location to another, e.g., from the gastrointestinal tract to the mesenteric lymph nodes (8). In a normal condition, bowel keeps an effective barrier to the microorganisms and toxins. This defense mechanism is composed of mechanical and chemical barriers such as acidity in the stomach, epithelial tight junctions, mucous layer (11), and GALT. GALT contains intraepithelial lymphocytes, Payer's patch, mesenteric lymph nodes, and IgA and secretory components (SC) (9). All of these are dependent on the enterocyte status. Although IgA and SC are excreted from the enterocytes, the place where they play their role is the luminal space. The spectra and population of the luminal microbes seemed to be changed depending upon the enterocytes status, which obviously affect the BT. Alverdy et al. (8) and Deitch et al. (24) reported that in cases of intravenous nutrition or oral administration of only EmD, the intestinal mucosa was atrophied, the colonies of cecal bacteria increased, the level of IgA in the intestine decreased, and eventually in two weeks, BT could be observed. In the present study, BT increased by experimental day 15 in all of the groups. Of notes, the numbers of bacteria in cecal contents significantly increased, with higher numbers

in group B and C than in group A. Briet et al. (7) have also reported increased counts of microbes after intestinal resection. The lower counts of CFU in group C than in group B, and clinical significance of 5-fold changes of CFU indicate that GLN may inhibit luminal microbes. This observation has not been reported so far.

Among the microbes, CFU of *E. coli* increased more in the lumen as well as in the translocated organs after bowel resection. These results suggest that *E. coli* is mainly responsible for BT, in accordance with the previous reports by Alverdy et al. (8) and Jackson et al. (26). Other species such as Proteus sp., Klebsiella sp. and Enterobacter sp. were also found in the translocated organs in the present study.

In conclusion, oral administration of GLN to the animal with short bowel suppressed the number of the luminal microbes, changed their proportions, and decreased consequent BT. Considering the accompanying evidences of the enterocytes' growth, the enforced mucosa by GLN supplementation seems to affect the luminal microbial environment. Further investigations are required to clarify the mechanisms of enhanced GALT by GLN.

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