Intestinal Bacterial Flora and Urea Cycle

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Rearing rats on low- and high-protein diets yielded no difference in the level of hepatic ammonia while urea production was assessed at a higher level in the high-protein diet group. The appearance of a large number of ammonia-producing nonsporeforming anaerobic bacteria in the jejunum of the rats reared on a high-protein diet seems to imply that the hepatic urea cycle was stimulated by the bacteria enhancing the generative reaction from ammonia to urea. These results are significant when viewed as the probable indication of the integral ecological function of intestinal flora in the homeostatic regulation mechanism, taking the regulation of the urea cycle as one example.

Key words: Intestinal bacterial flora; urea cycle; ornithine; nonsporeforming anaerobic bacteria

The intestinal bacterial flora is considered as functioning as an independent organism in maintaining ecological homeostasis in their interrelation with the host.

There are characteristic bacterial floras on the skin and mucous membranes where an organism interfaces with the outer world. These are composed of resident flora comprised of bacteria which are not eliminated by the host's defense mechanisms and which exhibit strong symbiotic relationships with the host, and transient flora maintaining weak symbiosis with the host. The composition pattern of the intestinal bacterial flora is disrupted by disorders of host factors such as the physiological mechanisms of the intestinal tract and immunological mechanisms, and by external factors such as the type of feed, and the administration of antibacterial chemotherapeutic drugs.

In this paper, the relationship between the fluctuations of the intestinal flora of rats reared on low- and high-protein diets and

the function of hepatic urea cycle is presented and discussed.

Materials and Methods

Male Wistar-strain rats, weighing 150-170 g, were fed 0%, 10% and 70% casein diets for 7 days. Germfree, Specific Pathogen Free (SPF), and conventional male mice (ICR strain) were obtained from CLEA Japan Inc. Germfree and SPF mice were housed in five cages and placed in flexible plastic isolators. Surveillance for contamination in the germfree mice consisted of periodic aerobic and anaerobic cultures of their feces for bacteria. All the mice were fed on sterilized food (CL-2) obtained from CLEA Japan Inc. and sterile water, ad libitum, and then sacrificed 9 weeks after birth.

The blood samples were taken from the portal vein and aorta under ether anaesthesia, and deproteinized with 5% sulfosalicylic acid. Livers were frozen quickly in a dry ice

acetone mixture. The frozen material was homogenized with five volumes of 2.5% salicylic acid. After centrifugation, the extract was used for the determinations. The small intestines were washed with 50 ml of cold physiological saline. The samples were deproteinized with 4 ml of 25% sulfosalicylic acid.

Urea was measured colorimetrically with diacetylmonoxime and thiosemicarbazide according to the method of Coulombe and Favreau (1).

Ammonia was determined by the indophenol method following diffusion in a Seligson apparatus (7).

Amino acids were determined by the amino acid autoanalyzer. The activities of the urea cycle enzymes were determined essentially according to the method of Schimke (6), except that the activity of argininosuccinate synthetase was determined by the method of Rochvansky and Ratner

(4). The method of Prescott and Jones (3) was used for the determination of the amount of citrulline, ornithine, and arginine as citrulline or urea, after the conversion of ornithine to citrulline with ornithine transcarbamylase and carbamyl phosphate, and the conversion of arginine to urea with arginase.

In bacteriological examinations, segments of jejunum and colon were homogenized with teflon grinders in sterile diluent. The diluent was prepared by adding 4.5 g of KH_2PO_4 , 6.0 g of Na_2HPO_4 , 0.5 g of L-cysteine, 0.5 g of Tween 80 and 0.1 g of agar to one liter of distilled water. The bacterial suspensions were further diluted in the sterile diluent in tenfold steps, and 0.1 ml samples of each dilution were spread on the surface of various agar culture media (Table 1). The aerobic cultures were made by streaking agar plates with various differential media designed to culture selectively certain types of aerobic

Table 1. Media for the isolation of intestinal bacteria

Medium	Incubation		Organism
	Atmosphere days		
For facultative anaerobes			
DHL agar	0	1-2	<i>Enterobacteriaceae</i>
TCBS agar	0	1-2	<i>E. faecalis</i> and <i>Vibrio</i>
NAC agar	0	1-2	<i>Pseudomonas aeruginosa</i>
No. 110 agar	0	1-2	<i>Staphylococcus</i>
Phenylethyl alcohol blood agar	0	1-2	<i>Streptococcus</i>
SF agar	0	1-2	<i>Streptococcus</i>
CS agar	0	3	<i>Candida</i>
Potato dextrose agar	0	3	Yeast
For obligate anaerobes			
GAM agar and brain heart			
Infusion blood agar	An	3-4	Total anaerobes
CW agar	An	3-4	<i>Clostridium</i>
<i>Bifidobacterium</i> agar	An	3-4	<i>Bifidobacterium</i>
Rogosa SL agar	An	3-4	<i>Lactobacillus</i>
Rifampin blood agar	An	3-4	<i>Eubacterium</i>
Brucella agar + 5% horse blood + 75 µg kanamycin + 2 µg vancomycin	An	3-4	<i>Bacteroides fragilis</i> and anaerobic gram positive cocci
<i>Bacteroides</i> agar	An	3-4	<i>Bacteroides</i>
Modified FM agar	An	3-4	<i>Fusobacterium</i>
Phenylethyl alcohol blood agar	An	3-4	Anaerobic gram positive cocci
Veillonella agar + 7.5 µg vancomycin + 0.1% Tween 80	An	3-4	<i>Veillonella</i>
0: aerobic An: anaerobic			

bacteria. The anaerobic culturing of the samples was performed immediately by ten-fold serial dilution with the diluent and the serially diluted material was incubated in an anaerobic globe box to which H₂, CO₂, and N₂ gases were added at 7%, 10%, and 83% levels, respectively. Anaerobic colonies which appeared on the culture media were selected from the agar plates and thrice serially transferred into agar culture medium for confirmation of the characteristics of anaerobic bacteria. Attempts were made to classify and identify the anaerobes isolated according to the manual published by the Virginia Polytechnic Institute Laboratory (2).

Results and Discussion

A quantitative and qualitative analysis of intestinal flora in the small and large intestines of rats reared on high- and low-protein diets revealed a marked difference in the species composing the flora of the jejunum. In the jejunum of rats reared on the high-protein diet, ammonia-producing non-sporeforming anaerobic bacteria such as *Peptostreptococcus*, *Peptococcus*, *Eubacterium lentum* and *Fusobacterium nucleatum* appeared in large numbers while in rats on the low-protein diet, the jejunal flora was greatly simplified with very few ammonia-producing bacteria being seen (Table 2). No striking differences in colonic bacterial composition

between low- and high-protein diets were observed (Table 3). In the high-protein diet group, the amount of ammonia in intestinal content and portal vein blood was assessed at levels significantly higher than in the low-protein diet group, but no difference could be seen in the level of ammonia in the liver (Fig. 1). Furthermore, the concentration of urea in intestinal content, portal vein blood, and liver was significantly higher in the high-protein diet group than in the low-protein diet group (Fig. 2).

These values are probably the result of the appearance of the urease positive ammonia-producing nonsporeforming anaerobic bacteria, such as *Peptococcus*, *Peptostreptococcus* and *Eubacterium lentum* in the jejunum of the high-protein diet group, stimulating the urea cycle of the liver and thus enhancing the generative reaction from ammonia to urea.

In the enterohepatic circulation of urea, urease in the intestinal bacterial flora breaks down urea, which is secreted into the gastrointestinal tract from the vessel, with the product, ammonia, returning to the liver via the portal vein to be synthesized into urea again.

The ammonia concentration in the portal vein of conventional rats was higher than in that of germfree rats. This result would suggest that urea synthesis in the liver of conventional animals may be regulated differently from that of germfree counterparts.

To explore this point, an experiment em-

Table 2. Changes occurring in intestinal bacterial flora of rats fed 70%, 10% and 0% casein diets

Jejunal bacterial flora	70% casein	10% casein	0% casein
<i>Escherichia coli</i>	4.1×10^7	—	1.0×10^2
<i>Enterococcus faecalis</i>	8.0×10^4	—	—
<i>Staphylococcus epidermidis</i>	4.8×10^3	—	—
<i>Candida</i>	1.4×10^3	2.0×10^5	—
<i>Clostridium clostridiiforme</i>	4.6×10^7	1.0×10^4	—
<i>Bacteroides intermedius</i>	—	1.0×10^6	2.0×10^2
<i>Eubacterium lentum</i>	1.0×10^8	—	—
<i>Fusobacterium nucleatum</i>	1.5×10^3	—	—
<i>Peptostreptococcus</i>	3.1×10^5	—	—
<i>Peptococcus</i>	1.0×10^6	—	—
<i>Lactobacillus</i>	1.2×10^7	—	—
<i>Actinomyces israelii</i>	2.0×10^8	—	—

Table 3. Changes occurring in colonic bacterial flora of rats fed 70%, 10% and 0% casein diets

Colonic bacterial flora	70% casein	10% casein	0% casein
<i>Escherichia coli</i>	1.2×10^8	4.0×10^6	1.0×10^6
<i>Proteus vulgaris</i>	1.4×10^6	—	—
<i>Proteus mirabilis</i>	—	2.0×10^4	—
<i>Enterococcus faecalis</i>	2.0×10^6	1.1×10^5	1.1×10^5
<i>Staphylococcus epidermidis</i>	2.0×10^3	5.0×10^4	1.0×10^3
<i>Candida</i>	8.2×10^2	8.8×10^6	1.7×10^5
<i>Bacteroides fragilis</i>	—	5.5×10^7	—
<i>Bacteroides vulgatus</i>	—	1.0×10^6	—
<i>Fusobacterium mortiferum</i>	—	—	3.0×10^3
<i>Bacteroides intermedius</i>	4.0×10^9	—	—
<i>Bacteroides asaccharolyticus</i>	—	5.7×10^3	3.1×10^4
<i>Eubacterium lentum</i>	8.0×10^9	5.0×10^2	1.0×10^8
<i>Eubacterium limosum</i>	—	—	2.0×10^6
<i>Bifidobacterium adolescentis</i>	—	3.6×10^7	7.0×10^6
<i>Clostridium butyricum</i>	—	3.8×10^7	2.0×10^8
<i>Peptostreptococcus</i>	—	6.1×10^5	—
<i>Lactobacillus</i>	1.2×10^8	—	—
<i>Actinomyces israelii</i>	8.0×10^2	1.0×10^7	—

Fig. 1. Effects of protein levels in diet on ammonia concentrations in aorta, intestinal content, portal vein and liver. P.V.: Portal vein, A.: aorta, L.: liver, In.C.: intestinal content, shaded column: 0% casein diet, hatched column: 10% casein diet, open column: 70% casein diet.

Fig. 2. Effects of the protein levels in diet on urea concentrations in aorta, intestinal content, portal vein and liver. P.V.: Portal vein, A.: aorta, L.: liver, In.C.: intestinal content, shaded column: 0% casein diet, hatched column: 10% casein diet, open column: 70% casein diet.

ploying germfree and conventional mice was undertaken to clarify the relationship between intestinal flora and the function of the urea cycle. The above mentioned consideration seems to be the case. A quantification of the urea cycle-related substances in germfree and conventional mice possessing bacterial flora revealed a higher concentration of ornithine in the liver of conventional mice

than in their germfree counterparts (Table 4).

It has been reported that the high concentration of ornithine observed in the liver of conventional mice with indigenous intestinal bacterial flora plays an important role in the regulation of urea synthesis (5).

These observations indicate that within enterohepatic circulation, the primary re-

Table 4. Comparison of the concentrations of the urea cycle-related substances in the liver of conventional and germfree mice. The samples from two animals were mixed for the determination of amino acids and acetylglutamate. Values are expressed as mean S.D. of 10 animals

	Conventional	Germfree
Number of animals	10	10
Urea $\mu\text{mol/g}$ liver	7.1 ± 1.3	6.8 ± 2.1
Ammonia $\mu\text{mol/g}$ liver	0.42 ± 0.07	0.36 ± 0.07
Ornithine nmol/g liver	700 ± 182	346 ± 122 ($p < 0.01$)
Citrulline nmol/g liver	83 ± 12	61 ± 26
Arginine nmol/g liver	49 ± 13	28 ± 6 ($p < 0.05$)
Acetylglutamate nmol/g liver	51 ± 12	38 ± 13

Fig. 3. Urea cycle.

Fig. 4. Enterohepatic circulation of ammonia.

sponse to the increase in urea synthesizing substrate for urea catabolism by intestinal bacterial flora is the increase in concentration of ornithine in the liver. And this in turn indicates the integral ecological function of the intestinal flora in the regulation of the urea cycle (Figs. 3 and 4), one example of the homeostatic regulation mechanism of an organism.

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