Modulation of CD8$^+$ Intraepithelial Lymphocyte Distribution by Dietary Fiber in the Rat Large Intestine[1]

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We studied whether ingestion of dietary fiber modifies the distribution of intraepithelial lymphocytes (IEL) in a physiological condition. Male WKAH rats were fed diets either with fiber (sugar beet fiber or crystalline cellulose, 100 g/kg diet each) or without fiber for 3 weeks. The number of CD8$^+$, CD4$^+$, and NKR-P1$^+$ IEL per epithelial layer in the crypt section of the cecum, proximal colon, and distal colon were scored by immunohistochemical staining. We found that the proportion of CD8$^+$ IEL was greater in the cecal mucosa and was gradually reduced toward the distal large intestine in general. In contrast, there was no difference in the proportion of CD4$^+$ and NKR-P1$^+$ IEL in the large intestine. Dietary sugar beet fiber, but not crystalline cellulose, increased the proportion of CD8$^+$ IEL, especially in the cecal mucosa, but not the CD4$^+$ and NKR-P1$^+$ IEL. Analysis of cecal organic acid concentration confirmed higher concentrations of acetate and butyrate, and lower concentration of succinate and isovalerate, in the cecum of the rats fed sugar beet fiber than other diets. These results indicate that ingestion of some dietary fiber modulates local cell proliferation of a progenitor of CD8$^+$ IEL or promotes homing of CD8$^+$ T cells into the large intestinal epithelium, most likely via the fermentation in the luminal contents. Exp Biol Med 227:1017–1021, 2002

Key words: dietary fiber; intraepithelial lymphocyte; large intestine

One of the effects of dietary fiber is mediated by modification of bacterial fermentation processes in the lumen of the large intestine (1). Fermentation generates short-chain fatty acids (SCFA) that are known to affect the activities of the colon. For example, butyrate is used as a primary energy source in colonic epithelial cells (2, 3) and it stimulates normal epithelial cell proliferation (4). Moreover, physiological concentrations of butyrate have been shown to induce apoptosis in some colonic carcinoma cell lines (5). Therefore, SCFA in the luminal contents of large intestine has an important role in epithelial homeostasis.

Intraepithelial lymphocytes (IEL) also have a critical role in intestinal epithelial homeostasis. Intraepithelial γδ T cells promote epithelial cell growth via secretion of keratinocyte growth factor (6). It has been demonstrated that abrogation of γδ T cells reduces epithelial cell turnover (7) and enhances azoxymethane-induced colorectal carcinogenesis and aberrant crypt foci (8), a preneoplastic biomarker for colorectal cancer (9). We previously demonstrated that a reduction of CD8$^+$ IEL induced by treatment with antiasialo GM1 serum (αAGM1) is accompanied by promotion of the number of aberrant crypts in the colorectum and that ingestion of sugar beet fiber (SBF) partially restores the number of CD8$^+$ IEL in the epithelial-lining cells of the colon (10). In addition, dietary SBF has a more protective effect than other dietary fibers on colorectal carcinogenesis in an animal model (11). Thus, we deduced that ingestion of SBF promotes an accumulation of CD8$^+$ IEL to survey abnormal epithelial cells. In this report, we focus on the distribution of IEL (CD8$^+$, CD4$^+$, and NKR-P1$^+$) in the large intestine and we investigate whether ingestion of dietary fiber modulates their proportion and localization among epithelial cells under physiological condition.

Materials and Methods

Animals and Diets. Male WKAH/HKmSlc rats (5 weeks old; Japan SLC, Hamamatsu, Japan) were housed in individual cages in a temperature-controlled (23° ± 2°C) room under a 12:12-hr light:dark photoperiod (light: 0800–2000 hr). The fiber-free (FF) diet contained sucrose, casein, corn oil, a mineral mixture, and a modified AIN-76 vitamin mixture (Table I). Rats were allowed free access to food and water throughout the experimental period. All rats were given the FF diet for 7 days. After acclimation period, 19 rats were divided into three dietary groups fed FF, cellulose-supplemented diet (CE; 100 g of cellulose/kg diet), or SBF-

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Table I. Composition of the fiber-free diet

Ingredient	Amount (g/kg diet)
Casein	250
Sucrose	646
Corn oil	50
Mineral mixture	40
Vitamin mixture	10
Choline bitartrate	4

Dietary fiber (cellulose or sugar beet fiber) was added to a fiber-free diet at the expense of whole diet (100 g/kg diet). Casein is purchased from ALACID, New Zealand Dairy Board, Wellington, New Zealand. Retinyl palmitate (7.66 μmol/kg diet) and ergocarciferol (0.0504 μmol/kg diet) was added to corn oil. The mineral mixture was prepared based on the AIN-76 Workshop held in 1989 (12).

supplemented diet (SBF; 100 g of sugar beet fiber/kg diet). Cellulose (microcrystalline cellulose PH-102) was purchased from Asahi Kasei Corporation (Osaka, Japan). SBF was donated by Nippon Beet Sugar MFG (Obihiro, Japan). The study was approved by the Hokkaido University Animal Use Committee, and the animals were maintained under the guidelines for the care and use of laboratory animals, Hokkaido University. After 3 weeks of feeding each diet, rats were sacrificed by decapitation, and the cecum, proximal, and distal colon were removed. Segments of large intestine were flushed with saline and frozen at –80°C. The cecal contents were frozen and stored at –40°C until further analysis.

Immunohistochemical Analysis. Frozen section from these samples were fixed at 4°C with Zamboni's fixative for CD4 and CD8 staining or periodate-lysine-paraformaldehyde for NKR-P1 detection. After being fixed, these sections were immersed in 3% hydrogen peroxide in methanol to reduce endogenous peroxidase activity. They were incubated for 1 hr with 10% normal rabbit serum to block nonspecific bindings. As primary antibodies, we used mouse anti-rat CD4 (clone W3/25), mouse anti-rat CD8 (clone OX-8), and mouse anti-NKR-P1 (clone 10-78). After incubation with the primary antibody, sections were incubated with rabbit anti-mouse immunoglobulin (Ig) G conjugated with biotin. They were then incubated with peroxidase-conjugated streptavidin 3,3′-diaminobenzidine tetrahydrochloride used as the chromogen. The specimens were counterstained with hematoxylin. To evaluate the number of $CD4^+$, $CD8^+$, and $NKR\text{-}P1^+$ IEL, both the number of immunoreactive cells in epithelial layer and total number of the epithelial cells in a well-shaped crypt section were scored for 10 crypts in each part of the large intestine. We then calculated the ratio of $CD4^+$, $CD8^+$, and $NKR\text{-}P1^+$ IEL against 100 epithelial-lining cells.

Organic Acid Concentration in Cecal Contents. The concentration of organic acids (succinate, lactate, acetate, propionate, butyrate, isovalerate, and valerate) in the rat cecal contents were measured by using HPLC (Shimadzu, Kyoto, Japan) according to Hoshi *et al.* (13). Briefly, sodium hydroxide aqueous solution containing crotonic acid as an internal standard was added to the cecal contents. After centrifugation, the fat-soluble substance in the supernatant was removed by extraction with chloroform. The aqueous phase was filtered through a membrane filter and applied to HPLC.

Statistical Analyses. All results are expressed as mean ± SEM. The data were analyzed by two-way analysis of variance (ANOVA; site and diet) for the distribution of $CD4^+$, $CD8^+$, and $NKR\text{-}P1^+$ intraepithelial cells. Statistical comparisons for body weight gain, food intake, colonic mucosal weight, and cecal mucosal weight cecal organic acid concentration were performed using Tukey's-Kramer's test at $P < 0.05$. All statistics have been done using JMP software (SAS Institute, Cary, NC)

Results

There was no difference in body weight gain (FF, CE, and SBF: 128 ± 2, 135 ± 3, and 135 ± 2 g/3 weeks, respectively) or mucosal weight of colorectum (FF, CE, and SBF: 1.17 ± 0.13, 1.28 ± 0.04, and 1.48 ± 0.06 g, respectively) among the three dietary groups. A significant difference was observed in total food intake during the 3 weeks test period. The total amount of food intake by SBF-fed rats (365 ± 6 g/3 weeks) was less than by CE-fed rats (384 ± 4 g/3 weeks), but greater than for FF-fed rats (341 ± 4 g/3 weeks). Cecal weight of SBF-fed rats (0.86 ± 0.03 g) was greater than that of the other groups (FF and CE: 0.65 ± 0.08 and 0.54 ± 0.06 g, respectively).

The number of $CD8^+$ IEL was highest in the cecal mucosa and was gradually reduced toward the distal part of the large intestine (Table II). On the other hand, the proportions of $CD4^+$ IEL and $NKR\text{-}P1^+$ IEL were similar in the surveyed area.

Dietary SBF promoted the accumulation of $CD8^+$ IEL, especially in the cecal mucosa. The effect of SBF ingestion on $CD8^+$ IEL accumulation became weaker toward the distal large intestine both in the number of the IEL in the crypt and the number of IEL/100 epithelial-lining cells. In contrast, ingestion of the nonfermentable fiber CE did not the proportion of $CD8^+$ IEL in the cecal mucosa. Ingestion of SBF and CE had no effect on the distribution of $CD4^+$ and $NKR\text{-}P1^+$ IEL in any site of the large intestine. These results indicate that ingestion of SBF strongly modifies the $CD8^+$ IEL distribution, but not $CD4^+$ and $NKR\text{-}P1^+$ IEL in the large intestine.

Cecum is the major fermenter in the large intestine of rodents. We measured the concentration of some organic acids in the cecal contents of rats fed these three diets (Table III). Acetate was the most abundant organic acid in cecum, and its concentration was higher in SBF-fed rats than in cecum of rats fed the control and CE diets. A similar result was obtained for butyrate. Similarly, concentrations of succinate and isovalerate were lower in the cecal contents of SBF-fed rats than for the other groups.

We further analyzed relationships between numbers of $CD8^+$ and $NKR\text{-}P1^+$ IEL and the concentrations of several organic acids in the cecum (Table IV). There was a signifi-

Table II. Number of intraepithelial lymphocytes (IEL) in the large intestine of WKAH/HkmSlc rats fed sugar beet fiber (SBF), crystalline cellulose (CE) or fiber-free (FF) diet

	No. of IEL/crypt section			No. of IEL/100 epithelial-lining cells		
	$CD8^+$	$CD4^+$	NKR-$P1^+$	$CD8^+$	$CD4^+$	NKR-$P1^+$
Cecum						
FF	1.32 ± 0.12	0.05 ± 0.02	0.07 ± 0.03	1.78 ± 0.18	0.06 ± 0.03	0.08 ± 0.04
CE	0.98 ± 0.09	0.02 ± 0.02	ND	1.32 ± 0.10	0.03 ± 0.02	ND
SBF	1.74 ± 0.18	0.02 ± 0.02	0.06 ± 0.02	2.26 ± 0.19	0.04 ± 0.02	0.07 ± 0.03
Proximal colon						
FF	0.54 ± 0.09	0.05 ± 0.02	0.07 ± 0.03	0.62 ± 0.12	0.06 ± 0.03	0.08 ± 0.04
CE	0.72 ± 0.10	0.03 ± 0.02	0.05 ± 0.02	0.90 ± 0.13	0.04 ± 0.03	0.06 ± 0.03
SBF	0.69 ± 0.15	0.09 ± 0.01	0.09 ± 0.04	0.84 ± 0.16	0.10 ± 0.02	0.10 ± 0.05
Distal colon						
FF	0.22 ± 0.03	0.03 ± 0.02	0.07 ± 0.02	0.27 ± 0.04	0.04 ± 0.03	0.08 ± 0.03
CE	0.18 ± 0.09	0.05 ± 0.03	0.03 ± 0.02	0.22 ± 0.11	0.06 ± 0.04	0.04 ± 0.03
SBF	0.20 ± 0.04	0.04 ± 0.02	0.04 ± 0.03	0.25 ± 0.05	0.05 ± 0.02	0.05 ± 0.03
Two-way ANOVA *P* value						
Diet	0.0202	NS	0.0161	0.0203	NS	NS
Site	<0.001	NS	NS	<0.0001	NS	NS
Diet x site	0.0049	NS	NS	0.0026	NS	NS

Dietary sugar beet fiber modified distribution of $CD8^+$ but not $CD4^+$ and NKR-$P1^+$ IEL. Basically, $CD8^+$ IEL frequency was reduced towards the distal large intestine. No difference of the distribution of $CD4^+$ IEL was observed among the dietary groups. Values are expressed as mean ± SEM, n = 6 (FF and CE), n = 7 (SBF).

Table III. Concentration of organic acids (μmol/g cecal contents) in the cecal contents of WKAH/HkmSlc rats fed sugar beet fiber (SBF), crystalline cellulose (CE) or fiber-free (FF) diet

	FF	CE	SBF
Succinate	1.79 ± 0.38^b	4.83 ± 0.69^a	0.18 ± 0.05^c
Lactate	0.86 ± 0.62	1.75 ± 1.19	0.11 ± 0.08
Acetate	77.7 ± 8.1^a	40.0 ± 4.9^b	91.1 ± 4.6^a
Propionate	21.8 ± 3.0^a	9.0 ± 1.1^b	15.3 ± 0.6^b
Butyrate	7.4 ± 1.2^b	5.5 ± 1.3^b	22.7 ± 1.9^a
Iso-valerate	1.54 ± 0.17^a	0.69 ± 0.08^b	0.14 ± 0.05^c
Valerate	2.15 ± 0.27^a	0.72 ± 0.16^b	1.33 ± 0.06^b

Values are expressed as mean ± SEM, n = 6 (FF and CE), n = 7 (SBF). Values in a row with different superscript letters, $P < 0.05$.

cant positive relationship between cecal $CD8^+$ IEL and cecal acetate and butyrate concentrations. In contrast, there was a negative correlation between cecal $CD8^+$ IEL and succinate concentration in the cecum. Cecal NKR-$P1^+$ IEL were positively correlated with cecal acetate and propionate concentrations.

Discussion

Comparison of IEL phenotypes and function between small and large intestine has received limited attention in rodents, despite many reports on the impact of various situations on the characteristics of IELs isolated from small intestine (14–16). In addition, there have been few histological analyses of the localization of IELs in intestinal crypts. Intestinal epithelium has a highly organized and well-defined architecture (17). *In situ* localization of specific cell types has the potential to provide useful insights about cell function. Previously, we reported that the proportion of $CD8^+$ IEL in the epithelial-lining cells is greater in the proximal than in the distal colon (10). In addition to confirming this observation, the present study revealed that the proportion of $CD8^+$ IEL in the cecum was much greater than in proximal colon (Table II).

This is the first report showing that dietary fiber is able to modify the proportion of $CD8^+$ IEL in crypt-basis and that the effect depends on the type of ingested dietary fiber. As expected, SBF, which promotes production of SCFAs in the cecal contents, increased the cecal $CD8^+$ IEL frequency in the epithelium (Table II). Interestingly, distribution of other IELs ($CD4^+$ and NKR-$P1^+$) was not affected by tissue region or by SBF. In addition, the proportion of cecal $CD8^+$ IEL was positively correlated with cecal concentration of acetate or butyrate (Table IV). These results indicate that dietary SBF selectively modifies $CD8^+$ IEL distribution, probably via the cecal fermentation pattern.

In contrast, the proportion of the cecal $CD8^+$ IEL has a negative regression with succinate concentration in the cecal contents (Table IV). Feeding certain type of indigestible saccharides leads to the accumulation of succinate. A high concentration of succinic acid was observed in rats fed a diet containing resistant starch prepared from amylomaise (18). Sakata *et al.* (19) reported that 75 m*M*/l of succinic acid infused into an isolated colon reduced crypt cell proliferation. The inhibitory effect of succinate on the epithelial proliferation may be related to $CD8^+$ IEL homing.

In our previous studies, we found that the administration of αAGM1, which reduces murine natural killer (NK) activity *in vitro* (20) and *in vivo* (21, 22), increased the 1,2-dimethylhydrazine-induced aberrant crypt foci in the rat colorectum (23). NK cells are considered to attack and eliminate abnormal cells (24). NKR-P1 (CD161) is one of

Table IV. Linear regression between IEL phenotype and cecal organic acid in WKAH/HkmSlc rats fed sugar beet fiber (SBF), crystalline cellulose (CE) or fiber-free (FF) diet

No. of IEL/crypt section			No. of IEL/100 epithelial-lining cells		
IEL phenotype	Organic acid	*P* value	IEL phenotype	Organic acid	*P* value
Cecal $CD8^+$			Cecal $CD8^+$		
	Succinate	−0.0016		Succinate	−0.0006
	Acetate	+0.0122		Acetate	+0.0060
	Butyrate	+0.0276		Butyrate	+0.0247
Cecal NKR-$P1^+$			Cecal NKR-$P1^+$		
	Acetate	+0.0414		Acetate	+0.0337
	Propionate	+0.0390		Propionate	+0.0381

The values show the probabilities having significantly positive (+) or negative (−) regression between IEL phenotype and organic acid concentration in the rat cecum ($P < 0.05$).

the C-type lectin molecules and a surface marker expressed on most of NK cells (25, 26). There were significant relationships between cecal NKR-$P1^+$ IEL distribution and several SCFAs (i.e., acetate or propionate), although the ratio of NKR-$P1^+$ IEL both per crypt section and per 100 epithelial-lining cells were below 0.1. Two-way ANOVA analysis revealed a significant difference of NKR-$P1^+$ IEL per crypt, but not per 100 epithelial cells between diets. In this study, 10 well-shaped crypt sections were analyzed in each part of the large intestine. There is a need to increase the number of crypt analyzed for more rigorous assessment of the affect of dietary fiber on the relative number of NKR-$P1^+$ cells.

The rationale for consideration of distribution of IEL both per crypt section and per 100 epithelial-lining cells is that diet may modify the number of the cells per crypt by affecting epithelial cell proliferation. In contrast, if diet affects the proliferation of selective subpopulation, cell number per 100 epithelial-lining cells will increase. The stimulatory effect of dietary SBF on $CD8^+$ IEL was sufficiently robust that the number of $CD8^+$ IEL per crypt and per 100 cells increased significantly.

The mechanism by which dietary SBF promotes increase in $CD8^+$ IEL population in the epithelial layer in the large intestine is unclear, although enhanced homing and stimulation of localized proliferation are most evident possibilities. For example, antigens on the surface of intestinal bacteria or increased concentrations of luminal SCFA(s) may stimulate epithelial chemokine(s) secretion, thereby stimulating $CD8^+$ IEL homing. The large intestine has a highly organized system for maintenance of its epithelial cell layer (17, 27) with stem cells in the base of the crypt and many chronogenic cells in the intermediate and differentiated cells at higher cell positions in crypt. The possibility that there is a difference between stem and differentiated epithelial cells in their abilities to attract IELs to the epithelial layer merits investigation. Such information may be useful for the prevention or treatment of diseases related to disruption of epithelial homeostasis.

Regarding the possibility that the increase in $CD8^+$ IEL is due to local proliferation of these cells in the cryptpatches (CPs), Ishikawa and colleagues (28) have shown a role for CP in the development of $CD8\alpha\alpha^+$ IEL. CPs are absent in mice with a defective cytokine-receptor γ chain gene (γc). When athymic γc-mutant mice were grafted with bone marrow or fetal liver cells from normal mice, CP and $CD8\alpha\alpha^+$ IEL appeared 2 weeks and 2 months after the graft, respectively. This observation indicates that CP contains a progenitor for $CD8^+$ IEL. Increase in organic acid concentration or changes of the luminal bacterial population may stimulate its proliferation.

Camerini *et al.* (29) reported that most of TCR-$\gamma\delta^+$ IELs isolated from large intestine are $CD4^-CD8^-$ cells as compared with TCR-$\gamma\delta^+$ IELs isolated from the small intestine, which are predominantly $CD8^+$. It is also interesting to study the influence of dietary factors on the physiological distribution of $CD8^+$ and TCR-$\gamma\delta^+$ IELs in the large intestine because TCR-$\gamma\delta^+$ T cells are closely related to intestinal epithelial homeostasis (6–8).

In conclusion, we demonstrated that dietary fiber has the ability to modulate the $CD8^+$ IEL distribution in the large intestine of rats. The effect is more marked in the cecum than the distal part of the large intestine. Furthermore, SBF, one of the fermentable dietary fibers, is more efficient than CE at increasing the $CD8^+$ population of IEL in the epithelial layer. Luminal fermentation is likely a critical event in this process, and SCFAs are candidates as the mediators of the observed response.

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