Characteristic Intestinal Microflora of Specific Pathogen-Free Mice Bred in Two Different Colonies and their Influence on Postnatal Murine Immunocyte Profiles

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Abstract: Cecal microflora of BALB/c mice originating from two different SPF-breeding colonies were compared. The analysis of cultivable bacteria in the ceca showed significantly higher numbers of total bacteria in BALB/cCrSlc (SLC mice) than in BALB/cA Jcl (JCL mice) ($p < 0.05$), which were mainly based on higher numbers and occurrence of *Peptococaceae*. *Bifidobacteria* were detected only in SLC mice. Feeding an oligosaccharide, raffinose, to the mice also induced different shifts in the composition of cecal microflora and the concentration of cecal organic acids. In the second experiment, hysterectomy-derived (HD) SLC mice were fostered to SPF lactating SLC mothers, or SPF lactating JCL mice, together with the mother's own natural birth (NB) pups in each isolator. HD mice fostered to SLC-mothers showed significantly higher percentages of T-cell receptor $\alpha\beta$ cells expressing a CD8 α homodimer ($p < 0.05$) and a CD8 $\alpha\beta$ heterodimer ($p < 0.001$) in the intraepithelial lymphocytes (IEL) compared with HD mice fostered to JCL-mothers. IEL profiles of HD mice corresponded well to those of NB mice that were breast-fed by the same mothers. Differences in the ratio of B220⁺ cells to Thy1.2⁺ cells in the splenocytes were also observed as a trend between both HD mice fostered to SLC or JCL mothers ($p = 0.06$). These results suggest that postnatal colonization of various characteristic intestinal microflora derived from SPF-breeding colonies results in differences in development of lymphocyte populations in the intestinal and systemic organs of mice.

Key words: immunity, intestinal microflora, intraepithelial lymphocytes, SPF mice

Introduction

It is considered that postnatal formation of intestinal microflora of mammals is influenced by bacterial contamination from the mother during lactation [5, 8]. It

has also been reported that the composition of intestinal microflora of adult mice was based on genetic factors in each mouse strain [7] including levels of major histocompatibility complex [11], and was not affected very much by bacterial contamination from

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the mothers [7]. Thus if mice, particularly inbred mice, are bred in barrier-sustained colonies, their intestinal microflora should be strongly affected by their mother's microflora because they are strictly isolated from the external environment. It is possible that the characteristic composition of microflora in each breeding colony might have different effects on characteristics of SPF mice. Microbes have various characteristics related to microbial metabolism, immunogens and pathogenicity in hosts. However, there are few reports showing differences in intestinal microflora of mice in SPF-breeding colonies, and also the influence of these differences on characteristics of SPF mice. In order to clarify the situation, we focused on whether different SPF-intestinal microflora affect development of immune organs because the immune system is susceptible to colonization of intestinal microbes. For example, germ-free animals are well known to show immunological underdevelopment of systemic and intestinal immune organs, such as the spleen, lymph nodes [4], Peyer's patches [10] and intraepithelial lymphocytes (IEL) [2, 14], compared with conventional animals.

In this study, we compared the cecal microflora of two BALB/c mice from different breeding colonies and also examined the composition of cecal microflora and the concentration of cecal organic acids after feeding indigestible oligosaccharide to both groups of mice. Lastly, we demonstrated that postnatal association with these 2 kinds of SPF-microflora in hysterectomy-derived mice induces development of different immunocyte profiles in the hosts.

Materials and Methods

Animals

SPF animals, female BALB/cCrSlc mice (SLC mice), female BALB/cAJcl mice (JCL mice), and pregnant mice, were purchased from Japan SLC (Hamamatsu, Japan) and CLEA Japan (Tokyo, Japan). The mice used for analysis of intestinal microflora were housed in plastic cages at $22 \pm 2^\circ\text{C}$ with a 12 h light-dark cycle under conventional conditions. The mice used for foster-nursing experiments were housed in vinyl isolators sterilized with 2% peracetic acid. Metal cages and water were sterilized by autoclaving at 121°C for 70 min. The isolators were placed in a room with a controlled 12 h light dark cycle at $24 \pm 1^\circ\text{C}$ with a relative humid-

ity of $55 \pm 5\%$. All mice were given sterilized diet and water *ad libitum*. All experiments were performed in accordance with the guidelines for the care and use of laboratory animals of The University of Tokyo.

Diets

The purified basal diet was composed of (g/kg) casein 200.0, maize starch 481.7, α,β -starch 90.0, sucrose 50.0, cellulose 50.0, soyabean oil 60.0, mineral mixture (AIN-76) 50.0, vitamin mixture (AIN-76) 13.0, choline chloride 2.3, and methionine 3.0 (1). The oligosaccharide diet was prepared by adding raffinose (Nippon Beet Sugar Mfg, Tokyo, Japan, purity > 995 g/kg DM) to the basal diet (50 g/kg diet) instead of maize starch. The diets were pelletized and vacuum-sealed in plastic bags by Funabashi Farm (Chiba, Japan), and were sterilized by γ -irradiation at 10 kGy.

Oligosaccharide feeding experiment

BALB/cCrSlc mice and BALB/cAJcl (6 weeks of age) were randomly allocated to two groups ($n=5$ per group, total 4 groups). Each group was fed the basal diet or the oligosaccharide diet for 2 weeks. After the mice were sacrificed by cervical dislocation, ceca removed from the mice were each subjected to analysis of intestinal microflora and organic acids.

Foster-nursing experiment: association with different SPF microflora

Ten pregnant BALB/cCrSlc mice were maintained under SPF conditions. After the pups were removed from the pregnant mice by hysterectomy, the pups were randomly fostered to BALB/cCrSlc lactating mothers ($n=5$) or BALB/cAJcl lactating mothers ($n=5$), together with the mother's own natural birth pups in each vinyl isolator. After both the hysterectomy-derived (HD) and natural birth (NB) pups were weaned at 4 weeks of age, they were kept in the same isolators until 6 weeks of age. Thereafter, HD and NB mice were sacrificed by cervical dislocation, and the small intestine, spleen and mesenteric lymph nodes (MLN) removed from these mice were each subjected to flow cytometric analysis.

Analysis of intestinal microflora

Bacteriological analysis of mice cecal contents was carried out according to the method of Mitsuoka [9]. Briefly, fresh samples were immediately diluted in 10-

fold steps with anaerobic phosphate buffer and 0.05 ml of each dilution was inoculated on two non-selective media (BL and TS agar) and three selective media (mLBS, DHL and TATAC agar). BL and mLBS agar plates were incubated at 37°C for 48 h in an anaerobic steel wool jar filled with oxygen-free CO₂, and TS, DHL and TATAC agars were incubated for 24–48 h aerobically. Bacterial groups were identified using colony and cell morphology, Gram staining, spore formation and aerobic growth.

Determination of cecal organic acids

Cecal contents were weighed and homogenized with a 20-fold volume of 0.2 N HCl. Pyroglutamic acid was used as the internal standard. Organic acids in the cecal contents were determined by high performance liquid chromatography using the postcolumn method with bromothymol blue as pH indicator (wave length for detection; 445 nm, column; RSpak KC-811, Showa Denko K.K., Tokyo, Japan).

Lymphocyte preparation

IEL were prepared as previously described [6]. In brief, contents of the small intestines of mice were thoroughly washed out with Hank's balanced salt solution (HBSS; Gibco BRL, Gaithersburg, MD). Each intestine was inverted, and individually transferred to a 50-ml conical tube containing 45 ml of HBSS supplemented with 5% (v/v) fetal calf serum (Sigma-Aldrich, Mo, USA). The tubes were shaken at 150 rpm in the horizontal position for 45 min at 37°C, and then were shaken several times by hand. Each resultant cell suspension was collected and filtered through a glass-wool column. Subsequently, the cells were suspended in 30% Percoll (Pharmacia Biotech, Uppsala, Sweden) solution and centrifuged at 400 × g for 20 min. Cells pelleted at the bottom of the tube were applied to Percoll discontinuous density gradient centrifugation. IEL were recovered at the 44 to 70% Percoll interface. Single-cell suspensions of splenocytes and MLN cells were prepared by mashing the organ with the end of a syringe, and passing each cell suspension through a polyester mesh.

Flow cytometry

Lymphocytes (5×10^5 cells) were incubated with fluorochrome-labeled or biotinylated monoclonal anti-

bodies (mAb) for 20 min in ice water after blocking Fc receptors with anti CD16/32 mAb (2.4G2; PharMingen, San Diego, CA). If necessary, fluorochrome-labeled streptavidins were used as the second antibodies. After staining, the cells were washed with HBSS containing 1% (w/v) fetal calf serum and 0.01% (w/v) sodium azide. Cytofluorometric analysis was performed by flow cytometry (FACSort, Becton Dickinson, Franklin Lakes, NJ). The following mAb were used: biotin-anti-TCR β mAb (H57-597; PharMingen), fluorescein isothiocyanate (FITC)-anti-TCR γ mAb (GL-3; Cedarlane, Hornby, Ontario, Canada), biotin-anti-TCR γ mAb (GL-3, Cedarlane), FITC-anti-CD4 mAb (H129.12; PharMingen), phycoerythrin (PE)-anti-CD8 α mAb (53-6.7; Gibco BRL), FITC-anti-CD8 β mAb (Y8.77; Seikagaku-Kogyo, Tokyo, Japan), PE-anti-B220 mAb (RA3-6B2, Gibco BRL), FITC-anti-Thy1.2 mAb (30-H12, PharMingen), FITC-streptavidin (Gibco BRL), PE-streptavidin (Gibco BRL), and RED 670-streptavidin (Gibco BRL); all were purchased from the sources indicated.

Statistical analysis

Results were expressed as mean values with standard deviations. Differences in bacterial counts and organic acid concentrations in the ceca of mice were analyzed using Scheffe's F post-hoc test, and differences in occurrences of microbes were analyzed using Fisher's exact probability test. Differences in lymphocyte profiles of mice were analyzed using Student's *t* test.

Results

Intestinal microflora of mice bred in different colonies

Differences in cecal microflora between SLC mice and JCL mice were compared in terms of cultivable bacteria (Table 1). SLC mice showed significantly higher numbers of total bacteria than JCL mice ($p < 0.05$). In particular, higher numbers and significantly higher occurrences of Peptococaceae in SLC mice were observed compared with those in JCL mice ($p < 0.05$). The percentage of Peptococaceae to total bacteria was $52 \pm 16\%$ in SLC mice, while that in JCL mice was below 4%. Bifidobacteria were detected only in SLC mice. There were no differences between groups in numbers of facultative aerobes such as Lactobacilli, Enterobacteriaceae and Streptococaceae. Feeding of the oligosaccharide diet

Table 1. Comparison of cecal microflora of BALB/cAJcl and BALB/cCrSlc fed basal diet or oligosaccharide diet

	Basal diet		Oligosaccharide diet	
	BALB/cAJcl	BALB/cCrSlc	BALB/cAJcl	BALB/cCrSlc
Total bacteria	9.1 ± 0.2 (100) ^a	9.6 ± 0.3* (100)	9.5 ± 0.1 (100)	9.9 ± 0.3 (100)
Bacteroidaceae	8.8 ± 0.2 (100)	9.1 ± 0.2 (100)	9.4 ± 0.2 [#] (100)	9.3 ± 0.4 (100)
Bifidobacteria	(0)	7.2 (40)	(0)	7.8 ± 0.4 (100)
Eubacteria	7.9 (40)	7.9 (40)	8.3 (20)	8.6 ± 0.6 (80)
Peptococaceae	7.6 (20)	9.3 ± 0.4 (100)*	6.6 (20)	9.6 ± 0.2 (100)
Clostridia	(0)	7.2 (40)	8.1 (40)	7.8 ± 0.4 (100)
Fusiform bacteria	(0)	(0)	8.0 ± 0.2 (60) [#]	(0)
Curved rods	7.6 (20)	(0)	7.6 (40)	(0)
Lactobacilli	8.6 ± 0.4 (100)	8.5 ± 0.2 (100)	8.0 ± 0.6 (100)	8.3 ± 0.4 (100)
Enterobacteriaceae	5.4 ± 0.5 (100)	5.6 ± 0.4 (100)	4.9 ± 0.9 (100)	4.4 ± 0.2 [#] (100)
Streptococaceae	6.6 ± 0.3 (100)	6.5 ± 0.5 (100)	6.6 ± 0.0 (100)	6.5 ± 0.7 (100)

The mice were fed purified basal diet or oligosaccharide diet (containing raffinose at 50 g/kg) for 2 weeks. a) Values are expressed as mean ± S.D. of bacterial counts (log no./g cecal contents). Figures in parentheses refer to frequency of occurrence (%). Significant difference * ($p < 0.05$) between BALB/cA and BALB/cCr fed basal diet, # ($p < 0.05$) between basal diet and oligosaccharide diet within the same mouse strain.

Table 2. Cecal organic acids of BALB/cAJcl and BALB/cCrSlc fed basal diet or oligosaccharide diet

	Basal diet		Oligosaccharide diet	
	BALB/cAJcl	BALB/cCrSlc	BALB/cAJcl	BALB/cCrSlc
Acetic acid	25.4 ± 7.5	23.1 ± 7.8	25.5 ± 8.8	29.2 ± 6.4
Propionic acid	3.3 ± 0.7	3.7 ± 0.8	4.8 ± 2.0	8.0 ± 1.9 [#]
<i>n</i> -Butyric acid	2.7 ± 0.7	3.1 ± 1.6	4.7 ± 3.2	9.2 ± 3.0 [#]
Formic acid	0.1 ± 0.1	1.1 ± 0.4*	0.0 ± 0.0	0.2 ± 0.3 [#]
Succinic acid	0.0 ± 0.0	0.1 ± 0.1	0.2 ± 0.4	0.2 ± 0.2
Lactic acid	0.3 ± 0.2	0.0 ± 0.0	0.2 ± 0.2	1.2 ± 1.1 [#]

Values are expressed as mean ± S.D. of organic acids (μmol/g cecal contents) of mice. * and #; see footnotes of Table 1.

(50 g raffinose/kg diet) to JCL mice for 2 weeks significantly increased numbers of Bacteroidaceae ($p < 0.05$) and occurrence of fusiform bacteria ($p < 0.05$). In contrast, SLC mice fed oligosaccharide diet showed increasing tendencies in the numbers or occurrences of various strict anaerobes such as Bacteroidaceae, bifidobacteria, eubacteria, Peptococaceae and clostridia, and a significant decrease in the number of Enterobacteriaceae ($p < 0.05$). On the other hand, the organic acid concentration in the ceca of mice fed a basal diet showed a significant difference only in formic acid concentration between SLC and JCL mice (Table 2). There were no significant differences in cecal organic acids of JCL-mice between mice fed the basal diet and the oligosaccharide diet. However, the oligosaccharide diet

fed to SLC mice induced significant increases of cecal propionic, butyric and lactic acid concentrations compared with those fed the basal diet ($p < 0.05$).

Effect of postnatal association with different SPF microflora on lymphocyte profiles of the mice

HD mice were fostered to SPF lactating SLC mothers or SPF lactating JCL mothers in each isolator together with NB pups. With this method, intestinal microflora of HD mice should have been transferred from foster mothers. There were marked differences in the IEL populations between SLC-NB mice and JCL-NB mice (Fig. 1). The SLC-NB mice showed higher percentages of TCRαβ IEL and lower percentages of TCRγδ IEL than the JCL-NB mice. The high percent-

Fig. 1. Comparison of intraepithelial lymphocyte profiles among natural birth and hysterectomy-derived BALB/c mice fostered to lactating mothers from different colonies. The hysterectomy-derived (HD) SLC mice were fostered to SPF lactating mothers together with the mother's own natural birth (NB) pups until 4 weeks of age. Data show means of each population in intraepithelial lymphocytes of NB and HB mice at 6 weeks of age. a) JCL; BALB/cAJcl. b) SLC; BALB/cCrSlc.

age of TCR $\alpha\beta$ IEL in the SLC-NB mice consisted of increased percentages of both CD8 $\alpha\alpha$ ⁺ cells and CD8 $\alpha\beta$ ⁺ cells. The percentages of CD8 $\alpha\beta$ ⁺ cells and CD8 $\alpha\alpha$ ⁺ cells in SLC-NB mice were 4-fold and 2-fold higher, respectively, than those in JCL-NB mice. There were few differences in the percentages of CD4⁺ TCR $\alpha\beta$ IEL between SLC-NB mice and JCL-NB mice. On the other hand, IEL profiles of HD mice show good agreement with those of NB mice that were breast-fed by the same mothers. HD mice fostered to SLC-mothers showed significantly higher percentages of TCR $\alpha\beta$ IEL expressing CD8 $\alpha\alpha$ ($p < 0.05$) and CD8 $\alpha\beta$ ($p < 0.001$) compared with HD mice fostered to JCL-mothers. Therefore, the ratio of TCR $\alpha\beta$ IEL to TCR $\gamma\delta$ IEL was significantly higher in SLC-fostered HD mice compared with JCL-fostered HD mice ($p < 0.05$: SLC-fostered HD mice, 2.4 ± 0.2 ; JCL-fostered HD mice, 0.4 ± 0.1). Moreover, the ratio of B220⁺ cells to Thy1.2⁺ cells in the spleen of SLC-fostered HD mice tended to be higher than that in JCL-fostered HD mice (Table 3).

Discussion

It is possible that the compositions of intestinal microflora in SPF experimental animals might differ

Table 3. Ratio of B220⁺ cells to Thy1.2⁺ cells in the spleen and mesenteric lymph nodes of hysterectomy-derived mice fostered to lactating mothers for different colonies

	Foster mother		<i>p</i>
	JCL ^{a)}	SLC ^{b)}	
Spleen	1.88 ± 0.39	2.33 ± 0.06	0.06
Mesenteric lymph nodes	0.42 ± 0.09	0.52 ± 0.07	0.11

Ratio of B220⁺ cells to Thy1.2⁺ cells in the spleen and mesenteric lymph nodes of hysterectomy-derived mice shown in Fig. 1 (means $\pm$ SD, $n=4$) *p* values were calculated using Student's *t* test. a, b) see footnotes in Fig. 1.

among breeding colonies, and this difference might have some effect on development of immune organs in the animals. In order to prove this hypothesis, cecal microflora of different colonies of BALB/c mice, BALB/cCrSlc and BALB/cAJcl, were compared. An analysis of cultivable bacteria in the ceca showed higher numbers and occurrences of Peptococaceae in SLC mice than in JCL mice. Bifidobacteria were also detected only in SLC mice. These results show that there was a major difference in composition of cecal anaerobes between JCL and SLC BALB/c mice. Raffinose is known to be a fermentable indigestible oligosaccharide and growth factor of bifidobacteria *in vitro* [15] and in humans *in vivo* [3]. Feeding this sugar to mice also induced different changes of various bacterial counts between the two mice colonies. Analysis of changes in cecal organic acids by oligosaccharide feeding also gave different results: a significant increase of some organic acids in SLC mice but no changes in JCL mice. The difference in metabolism of orally fed oligosaccharide by indigenous microflora suggests that not only cultivable bacterial counts in the intestine but also essential microbes composing microflora differed in SLC and JCL mice.

In the second experiment, HD mice were fostered to SPF lactating SLC mothers or SPF lactating JCL mothers together with the mother's NB pups. This method resulted in association of HD and NB mice with the intestinal microflora of the foster mother in the isolator. HD and NB mice acquired identical intestinal microflora because the BALB/cCr and BALB/cA mice used in the present study have identical genetic backgrounds. SLC-NB mice and SLC-fostered HD mice showed significantly higher percentages of TCR $\alpha\beta$ cells

expressing CD8 molecules in IEL compared with those of JCL-NB and JCL-fostered HD mice. This result suggests that SLC-flora induce expansion of CD8⁺ TCR $\alpha\beta$ IEL more strongly than JCL-flora. Moreover, the ratios of B220⁺ cells (B-cell marker) to Thy1.2⁺ cells (T-cell marker) in the splenocytes also differed between SLC-fostered HD mice and JCL-fostered HD mice.

It is known that the number of TCR $\alpha\beta$ IEL in germ-free mice is greatly reduced compared with conventional mice [2, 14]. The ratios of TCR $\alpha\beta$ cells to TCR $\gamma\delta$ cells in the IEL of the JCL-fostered HD mice in our results showed good agreement with those of germ-free mice reported previously (JCL-fostered HD mice, 0.4 ± 0.1 ; germ-free mice, 0.39 as reported in Reference No.13). These results suggest that JCL-flora might lack microbes able to induce expansion of CD8⁺ TCR $\alpha\beta$ IEL. It has been reported that mono-association with segmented filamentous bacteria (SFB) in germ-free mice induced marked expansion of CD8⁺ TCR $\alpha\beta$ IEL in the small intestine from germ-free levels to conventional levels [13]. In the present study, we were not able to clarify whether SFB colonized the intestinal lumens of JCL and SLC mice because SFB is a non-cultivable bacteria [12].

In conclusion, we showed that BALB/c mice bred in two different colonies have essentially different compositions of intestinal microflora in terms of not only bacterial counts but also metabolic responses to oligosaccharides. Moreover, postnatal association with each microflora in HD mice resulted in different development of lymphocyte populations in the intestinal and systemic immune systems. Specific indigenous microflora of each breeding colony might affect not only development of the immune system but also systemic metabolism and pathology. We consider that investigators should pay more attention to the possibility that results obtained from animal experiments might be influenced by characteristic microflora of each breeding colony.

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