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ORIGINAL ARTICLE

Change of Intestinal Microbiota with Elemental Diet and Its Impact on Therapeutic Effects in a Murine Model of Chronic Colitis

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Abstract Elemental diet (ED) has been used as an enteral nutritional therapy for Crohn's disease. However, the precise mechanisms of ED remain unclear. In interleukin-10 (IL-10)-deficient cell-transferred mice, we investigated the change of intestinal microbiota with ED using molecular terminal-restriction fragment length polymorphism (T-RFLP) analysis and culture method, and evaluated its influence on therapeutic effects of ED. ED significantly suppressed intestinal inflammation. The total amount of bacteria in colitis mice fed the regular diet was higher than in normal mice but decreased in colitis mice fed ED. T-RFLP profiles of the ED group markedly differed from those of the regular diet groups. The diversity of bacterial species in the ED group decreased to 60% of that found in the regular diet groups. Among the cultivated bacteria, the change in lactic acid bacteria composition was remarkable. *Lactobacillus reuteri* and *L. johnsonii* decreased and *Enterococcus faecalis* and *E. durans* increased in the ED group. The culture supernatant of *L. reuteri* isolates induced significant tumor necrosis factor-alpha (TNF-α) and IL-6 activity in RAW 264 cells, while the culture supernatant of *E. faecalis* and *E. durans* barely induced their activity. These data suggested that reduction in amount and diversity of intestinal microbiota and decrease of proinflammatory cytokines via a change in composition of lactic acid bacteria by ED seem to contribute to reduction of bowel inflammation in this model.

Keywords IL-10 deficient mice · Elemental diet · Microbiota · Inflammatory cytokines

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Introduction

Inflammatory bowel disease (IBD) is supposed to be the result of interactions among genetic, immune, and environmental factors [1]. Intestinal microbiota is an important environmental factor in the etiology of IBD. Antibodies against *Saccharomyces cerevisiae* (ASCA), *Pseudomonas fluorescens*-associated sequence I2 and the outer membrane porin protein C of *Escherichia coli* (OmpC) have been specifically detected in patients with Crohn's disease (CD) [2–6]. On the other hand, no inflammation or colitis is induced in certain murine and rodent models maintained under germ-free conditions [7, 8]. Among animal models, interleukin-10-deficient (IL-$10^{-/-}$) mice have been used to investigate the influence of microbiota on the development of colitis, because the effect of intestinal microbiota on the immune response to intestinal inflammation is associated with an apparently dysregulated production of Th1-type proinflammatory cytokines, similar to that observed in CD [9–12]. Some modifications of the enteric microbiota can attenuate colitis in this model [13, 14].

In Japan, elemental diet (ED; Elental®, Ajinomoto Co. Inc., Tokyo, Japan) has been used as the primary therapy for CD, showing an effectiveness equivalent to that of corticosteroids [15–18], but the precise mechanisms underlying the therapeutic effects of ED remain unclear. ED is a special formula that contains amino acids as the source of elemental nitrogen, together with other easily

digestible nutrients, minerals, and vitamins. Fat is present in a very small quantity. The intestinal microbiota is strongly influenced by nutrients; thus the intestinal microbiota of CD patients treated with ED is assumed to change.

The objectives of this study were: (i) to examine the effect of ED using IL-$10^{-/-}$ cell-transferred mice showing chronic inflammations and immunological characteristics with human CD [19], (ii) to characterize the differences in the intestinal microbiota between mice fed a regular diet and those fed the elemental diet, and (iii) to elucidate whether the change in intestinal microbiota contributed to the therapeutic effects of ED.

Materials and Methods

Animal Experiment

IL-$10^{-/-}$ cell-transferred colitis mice were used. Transfer of IL-$10^{-/-}$ cells was done using the methods described by Ikenoue et al. [19]. Briefly, spleen and mesenteric lymph node (MLN) cells from diseased male IL-$10^{-/-}$ mice were isolated and single-cell suspensions were prepared by mechanical dissociation. Erythrocytes were removed by hypotonic lysis. Female CB-17 SCID mice, 8–12 weeks of age, were injected intraperitoneally 1.0×10^7 cells suspended in 200 µl phosphate buffered saline (PBS). IL-$10^{-/-}$ cell-transferred and nontransferred mice were fed a regular diet (CRF-1, Charles River Japan, Inc., Tokyo, Japan) or elemental diet (dextrin 79.3%, amino acids 17.6%, soybean oil 0.6%, minerals 2.0%, vitamins 0.5%, by weight) for 1, 2, and 3 weeks. Three groups of mice were used: cell-transferred mice fed the regular diet (TRD group); cell-transferred mice fed the elemental diet (TED group); and nontransferred mice fed the regular diet (NRD group) as the healthy control. The mice were sacrificed, then their cecum and colon were removed and weighed. Cecal contents were used for the analysis of microbiota and short-chain fatty acids (SCFAs) while the colon was histologically examined. Each sample was identified by group name, breeding period, and mouse identification (ID) number.

Histological Analysis

Tissues samples were fixed in 10% phosphate-buffered formalin. Paraffin-embedded sections were stained with hematoxylin and eosin (H–E) for light microscopic examination.

Cell Lysis and DNA Isolation from Cecal Samples

DNA was isolated using Ultra Clean™ Soil DNA Isolation Kit (Mo Bio Laboratories Inc., Solana Beach, CA). The sample (0.05 g) was suspended in a bead solution containing 5 mg/ml lysozyme and 0.08 mg/ml *N*-acetylmuramidase, and incubated for 30 min at 37°C to lyse the cells. DNA was extracted and purified as described by Clement and Kitts [20] with some modifications.

PCR and T-RFLP Analysis

Polymerase chain reaction (PCR) amplification was performed as described by Kibe et al. [21]. Two pairs of primers were used: 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′) as the universal primer, and 27f and Lab-677r (5′-CACCGCTACACATGGAG-3′) as the specific primer set for lactic acid bacteria (LAB) designed by Heilig et al. [22]. 27f was labeled with 6-FAM (6-carboxyfluorescein, Applied Biosystems, Foster City, CA) for T-RFLP analysis. PCR was performed using a Thermocycler T Gradient (Biometra, Gottingen, Germany) in a reaction mixture (50 µl) containing 5 µl dissolved DNA (100 ng), 1.25 U TaKaRa Ex Taq™ (Takara, Shiga, Japan), 10 × Ex Taq™ buffer, 4 µl dNTP mixture (2.5 mM each), and 10 pmol of each primer. The amplification program used was as follows: preheating at 95°C for 3 min; 30 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 1.5 min; and final extension at 72°C for 10 min. The PCR products were purified by polyethylene glycol (PEG) precipitation method [23]. Purified PCR products were digested with 20 U of either *Hha*I or *Msp*I (Takara) in total volume of 10 µl at 37°C for 3 h. Length of the terminal-restriction fragments (T-RFs) was determined based on standard size markers GS500 ROX and 1000 ROX (Applied Biosystems) using the ABI PRISM™ 3100 genetic analyzer (Applied Biosystems) and GeneScan® analysis software (Applied Biosystems). A dendrogram analysis was performed using T-RFLP patterns by using BioNumerics software (Applied Maths Sint-Martens-Latem, Belgium). Distances between samples were represented graphically by constructing a dendrogram based on Jaccard's similarity coefficients and Ward's algorithm type. The position tolerance of band matching was 0.05%.

Analysis of Short-Chain Fatty Acids

A portion of cecal contents was deproteinated with perchloric acid and used to measure short-chain fatty acids (SCFAs). Cecal SCFA content was measured as follows: the sample was centrifuged and the supernatant was subjected to high-performance liquid chromatography as described by Teramoto et al. [24].

Culture and Isolation of Bacteria

Culturable bacteria in the sample obtained at the end of the experiment (week 3) were detected and isolated by the serial dilution method. Fresh cecal contents were serially diluted with prereduced dilution buffers, and 0.05 ml samples of 10^6, 10^7, and 10^8 dilutions were plated on nonselective BL agar (Nissui Seiyaku, Tokyo, Japan), EG agar (Merck, Darmstadt, Germany), and TS agar (Becton Dickinson, Franklin Lake, NJ); and 0.05 ml of 10^1,10^3, 10^5, and 10^7 dilutions were plated on selective LBS agar (Becton Dickinson) for *Lactobacillus*, and DHL agar (Nissui Seiyaku) for *Enterobacteria* [25]. BL, EG, and LBS agar plates were subsequently incubated at 37°C for 2 days under anaerobic conditions with CO_2 gas, while TS and DHL agar plates were subsequently incubated at 37°C for 1 day under aerobic conditions. Bacteria were identified by macroscopic observation of the colonies and microscopic examination of cells with Gram staining.

Isolation and Identification of Lactic Acid Bacteria (LAB)

Twenty-four strains of *Lactobacillus* growing on LBS agar plates and 16 strains found on TS agar plates and presumed to be *Streptococcus* or *Enterococcus*, were transferred with a sterile toothpick to 50 µl 10 mM Tris–HCl-1 mM ethylenediamine tetraacetic acid (EDTA) (pH 8.0) (TE buffer). One microliter of the suspension was smeared onto a glass slide for Gram staining. The remaining suspension was boiled for 15 min to lyse the cells and the lysate was used as template for the PCR. Nearly complete (1,500 bases) 16 S rRNA gene sequences of the strains investigated were amplified by PCR using universal primers 27f and 1492r. PCR products were sequenced using a BigDye Terminator cycle sequencing kit and the ABI PRISM^{TM} 3100 Genetic Analyzer (both from Applied Biosystems). The closest relatives of the isolates were identified through database searches, and the sequences of closely related species were retrieved from DNA Data Bank of Japan (DDBJ), Europian Molecular Biology Laboratory (EMBL), and Genbank.

Cytokines Induction of LAB Culture Supernatant in the Murine Macrophage Cell Line

Isolates identified as LAB were cultured in *Lactobacillus* MRS broth (Becton Dickinson) for 2 days at 37°C under anaerobic conditions. In vitro cytokines induction assays were carried out using LAB culture supernatant as described by Peña et al. [26] with some modifications. Briefly, media conditioned by LABs were tested for the ability to induce the production of TNF-α and IL-6 by the murine macrophage cell line RAW 264 (JCM RCB0535). RAW 264 macrophages were incubated in Dulbecco's Modified Eagles Medium (D-MEM; Sigma-Aldrich, Taufkirchen, Germany) supplemented with 10% fetal bovine serum (FBS; MP Biomedicals LLC, Morgan Irvine, CA), 20 units/ml sodium penicillinG, and 20 µg/ml streptomycin sulfate for 3 days and then exposed to 5% (v/v) LAB culture supernatant in 24-well flat-bottom plates. After 6 h, the culture supernatant was collected and TNF-α and IL-6 production was measured using a BD Opt EIA^{TM} Mouse TNF Mono/Mono Set (Becton Dickinson) and a BD Opt EIA^{TM} Mouse IL-6 enzyme-linked immunosorbent assay (ELISA) Set (Becton Dickinson), respectively.

Statistical Analysis

Data are presented as mean ± standard error (SE). Differences between two groups were evaluated using Student's *t*-test and Dunnett's test. The level of statistical significance was set at <0.05.

Results

Effect of ED on Inflammation in Colitis

We assessed the therapeutic effects of ED in IL-10 (−/−) cell-transferred mice. As shown in Fig. 1a, mice in the TRD group progressively lost weight during the 3 weeks after cell transfer. In contrast, mice in the TED group did not lose weight. At week 1, colon weight was similar in the three groups, but at weeks 2 and 3 it increased significantly due to inflammation in the TRD group, while in the TED group it was significantly lower than in the TRD group (Fig. 1b). At week 3, histological examination of the colon revealed massive inflammatory infiltrates, crypt hyperplasia, and marked mucin depletion of goblet cells in the TRD group, whereas in the TED group histological damage was markedly suppressed (Fig. 1c, H–E staining).

Weight and SCFAs Content in the Cecum

Cecal weight and SCFAs content measured at week 3 after cell transfer are shown in Table 1. Cecal weight in the TRD3W group was significantly greater than that in the NRD3W group, and in the TED3W group it was significantly smaller than in the TRD3W group ($P < 0.01$). We also measured cecal SCFAs content. Total SCFA concentration in the TRD3W group was about twofold that in the NRD3W group. Concentrations of malic acid, succinic acid, and lactic acid were especially higher in the TRD3W group compared with in the other two groups. Higher concentrations of propionic acid and valeric acid were characteristic of the TED3W group.

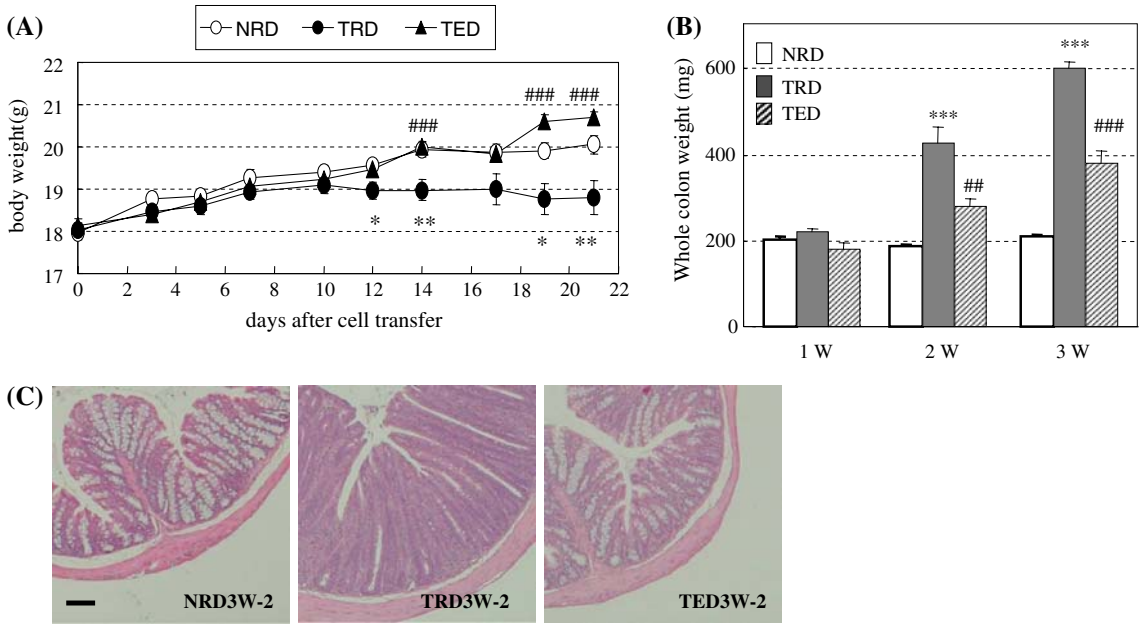

Fig. 1 Inhibitory effect of ED on inflammation in colitis. **a** Body weight. **b** colon weight after several breeding periods $^{***}P < 0.001$ versus NRD group, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus TRD group (*t*-test). **c** Histology of representative sections of the colon specimens; bar = 100 μm

Table 1 Weight and organic acid content in cecum at week 3 after cell transfer

Characteristic	NRD3W	TRD3W	TED3W
Cecum weight (mg)[a]	230 ± 8[b]	450 ± 16[b]	119 ± 22[b]
Organic acids (mg/g)[c]	23.896	50.244	26.128
Malic acid	0.862	6.266	1.090
Succinic acid	0.232	9.087	1.184
Lactic acid	6.453	12.704	3.439
Formic acid	1.338	3.380	2.754
Acetic acid	9.407	13.926	12.773
Propionic acid	1.335	2.556	2.951
i-Butyric acid	0.842	0.115	0.323
Butyric acid	3.225	1.935	0.896
i-Valeric acid	0.055	0.070	0.312
Valeric acid	0.146	0.205	0.406

[a] Mean ± standard deviation (SD)

[b] $P < 0.01$ versus the other two groups

[c] Concentration of cecum contents

T-RFLP

Remarkable differences in T-RFLP profiles were observed between the TED group and the other two groups, while the difference between NRD and TRD groups was quite small (Fig. 2). In the TED group, many T-RFs disappeared, the proportion of these T-RFs decreased, and a few new T-RFs emerged or the proportion of specific T-RFs increased. A cluster analysis done by combining T-RFLP profiles derived from *Msp*I and *Hha*I revealed that all samples from the TED group distributed within its cluster while most samples from the NRD and TRD groups were in the same cluster (Fig. 3). The difference between the TED group and NRD group or TRD group was more marked than between the NRD group and TRD group. The similarity index increased in relation to the breeding period. The number of T-RFs in the TED group decreased to 60% of that in the other groups (Fig. 4). The alteration in the composition of LAB in the TED group was also confirmed by T-RFLP profiles. The size of some T-RFs obtained with universal and LAB primers coincided, indicating that LAB accounted for a large proportion of the bacterial population in this murine intestinal microbiota.

Cultivation of Predominant Bacteria

The population of major groups of cecum-derived cultivated bacteria is shown in Table 2. Among anaerobic bacteria, the genera *Bacteroides* and *Clostridium* predominated, and their population did not differ among the three groups. On the other hand, the population of *Enterobacteria* and LAB differed among the three groups. Especially, the difference in LAB composition, including the genera *Lactobacillus*, *Streptococcus*, and *Enterococcus*, was significantly distinct. The population of *Lactobacillus* in the cecum was approximately 2×10^9 cells/g in the NRD3W and TRD3W groups and approximately 1×10^7 cells/g in the TED3W group, whereas the genus *Enterococcus* was detected only in the TED3W group.

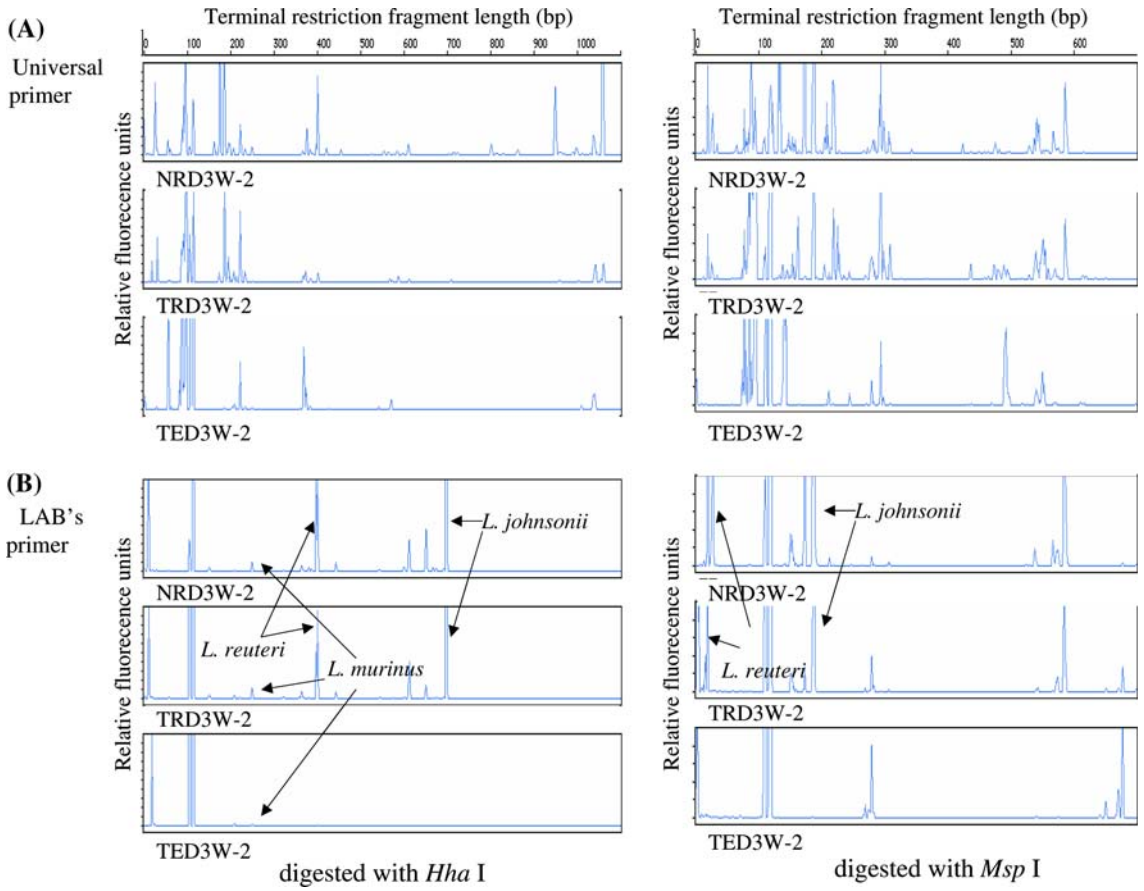

Fig. 2 T-RFLP pattern of 16 S rDNA from the cecum digested with *Hha*I (left) and *Msp*I (right). **a** 16 S rDNA was amplified with universal primers. **b** LAB specific primers. Arrows indicate the *Lactobacillus* spp. determined from the DNA sequence data of the isolates listed in Table 3

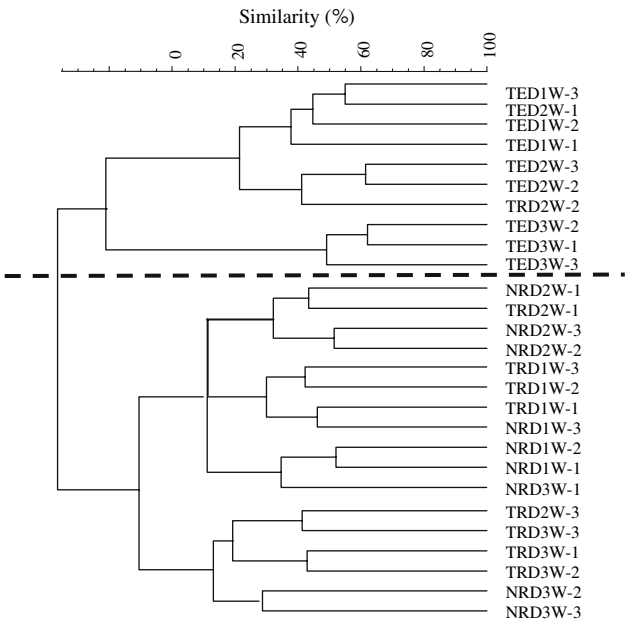

Fig. 3 Dendrogram illustrating the relatedness of T-RFLP patterns obtained with universal primers in each group

Isolation and Determination of LAB Species

LAB species from colonies isolated on agar plates were identified to clarify the change in LAB (Tables 3 and 4). In TRD3W mice, most *Lactobacillus* isolates were identified as *Lactobacillus reuteri* by 16 S rRNA gene sequence and three isolates were identified as *L. johnsonii*. In the TED3W mice, most of the *Lactobacillus* isolates were identified as *L. murinus*. As for the genera *Streptococcus* and *Enterococcus*, most isolates in the TED3W group were identified as *Enterococcus faecalis* and *E. durans*.

Cytokines Induction in RAW264 Cells by LAB Culture Supernatant

The culture supernatant of all *L. reuteri* isolates obtained from mice fed the regular diet significantly induced TNF-α (14,200 ± 2,300 pg/ml, $P < 0.001$) and IL-6 (643 ± 91 pg/ml, $P < 0.001$), whereas the culture supernatant of *L. murinus* isolates obtained from the TED3W group induced half the activity of TNF-α and a quarter of the activity of IL-6

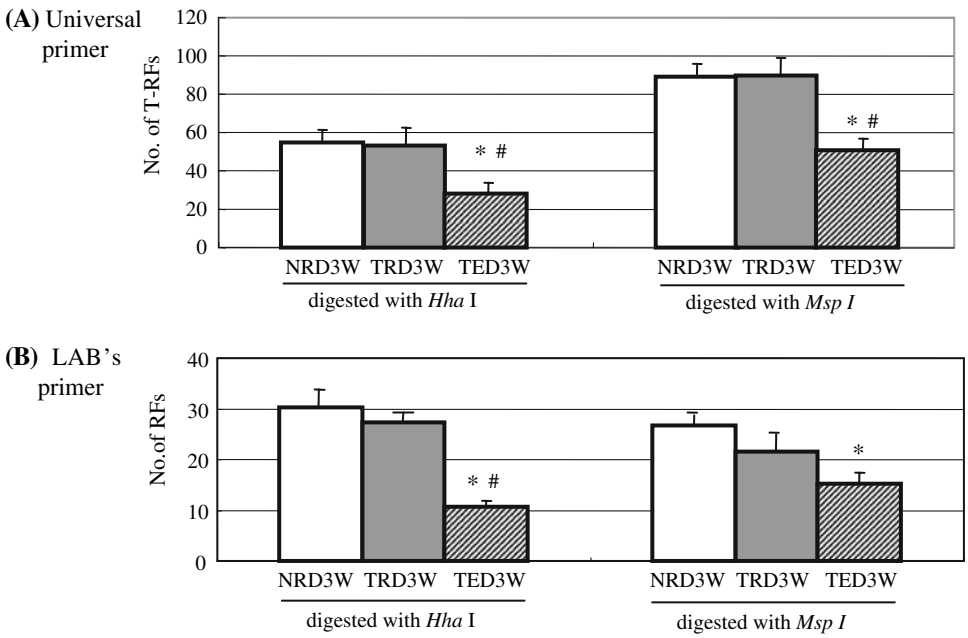

Fig. 4 Number of T-RFs digested with *Hha*I (left) and *Msp*I (right) at week 3. **a** primers. **b** LAB specific primers. $^{*}P < 0.05$ versus NRD3W group, $^{\#}P < 0.05$ versus TRD3W group. (*t*-test)

Table 2 Population of major cecum-derived bacterial groups at week 3 after cell transfer

Group	Cultivated bacterial count[a]		
	NRD3W	TRD3W	TED3W
Enterobacteria	5.76 ± 0.45	7.26 ± 0.29[b]	8.93 ± 0.40[c]
Streptococcus	8.20 ± 0.36	8.03 ± 0.57	7.60 ± 0.57
Enterococcus	Not detected	Not detected	7.03 ± 0.15
Lactobacillus	9.40 ± 0.36	9.46 ± 0.38	7.07 ± 0.25[C]
Bacteroides	10.90 ± 0.26	10.93 ± 0.65	10.87 ± 0.55
Clostridium	10.20 ± 0.78	10.80 ± 0.72	10.27 ± 0.72

[a] Mean log/g wet contents ± SD

[b] $P < 0.05$ versus NRD3W group

[c] $P < 0.05$ versus NRD3W group and TDR3W group

Table 3 Species distribution of *Lactobacillus* from colonies isolated on LBS agar plates

TRD3W		TED3W	
Species	Number of isolates	Species	Number of isolates
Lactobacillus reuteri	21	*Lactobacillus murinus*	23
Lactobacillus johnsonii	3	*Lactobacillus reuteri*	1

compared with *L. reuteri* (Fig. 5). On the other hand, the culture supernatants of *E. faecalis* and *E. durans* isolates obtained from the TED3W group did not induce a significant production of TNF-α, and IL-6 was not detected at all.

Table 4 Species distribution of *Streptococcus–Enterococcus* from colonies isolated on TS agar plates

TRD3W		TED3W	
Species	Number of isolates	Species	Number of isolates
Streptococcus alactolytieus	11	*Enterococcus faecalis*	10
Streptococcus parasanguinis	3	*Enterococcus durans*	5
Enterococcus faecalis	2	*Streptococcus alactolytieus*	1

Discussion

ED therapy has been established as a primary treatment for CD patients in Japan and pediatric CD patients in Western countries. These effects are reported by some randomized controlled trials [27–30]. However, the therapeutic mechanisms have not been fully clarified.

We used IL-$10^{-/-}$ cell-transferred mice [19] to elucidate the effect of ED treatment. This model shows severe symptoms in short term with a simple procedure that is easy to manipulate and also shows the same Th-1-predominant immune abnormality as IL-$10^{-/-}$ mice. In this study, cell-transfer-induced colonic inflammation and colon weight increased according to severity of inflammation. ED significantly inhibited colonic inflammation macroscopically and microscopically (Fig. 1). In addition, it was observed that plasma level of serum amyloid A (SAA), which is elevated in case of systemic inflammation, significantly decreased in the TED group (data not shown).

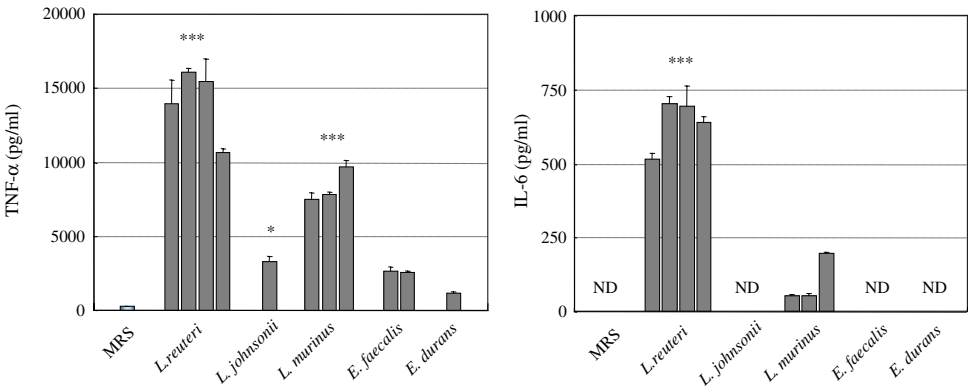

Fig. 5 Induction of TNF-α and IL-6 in RAW264 cells by LAB culture supernatant. $*P < 0.05$, $***P < 0.001$ versus MRS group. (Dunnett's test)

We speculated that modifications of the intestinal microbiota in mice fed the ED might be associated with inhibition of colitis in this model. Therefore, we examined the changes in the intestinal microbiota of these mice.

The role of the intestinal microbiota in the pathogenesis of CD has been mainly investigated in cultured bacteria derived from feces. However, no conclusions have been obtained so far because of the heterogeneity and complexity of the human gut microbiota.

In this model, we could control the diet and examine its effect on the intestinal microbiota. Figure 3 shows close similarity of the microbiota among the three mice of each group, indicating that this model is appropriate for analysis of the intestinal microbiota.

Cecal weight in the TRD3W group that exhibited severe colitis was significantly greater than that in the NRD3W group. Additionally, total SCFAs concentration in the TRD3W group was about twice the concentration in the NRD3W group. These findings suggested that the total amount of bacteria was increased in colitis mice. The reason is unknown, although it was suspected that antibacterial properties such as defensins might be decreased in this model, as reported in CD patients and colitis mice [31, 32]. The high ratio of succinic acid and lactic acid observed in the TRD3W group may aggravate colitis [33, 34]. On the other hand, cecal weight in the TED3W group was significantly lighter than that in the NRD3W and TRD3W groups. The cecum contains undigested components and their fermented bacteria. It is well known that ED is fully absorbed in the ileum and that the amount of undigested components is significantly reduced. Therefore, reductions of total amount of intestinal bacteria and its metabolites were strongly expected. Reductions of fecal microbiota and SCFA concentration using standard enteral formula were reported in healthy subjects [35–37]. On the other hand, the change of intestinal microbiota by enteral nutrition has not been sufficiently investigated in clinical studies of IBD and inflammatory bowel syndrome (IBS). In pediatric CD patients, profound modification of fecal microbiota was observed after exclusive enteral nutrition for 8 weeks with a polymeric formula (Modulen IBD®, Nestlé) [38]. Elemental diet (Vivonex Plus® Novaritis Nutrition Corp. Minneapolis, MN) proved to be highly effective in normalizing abnormal lactulose breath caused by bacterial overgrowth in IBS patients [39].

Total concentration of SCFAs in the TED 3 week group was low compared with in the TRD3W group and similar to that in the NRD3W group, but the profiles of SCFAs differed. High ratio of acetic acid and propionic acid, recently reported to have anti-inflammatory effects [40], was found in the TED3W group.

To further investigate the alteration of microbiota by ED, we used the T-RFLP method [41]. This method is relatively simple, easy, and reproducible compared with culture methods [42]. It is also advantageous that T-RFs are indicative of the genus or species of bacteria based on differences in 16 S rRNA sequence of each bacterium and allows the search of candidates genus or species from databases. In this study, T-RFLP profiles of the TED group differed markedly from those of the NRD and TRD groups. The diversity of intestinal microbiota in the TED group was 60% of that found in the NRD and TRD groups (Fig. 4). Some of the T-RFs that disappeared in the TED group were identified as *Lactobacillus* spp. by LAB specific primers but the others were unidentified. We also examined the gut microbiota using the culture method. We found that the genera *Bacteroides* and *Clostridium* predominated and that the population of these anaerobes was similar in the three groups while the population of *Enterobacteria* and LAB differed. As for the change of LAB, a decrease of *L. reuteri* and *L. johnsonii* and an increase of *E. faecalis* and *E. durans* were observed in the TED group.

LAB including the genera *Lactobacillus* and *Enterococcus* are used as probiotics. Probiotics exert various immunoactivator or immunomodulator effects. Some *Lactobacillus* strains have been tested for treatment or prevention of recurrence for IBD [8, 43, 44]. It is reported [24] that in IL-$10^{-/-}$ mice *Lactobacillus* spp. differ from those found in other gene-deficient mice and that they stimulate the murine macrophage cell line RAW 264.7 in

different ways. In IBD, macrophages are the primary source of TNF-α that is currently the main target of immunotherapy for CD. Therefore, we investigated the cytokine-promotion activity of LAB isolates using the murine macrophage cell line RAW 264 (Fig. 5). The culture supernatant of *L. reuteri* recovered from the TRD groups induced production of TNF-α and IL-6, while that of *E. faecalis* and *E. durans* isolated from the TED group did not, suggesting that some resident *Lactobacillus* spp. may be a risk factor for inflammation and that modification of the composition of LAB population by ED contributed to the reduction of inflammation.

It is strongly suggested that loss of tolerance towards resident intestinal microbiota occurs more commonly in animal IBD models than in humans [45]. In this model, transfer of lymphocytes from diseased IL-$10^{-/-}$ mice provoked an inflammatory reaction against the resident intestinal microbiota in SCID mice. In the CD4 + CD45RB high T cell transfer mice [46], colitis developed under specific pathogen-free (SPF) conditions plus segmented filamentous bacteria (SFB), but not under germ-free conditions. Investigation of SFB in this model will be needed in the future. In a T cell receptor alpha-chain-deficient model, Kishi et al. [47] reported that mice fed ED showed no pathologic features of IBD and that pathogenic *Bacteroides vulgatus* were eliminated by ED.

As mentioned above, various microbiological changes were observed after ED feeding in this IL-$10^{-/-}$ transfer mice: (i) reduction of population and diversity of the microbiota, (ii) change of SCFAs concentration and profile, and (iii) modification of the composition of LAB population that led to a reduction of inflammatory cytokines in the macrophage cell line in vitro. Modification of the microbiota by ED may be responsible, at least in part, for the suppression of the inflammatory response in this model. We expect to confirm these changes of the intestinal microbiota in patients with Crohn's disease treated with ED.

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