Bifidobacterium breve alters immune function and ameliorates DSS-induced inflammation in weanling rats

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BACKGROUND: *Bifidobacterium breve* M-16V (M16V) is a probiotic bacterial strain with a long tradition of use in neonatal intensive care units in some countries. Previous study showed that the effects of M16V administration on gene expression were greater during the weaning period than in the neonatal period and were greater in the colon than in the small intestine and spleen, suggesting that M16V has anti-inflammatory effects. In this study, we evaluated the effects of inflammation during the weaning period and the effects of M16V on normal and inflammatory conditions.

METHODS: From postnatal day (PD) 21 to 34, weanling rats were administered of 2.5×10^9 of M16V daily, and colitis was induced by administration of 2% dextran sulfate sodium from PD28 to 35. Colitis severity, immune function, and microbiota were investigated.

RESULTS: Colitis caused a reduction in body weight gain, colon shortening, poor nutritional status, anemia, changes in blood and spleen lymphocyte populations, spleen T-cell malfunctions, and alterations in colon microbiota. M16V administration improved some but not all of the changes induced by colitis.

CONCLUSION: M16V could suppress inflammation and, therefore, can be considered a safe strain to use not only during the neonatal period but also the weaning period.

Following delivery, mammalian neonates come out from a germ-free uterus to a highly contaminated extrauterine environment, in which they are exposed to many bacteria and antigens (1). During this process, trillions of microbes come to inhabit the mammalian neonatal intestine, and a complex symbiosis system termed microbiota is formed (1). Microbiota is considered important for normal immunological development of the host. For example, reports have shown reduced diversity in the intestinal microbiota and an association of reduced beneficial microbes, such as *lactobacilli* and *bifidobacteria*, with increased risk of allergic disease (2). Generally, *bifidobacteria*, a beneficial microbe, are more dominant in the intestinal microbiota of breastfed infants than in formula-fed infants (1,3). Additionally, the immune function of breastfed infants is reportedly different from that of formula-fed infants (4), and as a result, breastfeeding is thought to protect

infants from diseases such as allergy and pneumonia (4). Consequently, numerous trials testing the benefits of probiotics against neonatal immunological development have been conducted (5). *Lactobacilli* and *bifidobacteria* are commonly used as probiotics; however, *bifidobacteria* are reported to be more predominant than *lactobacilli* in human neonatal intestines (3). There are many species within the *bifidobacteria* genus, among which, *Bifidobacterium breve* (*B. breve*) is one of the most abundant in human neonates (3).

B. breve M-16V (M16V) is a probiotic strain that was isolated from the fecal sample of a healthy baby (6). This strain has been used for low-birth-weight infants in neonatal intensive care units in Japan for more than 20 y and proves to be a suitable probiotic strain for routine use in preterm neonates in Australia (7). M16V is effective in promoting the colonization of *bifidobacteria* in low-birth-weight infants (8) and showed antiallergic effects in mice (9,10) and humans (11). Ohtsuka *et al.* (12) evaluated the effects of M16V on different developmental periods (neonatal vs. weaning period) and tissues (small intestine, large intestine, and spleen) using microarrays. Their study showed M16V to be more effective during the weaning than the neonatal period and showed the strongest effect on the large intestine compared with the small intestine and spleen. They also showed that M16V appears to exert anti-inflammatory effects. Inflammation causes various metabolic disorders (13). Several studies regarding inflammation, such as necrotizing enterocolitis, during the neonatal period have been conducted, and rigorous studies are necessary due to the high mortality caused by necrotizing enterocolitis (14). However, studies are lacking regarding inflammation during the weaning period. In this study, we induced inflammation in the colon by administration of dextran sulfate sodium (DSS) and evaluated the effects of inflammation during the weaning period and the effects of M16V under normal and inflammatory conditions using weanling rats.

RESULTS

Severity of Colitis and Nutritional Status

Disease activity index (DAI) scores were significantly higher in the DSS group than in the control group from days 3 to 7 (postnatal day (PD) 31–35) after colitis induction, and M16V significantly repressed DAI scores (except for PD 33) during

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that period (Figure 1a). Colitis repressed body weight gain and shortened the colon length (Figure 1b,c). M16V could not improve the reduction in body weight gain, but it did repress colon length shortening. Colitis also suppressed plasma albumin levels (Figure 1d), indicating a deterioration in nutritional status. M16V significantly suppressed the reduction in plasma albumin induced by colitis (Figure 1d), although there were no differences in body weight reduction (Figure 1b) and in total diet intake between DSS and DSS+M16V groups (data not shown). According to histological analyses, M16V repressed the inflammation severity and inflammation extent, however, could not suppress the

Figure 1. The effects of colitis and *B. breve* M-16V administration on (a) the disease activity index (DAI) (control, open circle; M16V, filled circle; open triangle, DSS; filled triangle, DSS+M16V), (b) body weight change (control, white bar; M16V, black bar; DSS, gray bar; DSS+M16V, hatched bar), (c) colon length (control, white bar; M16V, black bar; DSS, gray bar; DSS+M16V, hatched bar), (d) the plasma albumin (control, white bar; M16V, black bar; DSS, gray bar; DSS+M16V, hatched bar), and (e–h) colon histological photographs in weanling F344 rats. (e–h) Representative (shows the near average scores of each group) histological photographs of the distal colon of weanling rats (magnification: $\times 4$). (e) Control group (total score = 0), (f) M16V group (total score = 0), (g) DSS group (inflammation severity score = 12.0, inflammation extent score = 12.0, crypt damage score = 14.7, total score = 38.7), (h) DSS+M16V group (inflammation severity score = 10.5, inflammation extent score = 10.5, crypt damage score = 11.5, total score = 32.5). Data are presented as the mean $\pm$ SEM; $n = 10$, control group and M16V group; $n = 12$, DSS group and DSS+M16V group; $^*P < 0.01$, $^{**}P < 0.0001$ vs. control group. $^{\dagger}P < 0.05$; $^{\ddagger}P < 0.01$ vs. DSS group.

crypt damage (Table 1). As a result, M16V tend to suppress total inflammation score ($P = 0.077$). Representative histological images (shown near the average scores of each group) are given in Figure 1e–h. Colitis also reduced blood erythrocytes and Hb (Figure 2b,c). Although colitis did not affect blood leukocyte counts (Figure 2a), colitis altered leukocyte populations in blood and spleen (Figure 2e,g,i). Colitis increased monocyte proportion in blood leukocytes and granulocyte proportion in spleen leukocytes, and decreased lymphocyte proportion in spleen leukocytes. M16V tended to suppress erythrocyte reduction ($P = 0.058$), significantly suppressed the Hb reduction in blood, and significantly suppressed monocyte increase in blood leukocytes induced by colitis. Colitis significantly induced inflammatory cytokines (IL-1 α , IL-1 β , and tumor necrosis factor α (TNF- α)), chemokines (Fractalkine, monocyte chemoattractant protein 1 (MCP-1)), monocyte/macrophage marker (CD11b), and the anti-inflammatory cytokines (IL-10 and TGF- β_2) in colon tissue (Figure 3). Under inflammatory conditions, M16V significantly suppressed IL-1 β , Fractalkine, and CD11b, but it did not suppress other cytokines (IL-1 α , IL-10, and TNF- α) or chemokine (MCP-1). Under normal conditions, M16V did not alter DAI, body weight change, colon length, plasma albumin levels, histological score, blood cell counts, blood and spleen leukocyte populations, colon cytokine, or colon chemokines.

Lymphocyte Populations and T-Cell Subsets in the Blood and Spleen

Colitis significantly increased the proportions of natural killer (NK) cells in blood lymphocytes and of T cells in the spleen lymphocytes and significantly decreased NK cell and NKT cell proportions in the spleen lymphocytes (Figure 4). Colitis significantly increased helper T (Th; CD3 $^{+}$ CD4 $^{+}$) cell subsets and decreased cytotoxic T (Tc; CD3 $^{+}$ CD8 $^{+}$) cell subsets among spleen T cells (Figure 5), thereby changing the Th/Tc ratio among spleen T cells significantly (control, 1.44 ± 0.02 ; M16V, 1.40 ± 0.02 ; DSS, $1.74 \pm 0.05^{**}$; DSS+M16V, 1.66 ± 0.10 ; $^{**}P < 0.0001$ vs. control). Lymphocyte cell subsets altered by colitis in the blood and spleen were not affected by M16V. Under normal conditions, M16V significantly increased NK cell proportions in blood lymphocytes compared with the control group, but it did not affect other cell proportions among lymphocytes and T-cell subsets (Figures 4 and 5).

Table 1. Histological scores of colon tissues in weanling rats

Group	Inflammation severity (0–12)	Inflammation extent (0–12)	Crypt damage (0–16)	Total score (0–40)
Control	0	0	0	0
M16V	0	0	0	0
DSS	$11.92 \pm 0.08^{**}$	$11.92 \pm 0.08^{**}$	$14.50 \pm 0.47^{*}$	$38.33 \pm 0.59^{*}$
DSS + M16V	$10.17 \pm 1.15^{\dagger}$	$10.58 \pm 0.75^{\dagger}$	11.75 ± 1.60	32.50 ± 3.46

Data are expressed as mean $\pm$ SEM (control and M16V, $n = 4$; DSS and DSS + M16V, $n = 6$). $^{*}P < 0.05$; $^{**}P < 0.01$ vs. control; $^{\dagger}P < 0.05$ vs. DSS group.

Figure 2. The effects of colitis and *B. breve* M-16V administration on blood parameters, leukocyte proportions in blood and spleen of weanling F344 rats. (a) White blood cells (WBC), (b) red blood cells (RBC), (c) blood Hb, (d) granulocytes in blood WBC, (e) monocytes in blood WBC, (f) lymphocytes in blood WBC, (g) granulocytes in spleen WBC, (h) monocytes in spleen WBC, and (i) Lymphocytes in spleen WBC. Data are presented as the mean $\pm$ SEM; $n = 10$, control group and M16V group; $n = 12$, DSS group and DSS+M16V group; * $P < 0.05$; ** $P < 0.001$ vs. control group; † $P < 0.05$ vs. DSS group.

Systemic Immune Function

Colitis significantly suppressed most of the T-cell cytokines evaluated in spleen leukocytes (IFN- γ , IL-2, IL-4, IL-10, and IL-17), tend to suppress IL-6 ($P = 0.058$), and had no effect on TGF- β_1 (TGF- β_2 were not detected in all groups). M16V significantly suppressed IFN- γ reduction and tended to suppress IL-2 reduction ($P = 0.071$) induced by colitis but had no effect on the other cytokines (IL-4, IL-10, IL-17, and TNF- α ; Figure 6). Under normal conditions, M16V significantly suppressed spleen IL-10 and IL-17 production than in the control group, but it did not affect other cytokines (IFN- γ , IL-2, IL-4, and TNF- α ; Figure 6).

Colon Microbiota

Colitis significantly increased *Bifidobacteria* and *Enterobacteriaceae* and significantly reduced *B. breve* and *Lactobacillus* in the colon content. Under inflammatory conditions, M16V significantly recovered the reduction in *B. breve* and the increase in *Enterobacteriaceae*. However, M16V did not alter the colitis-induced changes in *Bifidobacteria* or

Lactobacillus. Under normal conditions, M16V significantly increased *B. breve* but did not alter other bacteria (Figure 7).

DISCUSSION

In this study, we examined the influences of inflammation and the effects of *B. breve* M-16V on normal and inflammatory conditions during the weaning period. After delivery, infants show a polarization of Th cells toward a dominance of Th2 cells, and immune maturation occurs by skewing Th1 and regulatory T (Treg) cells during development (15). If normal immune maturation fails, allergy and autoimmune diseases can arise (15). Inflammation causes many metabolic and immune disorders (13). However, reports on the influence of inflammation during the weaning period are limited (16,17). In our study, inflammation in the colon hindered growth (reduced body weight gain and colon shortening) and was associated with poor nutritional status (low plasma albumin), anemia, and altered peripheral immune cell populations and systemic immune functions (Figures 1, 2, and 4–6). Colitis increased monocyte proportion in blood leukocytes (Figure 2e). In accordance with this result, monocyte/

Figure 3. The effects of colitis and *B. breve* M-16V administration on colon cytokines, chemokines, and monocyte/macrophage marker in weanling F344 rats. (a) IFN- γ , (b) IL-1 α , (c) IL-1 β , (d) IL-6, (e) IL-10, (f) IL-17A, (g) TNF- α , (h) TGF- β_1 , (i) TGF- β_2 , (j) Fractalkine, (k) monocyte chemoattractant protein (MCP)-1, (l) CD11b (also known as integrin α M). Data are presented as the mean $\pm$ SEM; $n = 10$, control group and M16V group; $n = 12$, DSS group and DSS+M16V group; * $P < 0.05$; ** $P < 0.01$ vs. control group; † $P < 0.05$; ‡ $P < 0.01$ vs. DSS group.

macrophage marker (CD11b), monocyte/macrophage tropic chemokine Fractalkine (18), and monocyte chemoattractant chemokine MCP-1 were increased (Figure 3j–l). These results suggest that monocytes/macrophages are recruited in the blood and inflamed tissues. Colitis altered the lymphocyte cell population also (Figure 4) by significantly increasing the blood NK

cell proportion and decreasing the spleen NK cell and NKT cell proportions (Figure 4a,d,e), indicating that pooled NK and NKT cells are recruited in the blood and inflamed tissues. Reportedly, colitis increased NK and NKT cells in inflamed colon lamina propria by inflammation-induced IL-15 (19). Colitis also increased the T-cell population in spleen lymphocytes (Figure 4f) by

Figure 4. The effects of colitis and *B. breve* M-16V administration on subset proportions among blood and spleen lymphocytes of weanling F344 rats. (a–c) blood, (d–f) spleen, (a, d) natural killer (NK; CD3⁺CD161^{high}) cells, (b, e) NKT (CD3⁺CD161⁺) cells, (c, f) T cells (CD3⁺CD161⁺). Data are presented as the mean $\pm$ SEM; $n = 10$, control group and M16V group; $n = 12$, DSS group and DSS+M16V group; * $P < 0.05$; ** $P < 0.01$ vs. control group.

Figure 5. The effects of colitis and *B. breve* M-16V administration on T-cell subsets among blood and spleen T cells of weanling F344 rats. (a–c) blood, (d–f) spleen, (a, d) helper T (Th; CD3⁺CD4⁺) cells, (b, e) cytotoxic T (Tc; CD3⁺CD8⁺) cells, (c, f) regulatory T (Treg; CD4⁺CD25⁺Foxp3⁺) cells. Data are presented as the mean $\pm$ SEM; $n = 10$, control group and M16V group; $n = 12$, DSS group and DSS+M16V group; * $P < 0.0001$ vs. control group.

significantly altering Th and Tc cell populations (increasing Th but decreasing Tc cell proportions) (Figure 5d,e); as a result, the Th/Tc ratio was significantly altered (control, 1.44 ± 0.02 vs. DSS, 1.74 ± 0.05 ; $P < 0.0001$), although blood T-cell populations were not affected. Lee and Woodward (20) reported that a low protein diet induced a higher CD4⁺/CD8⁺ ratio (Th/Tc ratio) among T cells in the spleen (but not blood) of weanling mice, indicating that an increased spleen Th/Tc ratio also reflects a poor nutritional status. The spleen Th cell population increased; however, Th cell

cytokine production in spleen leukocytes was significantly lower under inflammatory conditions (Figure 6). These results suggest that inflammation altered T-cell function or inhibited T-cell functional maturation. A previous study showed that DSS-induced colitis caused thymus involution and altered thymus function (21). Thymus development depends on growth hormones, which in turn depend on protein nutritional status. Accordingly, colitis induced protein malnutrition, which caused thymus malfunction, which in turn altered T-cell function in the spleen.

As described above, a previous study using microarray analysis suggests that M16V might exhibit an anti-inflammatory function (12). Thus, in this study, we evaluated the effects of M16V on inflammatory conditions during the weaning period. Surprisingly, under normal conditions, M16V significantly suppressed spleen anti-inflammatory cytokine IL-10 production (Figure 5e). However, M16V also suppressed inflammatory cytokine IL-17 production under the same conditions (Figure 5f). These results suggest that M16V could alter systemic T-cell immune functions during the weaning period. In comparison, under inflammatory conditions, M16V significantly improved inflammation-induced changes (DAI, colon shortening, blood albumin and Hb reductions, and histological changes; Figures 1 and 2 and Table 1). Moreover, M16V significantly suppressed inflammatory cytokine (IL-1 β) and monocyte/macrophage-related molecules (Fractalkine and CD11b) in the colon induced by colitis (Figure 3). Tomosada et al. (22). reported that M16V interacted with TLR2, upregulated the expression of A20 (a ubiquitin-editing enzyme) in intestinal epithelial cells, and modulated the subsequent TLR4 activation by reducing the activation of MAPK and NF- κ B pathways. Our results might have resulted

from the same mechanisms. M16V administration significantly suppressed or tended to suppress the colitis-induced reduction of several spleen T-cell cytokines, such as IFN- γ ($P < 0.05$) and IL-2 ($P = 0.071$) (Figure 6). Interestingly, when compared under normal conditions, although M16V significantly suppressed spleen IL-10 and IL-17 production, M16V did not worsen the colitis-induced cytokine reduction (Figure 6). That is to say, M16V alters systemic immune function, however, does not promote local inflammation. This mechanism is still unknown; however, it is important that at least M16V would not worsen colitis.

Colitis altered microbiota in colon. In the DSS group, *Bifidobacteria* and *Enterobacteriaceae* were increased significantly, but *B. breve* and *Lactobacilli* were decreased significantly (Figure 7). Generally, intestinal microbiota is involved in colitis (23). *Enterobacteriaceae* were increased and *Lactobacilli* were decreased in gut microbiota of inflammatory bowel disease patients and in an animal colitis model (23,24). Our results are in agreement with these previous reports. However, *Bifidobacteria*, generally known as beneficial bacteria, also increased in the DSS group. A recent study showed that *Bifidobacteria* in mucosal biopsy of active ulcerative colitis

Figure 6. The effects of colitis and *B. breve* M-16V administration on spleen T-cell cytokines in weanling F344 rats. (a) IFN- γ , (b) IL-2, (c) IL-4, (d) IL-6, (e) IL-10, (f) IL-17, (g) TNF- α , (h) TGF- β_1 . Data are presented as the mean $\pm$ SEM; $n = 10$, control group and M16V group; $n = 12$, DSS group and DSS+M16V group; * $P < 0.05$; ** $P < 0.01$ vs. control group. † $P < 0.05$ vs. DSS group.

patients were increased (25). Conversely, *B. breve* was significantly decreased in the DSS group, indicating that colitis might change the composition of *Bifidobacterium* species (Figure 7). Under inflammatory conditions, M16V administration significantly improved the reduction of *B. breve* and the increase of *Enterobacteriaceae* (Figure 7). Under normal conditions, M16V administration did not significantly change the microbiota in the colon except for *B. breve* (Figure 7). This result is in agreement with a previous study showing that M16V administration did not change weanling rat microbiota (12).

Recent accumulating evidence has indicated that some probiotic bacteria improve the inflammatory status (23,26). Previous study reported that several probiotic strains ameliorate colitis by inducing Treg cells (26). Thus, in this study, we also investigated Treg in blood and spleen, and Treg-related cytokine TGF- β in spleen and colon. Colitis increased TGF- β_2 in colon, however, did not change Treg proportions in blood or spleen lymphocytes and did not change TGF- β_1 production in colon and spleen (Figure 3, 5 and 6). Zakostelska *et al.* (26) reported that administration of *Lactobacillus casei* DN-114 001 increased the proportion of Tregs in the mesenteric lymph nodes (MLNs), although the proportions in spleen and Peyer's patches did not change. And Liu *et al.* (27) also reported that the Treg increase by administration of *Lactobacillus reuteri* DSM 17938 was found in the ileum and MLNs. These studies suggest that the Treg induction caused by probiotics might occur mainly in the MLNs and lamina propria (26,27).

In conclusion, colitis causes poor nutritional status and as a result inhibits physical and immune development in weaning period, therefore, suppressing colitis in this period is important,

and our results suggest that M16V can suppress inflammation. Thus, this strain is considered a safe strain to use not only in neonatal infants but also in weaning children. However, to expand the applications of this strain, further studies are necessary to clarify the mechanisms that increase blood NK cells and reduce spleen cytokines (IL-10 and IL-17) induced by M16V administration under normal conditions. Moreover, further studies focusing on the effects of M16V on other immune organs such as lamina propria and MLNs that were not investigated in this present study are needed.

METHODS

Experimental Design

All animal experiments were approved by the Institutional Animal Care and Use Committee of Morinaga Milk Industry Co., Ltd. and were performed in accordance with the Guide for the Care and Use of Laboratory Animals of Morinaga Milk Industry Co., Ltd. Pregnant F344/N rats (12 wk old at mating, $n = 6$) were obtained from Japan SLC Inc. (Shizuoka, Japan). After birth, neonatal rats were reared by their mothers until PD 20 (around weaning day of rats). After weaning, weanling rats were housed individually in cages under temperature-controlled ($23 \pm 2^\circ\text{C}$) conditions and a 12/12-h light/dark cycle and were allowed free access to a commercial diet and water. Weanling rats were divided into four groups: the first and second groups consisted of 10 rats each given autoclaved tap water as drinking water from PD 28 to 35. The third and fourth groups consisted of 12 rats each given DSS (molecular weight 36,000–50,000; MP Biomedicals, Santa Ana, CA) dissolved in drinking water at a concentration of 2% from PD 28 to 35 to induce colitis according to Fitzpatrick *et al.* (16) and Vicario *et al.* (17). They investigated about appropriate DSS concentration and treatment period. We decided DSS concentration as 2% and treatment period as 7 d based on their studies. M16V was obtained from a stock culture from the Morinaga Culture Collection (Morinaga Milk Industry, Kanagawa, Japan). This strain was deposited into the Belgian Co-Ordinated Collections of Micro-organisms as strain LMG23729 (6). M16V (2.5×10^9 colony forming units) plus starch were suspended in phosphate-buffered saline (PBS), and the suspended solution was administered once daily by gavage to the rats in the second (M16V group) and fourth groups (DSS+M16V group). As a control, an equal volume of starch-suspended PBS was administered to rats in the first (control group) and third groups (DSS group). Oral administration of M16V or control solution was started 7 d before induction of colitis and was continued for 14 d, until 1 d before sacrifice (PD21–34). At PD35, rats were sacrificed by deep anesthesia using sevoflurane (Mylan, Cecil Township, PA). Blood samples were collected from the inferior vena cava using an EDTA-coated syringe. Leukocytes, erythrocytes, and Hb were analyzed using a KX-21NV (Sysmex, Kobe, Japan). Following the cell counts and leukocyte preparation, the remaining blood was centrifuged (1,200g, 25°C , 10 min) to obtain plasma. The spleen was removed aseptically and preserved in cold sterile Hanks' balanced salt solution. The colon was removed and its length measured without stretching. The colon was opened, and its content was collected and was flushed with cold sterile PBS. A segment (2 cm) of the colon for cytokines, chemokines,

Figure 7. The effects of colitis and *B. breve* M-16V administration on microbiota in the colon content of weanling F344 rats. (a) *Bifidobacterium*, (b) *Bifidobacterium breve*, (c) *Lactobacillus*, and (d) *Enterobacteriaceae*. Data are presented as the mean $\pm$ SEM; $n = 10$, control group and M16V group; $n = 12$, DSS group and DSS+M16V group; * $P < 0.05$; ** $P < 0.01$ vs. control group; † $P < 0.05$; ‡ $P < 0.01$ vs. DSS group.

Table 2. Disease activity index calculated by summing the scores for stool consistency and occult blood/gross bleeding and dividing by 2

Score	Stool consistency	Occult blood/gross bleeding
0	Normal	Negative
1	Loose stool	Negative
2	Loose stool	Positive
3	Diarrhea	Positive
4	Diarrhea	Gross bleeding

and macrophage marker analyses, was removed located 2.5 cm from the anus, and refrigerated immediately. And a segment (2 cm) of the colon from anus was removed for histological analyses or mRNA analyses. The colon tissue for histological analyses was fixed in 10% formaldehyde neutral buffer immediately, and the colon tissue for RNA analyses was stocked in RNAlater (Ambion, Ausitin, TX) and refrigerated until use.

Disease Activity Index

The DAI was determined using a five-point severity scale ranging from 0 to 4, according to the method of Fitzpatrick *et al.* (16) (Table 2). The DAI was calculated by summing the scores for stool consistency and occult blood/gross bleeding and then dividing by 2. The presence of occult blood was determined using a hemoccult fecal blood kit (Shionogi, Osaka, Japan).

Table 3. Severity scale to estimate histological damage

Feature scored	Score	Description
Inflammation severity	0	None
	1	Mild
	2	Moderate
	3	Severe
Inflammation extent	0	None
	1	Mucosa
	2	Mucosa and submucosa
	3	Transmural
Crypt damage	0	None
	1	One-third of crypt damaged
	2	Two-thirds of crypt damaged
	3	Crypts lost, surface epithelium present
	4	Crypts and surface epithelium lost
Percent involvement	0	0
	1	1–25
	2	26–50
	3	51–75
	4	76–100

Colonic Histology Evaluation

Histological damage was determined on a 40-point severity scale, according to the method of Fitzpatrick *et al.* (16) (Table 3). Total colonic scores were determined by multiplying the three different histological features (inflammation severity, inflammation extent, and crypt damage). Six areas on each histology slide were evaluated, and a mean histology score was determined for each slide. The evaluation was done in the Histo Science Laboratory (Tokyo, Japan), and the investigator was unaware of the treatment group.

Colon Homogenate

Colon tissue samples were homogenized twice for 30 s using a polytron homogenizer (PT 10–35 GT, Kinematica) in ice-cold PBS. Homogenate samples were centrifuged (10,000g, 4 °C, 10 min), and the resulting supernatants collected and stored at –80 °C until analysis.

Preparation of Peripheral Blood and Spleen Leukocytes

Erythrocytes in peripheral blood were removed by incubating blood samples for 5 min with Pharm Lyse (BD Biosciences, San Jose, CA) at room temperature to prepare peripheral blood leukocytes. The isolated spleen was minced in Hanks' balanced salt solution. Single-cell suspensions were prepared by gently sieving the spleen through a 70-µm cell strainer. Erythrocytes were removed using Pharm Lyse. Cell suspensions were washed twice with cold Hanks' balanced salt solution to obtain leukocytes.

Flow Cytometric Analysis

Peripheral blood and spleen leukocytes were pretreated with a mAb that blocks Fc receptor binding (D34–485) for 10 min at 4 °C. Cell surfaces were subsequently stained with the following mAbs: allophycocyanin-labeled antirat CD3 (1F4), FITC-labeled antirat CD4 (OX-35), peridinin-chlorophyll-protein complex-labeled antirat CD8 (OX-8), phycoerythrin-labeled antirat CD161 (10/78), and phycoerythrin-labeled antirat CD25 (OX-39) (All from BD Biosciences) for 30 min at 4 °C and then washed twice with Cell Wash (BD Biosciences) reagent containing 2% fetal calf serum. Cells were resuspended and fixed in CellFIX (BD Biosciences). For intracellular Foxp3 staining, cells were fixed and permeabilized using the Fixation/Permeabilization set (eBioscience, San Diego, CA). Following fixation, cells were stained with allophycocyanin-labeled antimouse/rat Foxp3 (FJK-16s; eBioscience), and labeled cells were analyzed using a flow cytometer (FACSCanto, BD Biosciences). Flow cytometric data were analyzed using FlowJo software (Tree Star, Ashland, OR). Cell populations were evaluated according to previous studies (28,29). The detailed gating strategy for cell population is shown in Supplementary Figures S1 and S2 online.

Cell Culture

Spleen leukocytes were cultured in triplicate in 96-well flat bottom plates at 1×10^6 cells/ml in a final volume of 200 µl. The culture medium consisted of RPMI-1640 medium containing 10% fetal

Table 4. PCR primers for the detection of *Bifidobacterium*, *Bifidobacterium breve*, *Lactobacillus*, and *Enterobacteriaceae*

Target bacteria	Probe	Sequence (5' to 3')	Product size (bp)	Annealing temperature (°C)	Strain for standard curve	References
<i>Bifidobacterium</i>	g-Bifid-F	CTCCTGGAAACGGGTGG	552	55	JCM 1217	Matsuki <i>et al.</i> (32)
	g-Bifid-R	GGTGTCTTCCCGATATCTACA				
<i>B. breve</i>	BiBRE-1	CCGGATGCTCCATCACAC	288	55	JCM 1192	Matsuki <i>et al.</i> (33)
	BiBRE-2	ACAAAGTGCCTTGCTCCCT				
<i>Lactobacillus</i>	F-Lacto	GAGGCAGCAGTAGGGAATCTTC	126	60	JCM 1131	Delroisse <i>et al.</i> (34)
	R-Lacto	GGCCAGTTACTACCTCTATCCTTCTTC				
Enterobacteriaceae	En-Isu3F	TGCCGTAACCTCGGGAGAAGGCA	428	60	JCM 1649	Matsuda <i>et al.</i> (35).
	En-Isu3R	TCAAGGCTCAATGTTCAGTGTC				

JCM, Japan Collection of Microorganisms.

calf serum, 2 mmol/l L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (all from Invitrogen, Carlsbad, CA). Cultures were incubated for 48 h at 37 °C under a 5% CO₂ atmosphere with 5 µg/ml concanavalin A (ConA; Sigma Aldrich, St Louis, MO). For TGF-β quantification, spleen leukocytes were cultured as described previously (30). Briefly, spleen leukocytes were suspended in serum-free AIM-V medium (Invitrogen) containing 2 mmol/l L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin and incubated 72 h at 37 °C under a 5% CO₂ atmosphere with ConA. Culture supernatants were collected and stored at -80 °C until analysis.

ELISA, Milliplex, and Protein Assay

The plasma albumin concentration was measured using the Rat Albumin ELISA kit (AKRAL-120, Shibayagi, Gunma, Japan). Cytokines (except for TGF-β₁) and chemokines were measured in colon tissue homogenates and spleen cell cultures using the Milliplex Rat Cytokine/Chemokine Panel (RCYTO-80K; Millipore, Billerica, MA). TGF-β₁ in colon tissue homogenates and spleen cell cultures were measured using ELISA kits (TGF-β₁, MB100; TGF-β₂, DB250; R&D Systems, Abingdon, UK). CD11b (macrophage marker, also known as integrin αM) in colon tissue homogenates was measured using ELISA kit (SEB685Ra; Cloud-Clone, Houston, TX). Protein quantification in colon homogenate was done using Quick Start Protein Assay (Bio Rad, Hercules, CA).

DNA Extraction From Colon Contents

DNA was extracted from colon content samples as described previously (31). Briefly, colon content samples (20 mg) were washed three times in 1 ml PBS and centrifuged 13,000 rpm for 5 min. Fecal pellets were resuspended in 450 µl extraction buffer (100 mmol/l Tris-HCl, 40 mmol/l EDTA, pH 9.0) and 50 µl 10% sodium dodecyl sulfate. Glass beads (0.1 mm diameter, 300 mg) and 500 µl buffer-saturated phenol were added to the suspension, and the mixture was vortexed vigorously for 30 s using a FastPrep FP 100A (Funakoshi, Tokyo, Japan). After centrifugation at 13,000 rpm for 5 min, 400 µl supernatant was extracted using phenol-chloroform, and 250 µl supernatant was precipitated using isopropanol. Purification was conducted using the High Pure PCR Template Preparation Kit (Roche, Basel, Switzerland). Purified DNA was suspended in 200 µl Tris-EDTA buffer (pH 8.0).

Real-Time PCR for Quantitative Determination of Bacteria

Previously reported primers used to detect bacteria (*Bifidobacterium* (32), *B. breve* (33), *Lactobacillus* (34), and *Enterobacteriaceae* (35)) were employed in this study, and these primers are listed in Table 4. Real-time PCR analysis was performed using a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) with SYBR Premix Ex Taq (Takara Bio, Shiga, Japan). The amplification program consisted of 1 cycle of 95 °C for 20 s, followed by 40 cycles of 95 °C for 3 s, 55 or 60 °C for 30 s (Table 2), and 72 °C for 30 s to quantify *Bifidobacterium*, *B. breve*, and *Enterobacteriaceae*. To quantify *Lactobacillus*, the amplification program consisted of one cycle of 95 °C for 30 s, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. To determine the number of cells in each genus and strain, DNA extracts from the following strains were used as standards: Japan Collection of Microorganisms (JCM) 1217 (*B. longum* type strain) for *Bifidobacterium*, JCM 1192 (*B. breve* type strain) for *B. breve*, JCM 1131 (*Lactobacillus gasseri* type strain) for *Lactobacillus*, and JCM 1649 (*Escherichia coli* type strain) for *Enterobacteriaceae*.

Statistical Analyses

Values are expressed as means with their standard errors. The significances of the differences were determined using *t*-test (except for DAI and colonic histological score) (JMP software; SAS Institute, Cary, NC). The significances of the differences in DAI and colonic histological score were determined using Wilcoxon/Kruskal-Wallis test (JMP software). In this study, the first aim is to find the parameter affected by colitis in weanling period. And the second aim is to evaluate the effects of M16V on parameters affected by colitis in normal and inflammatory conditions. To avoid multiplicity concerns, comparisons between the DSS and the DSS+M16V groups and between Ctrl and M16V groups were conducted when significant differences

between the Ctrl and DSS groups were observed. A *P* value < 0.05 was considered to indicate statistical significance.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/pr>

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