INFLAMMATORY BOWEL DISEASE

Propionibacterium freudenreichii component 1,4-dihydroxy-2-naphthoic acid (DHNA) attenuates dextran sodium sulphate induced colitis by modulation of bacterial flora and lymphocyte homing

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Background and aim: 1,4-Dihydroxy-2-naphthoic acid (DHNA), a bifidogenic growth stimulator from *Propionibacterium freudenreichii*, is thought to have a beneficial effect as a prebiotic; however, its in vivo effect on intestinal inflammation remains unknown. The aim of this study was to determine whether oral administration of DHNA can ameliorate dextran sodium sulphate (DSS) induced colitis and to determine the possible underlying mechanisms.

Method: Colitis was induced in mice by treatment with 2.0% DSS for seven days. DHNA (0.6 or 2.0 mg/kg) was given in drinking water prior to (preventive study) or after (therapeutic study) DSS administration. Colonic damage was histologically scored, and mucosal addressin cell adhesion molecule 1 (MAdCAM-1) expression and $\beta 7$ positive cell infiltration were determined by immunohistochemistry. mRNA levels of proinflammatory cytokines (interleukin (IL)-1 β , IL-6 and tumour necrosis factor α (TNF- α)) were determined by quantitative real time polymerase chain reaction. In addition, bacterial flora in the caecum, concentrations of short chain acids, and luminal pH were examined.

Results: DHNA improved survival rate and histological damage score in mice administered DSS in both the preventive and therapeutic studies. DHNA significantly attenuated the enhanced expression of MAdCAM-1, the increased $\beta 7$ positive cell number, and the increased mRNA levels of IL-1 β , IL-6, and TNF- α in DSS treated colon. In addition, the decreased number of *Lactobacillus* and *Enterobacteriaceae* induced by DSS was recovered by DHNA. Preventive effects on decrease in butyrate concentration and decrease in pH level in mice administered DSS were also observed in the DHNA preventive study.

Conclusion: DHNA, a novel type of prebiotic, attenuates colonic inflammation not only by balancing intestinal bacterial flora but also by suppressing lymphocyte infiltration through reduction of MAdCAM-1.

Various aetiologies of ulcerative colitis (UC) and Crohn's disease (CD) have been proposed, including inappropriate inflammatory response to a luminal pathogen or abnormal luminal constituent, autoimmunity, and abnormal immune response to normal luminal contents, such as normal intestinal bacterial flora or dietary antigens.¹ Recently, there has been increasing experimental evidence that the intestinal bacterial flora plays an important role in the pathogenesis of inflammatory bowel diseases (IBD)^{2–5} whereas some genus bacteria in intestinal bacterial flora, such as *Bifidobacterium* and *Lactobacillus*, are known to be effective for improvement of intestinal inflammation.^{6–7} Thus much interest has been shown in modification of the balance of intestinal bacterial flora for IBD therapy.

Some clinical trials in which prebiotics such as lactosucrose, Plantago ovata seeds, and germinated barley food-stuff preparations were administered to IBD patients have been carried out to modify the intestinal bacterial flora.^{8–10} Prebiotics are generally defined as indigestible food ingredients that selectively stimulate the growth and activity of specific species of beneficial bacteria in the colon.¹¹ The mechanism of action of prebiotics is thought to be stimulation of the growth of specific species of bacteria in the gut, such as *Bifidobacteria* or *Lactobacilli*, resulting in suppression of the growth of injurious species by decreasing the luminal pH

and by blocking their epithelial attachment. In addition, increased substrate availability and increased bacterial fermentation, with resultant short chain fatty acids (SCFAs), especially butyrate production, may enhance mucosal barrier function and induce reduction of the pH value.^{11–12} These molecules, especially butyrate, are known to suppress the activity of nuclear factor κB , which acts as the major transcription factor in an inflammatory condition.^{13–14} Although administration of prebiotics is an attractive way to treat intestinal inflammation, accumulation of experimental evidence for its usefulness is not as great as that for the usefulness of probiotics. Moreover, there have been few studies on how prebiotics modulate intestinal inflammation through the mucosal immune system.¹⁵

In IBD, active lesions of the gut are characterised by marked infiltration of inflammatory cells. Endothelial cell adhesion molecules (ECAMs) such as intercellular adhesion

Abbreviations: DHNA, 1,4-dihydroxy-2-naphthoic acid; DSS, dextran sodium sulphate; IL, interleukin; TNF- α , tumour necrosis factor α ; UC, ulcerative colitis; CD, Crohn's disease; IBD, inflammatory bowel diseases; SCFA, short chain fatty acid; ECAM, endothelial cell adhesion molecule; ICAM-1, intercellular adhesion molecule 1; VCAM-1, vascular cell adhesion molecule 1; MAdCAM-1, mucosal addressin cell adhesion molecule 1; DW, distilled water; H&E, haematoxylin-eosin; PBS, phosphate buffered saline; PCR, polymerase chain reaction

molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and mucosal addressin cell adhesion molecule 1 (MAdCAM-1) play essential roles in leucocyte infiltration.^{16–17} Among them, MAdCAM-1, an Ig superfamily adhesion molecule, is selectively expressed in gut associated lymphoid tissue.^{18–21} It has been reported that inflammation of the intestine is associated with enhanced expression of MAdCAM-1 in both experimental animals and humans,^{20–22} and we have demonstrated that experimental colitis in mice and rats was ameliorated by functional blocking of MAdCAM-1.^{22–23} However, there has been no report on the effect of administration of prebiotics on MAdCAM-1 expression in the inflamed intestine.

1,4-Dihydroxy-2-naphthoic acid (DHNA) has been revealed to be the main component of bifidogenic growth stimulator, which is isolated from the culture broth of *Propionibacterium freudenreichii* ET3, and this component promotes specific proliferation in vitro of the genus *Bifidobacterium*.²⁴ However, the in vivo effect of this component on intestinal inflammation remains unknown.^{24–25} The aim of this study was therefore to determine whether oral administration of DHNA can attenuate colonic mucosal inflammation, using a mouse model of colitis induced by dextran sodium sulphate (DSS), that histologically resembles human UC,²⁶ particularly focusing on alterations in the composition of faecal flora, expression of MAdCAM-1, and lymphocyte infiltration.

MATERIALS AND METHODS

Animals and experimental protocol

Specific pathogen free five week old female C57BL/6NCrj mice were purchased from Charles River Japan Inc. (Yokohama, Japan). Mice were kept on a MF diet (Oriental Yeast Co. Ltd, Tokyo, Japan), and their care and use were in accordance with the guidelines of the National Defense Medical College. Two different protocols for administration of DHNA (prevention and therapy) were carried out in DSS induced colitis. DHNA was purchased from Wako Co. (Osaka, Japan). DHNA was dissolved in dimethyl sulphoxide (0.5 ml) and added to a vehicle (distilled water (DW) with 1% ascorbic acid, w/v). DSS (molecular weight 40 000) was obtained from ICN Biochemicals (Cleveland, Ohio, USA). In the prevention study, DHNA (0.6 or 2.0 mg/kg) or the vehicle (DW with ascorbic acid) was given for 14 days in free drinking water. Thereafter, 2.0% DSS was given in drinking water for seven days for induction of colitis (a total 21 days for the experimental period). As a DHNA therapy study, 2.0% DSS was given in drinking water for seven days and then DHNA or vehicle was given for seven days in free drinking water (a total of 14 days for the experimental period). Survival rates in the DHNA prevention group and in the therapy group were checked after the experimental period.

Assessment of colonic damage and immunohistochemistry for $\beta 7$ integrin positive cells and MAdCAM-1

Under pentobarbital anaesthesia, a segment of the proximal colon was fixed in 10% buffered formalin and embedded in paraffin, and 4 μ m longitudinal sections were stained with haematoxylin and eosin (H&E). Histological damage was assessed by the method of crypt scoring described by Cooper *et al*²⁷: grade 0, intact crypt; grade 1, loss of the basal one third of the crypt; grade 2, loss of the basal two thirds of the crypt; grade 3, loss of the entire crypt with the surface epithelium remaining intact; and grade 4, loss of both the entire crypt and surface epithelium. Another part of the removed colon was fixed for 12 hours at 4°C in periodate-lysine-paraformaldehyde. Subsequently, these tissues were washed and dehydrated for 12 hours with phosphate buffered saline

Figure 1 Effects of 1,4-dihydroxy-2-naphthoic acid (DHNA) prevention and therapy on survival rate of dextran sodium sulphate (DSS) treated mice. In the DHNA prevention group, DHNA (0.6 or 2.0 mg/kg) or a vehicle (distilled water (DW) with ascorbic acid for a control) was administered for 14 days, and then 2.0% DSS was given for seven days to induce colitis (a total 21 days for the experimental period). In the DHNA therapy group, 2.0% DSS was given for seven days, and then DHNA (0.6 or 2.0 mg/kg) or a vehicle (for control) was given for seven days (a total of 14 days for the experimental period). Survival rates in both groups were examined after the experimental periods. Results are expressed as means (SEM) from three series of experiments (10 animals in each group for each series at the beginning). * $p < 0.05$ compared with the DSS treated control group.

(PBS) containing 10% or 20% sucrose. After fixation, they were embedded in Tissue-Tek OCT compound (Sakura Finetek Inc., Tokyo, Japan) and frozen in liquid nitrogen. Cryostat sections of frozen tissue were cut at 7 μ m. Immunohistochemistry was performed by the labelled streptavidin biotin technique using monoclonal antibodies that react to MAdCAM-1 (MECA367, rat IgG2a; PharMingen, San Diego, California, USA) and $\beta 7$ integrin (M293, rat IgG2a; PharMingen) as previously described.²² An isotype matched IgG was used as a negative control. After treatment with biotinylated goat antirat IgG (PharMingen), tissues were visualised by streptavidin fluorescein isothiocyanate. The MAdCAM-1-positive area in tissue sections was quantified as positive area per millimetre of muscularis mucosa. The number of $\beta 7$ integrin positive cells was expressed per millimetre of muscularis mucosa.

Quantitative real time PCR for inflammatory cytokines

Colonic tissues were cut into pieces and homogenised in lysis buffer included in an RNeasy Mini kit (Qiagen, Hilden, Germany). After isolation, total RNA (1 μ g) was used for reverse transcription using Random 9mers (dp: 5'-NNNNNNNNN-3') and an RNA PCR kit (AMV) version 3.0 (TaKaRa, Shiga, Japan). Taqman PCR reactions were performed on complementary DNA samples using Taqman Universal PCR Master Mix (Applied Biosystems, California, USA) and an ABI PRISM 7000 Sequence Detection System. Taqman probes and primers for tumour necrosis factor α (TNF- α), interleukin 1 β (IL-1 β), interleukin 6 (IL-6), and 18S rRNA were developed as TaqMan(R) Gene Expression Assays by Applied Biosystems. Target mRNA was achieved using the comparative cycle threshold method of relative quantification. (The calibrator sample was isolated from

Figure 2 (A) Representative illustrations of the proximal colon of dextran sodium sulphate (DSS) colitis (DSS alone prevention control; upper panels) and 1,4-dihydroxy-2-naphthoic acid (DHNA) prevention groups (0.6 mg/kg) (lower panels). Haematoxylin-eosin (H&E) stainings of the proximal colon (left panels), immunohistochemical staining of mucosal addressin cell adhesion molecule 1 (MAdCAM-1) expression in microvessels (middle panels), and β7 positive cells in the colonic mucosa (right panels) ($\times 100$). (B) Representative illustrations of the proximal colon of DSS colitis (DSS alone therapy control; upper panels) and DHNA therapy groups (0.6 mg/kg) (lower panels). H&E stainings of the proximal colon (left panels), immunohistochemical staining of MAdCAM-1 expression (middle panels), and β7 positive cells (right panels) ($\times 100$). In DSS controls, crypt distortion or loss of crypts with marked cell infiltration and erosion were observed. Histological damage was greater in the DSS therapy group than in the DSS prevention control group. DHNA ameliorated these histological changes in both groups. Abundant MAdCAM-1 positive microvessels and β7 positive cell infiltration were observed in the lamina propria and in the submucosa of DSS treated animals, and these increases were inhibited by DHNA prevention and therapy.

healthy C57/BL6 mice with 18SrRNA used as the internal control.). All samples were assayed in duplicate.

Examination of caecal bacterial flora

Caecal contents were obtained directly from the colonic lumen. Samples were homogenised and serially diluted in

PBS or sterilised anaerobic buffer solution for incubation under aerobic or anaerobic conditions.²⁸ After serial dilution of the caecal suspensions with appropriate buffers, 50 μl of the diluent were spread onto the following selective media: TOS agar (Yakult Pharmaceutical Ind. Co., Tokyo, Japan) for selective isolation of *Bifidobacterium*; Bacteroides agar (Nissui

Figure 3 (A) Effect of 1,4-dihydroxy-2-naphthoic acid (DHNA) administration on histological damage score in dextran sodium sulphate (DSS) treated mice. Histological damage score was evaluated in paraffin sections of the proximal colon stained with haematoxylin-eosin on a 0–4 scale using the crypt scoring system (see materials and methods). Histological damage score was calculated in each segment and averaged in proportion to the length of muscularis mucosa. Prevention: DHNA prevention protocol against DSS. Therapy: DHNA therapy protocol against DSS. * $p < 0.05$ versus normal controls, † $p < 0.05$ versus DSS treated controls, ‡ $p < 0.05$ versus DHNA prevention protocol. (B) Effect of DHNA administration on expression area of mucosal addressin cell adhesion molecule 1 (MAdCAM-1) in colonic mucosa. The expression area of MAdCAM-1 was measured by NIH image and expressed as area/mm of muscularis mucosa. Normal: non-treated control mice. * $p < 0.05$ versus normal controls, † $p < 0.05$ versus DSS treated controls, ‡ $p < 0.05$ versus DHNA prevention protocol. (C) Effect of DHNA administration on the number of $\beta 7$ integrin positive cells in colonic mucosa. The number of cells was expressed as positive cells/mm muscularis mucosa. DW, distilled water. * $p < 0.05$ versus normal controls, † $p < 0.05$ versus DSS treated controls. Results are expressed as means (SEM) ($n = 10$).

Pharmaceutical Co., Tokyo, Japan) for isolation of *Bacteroidaceae*; LBS agar (Becton Dickinson and Co., Cockeysville, Maryland, USA) supplemented with 0.8% (W/V) Lab Lemco Powder (Oxoid Ltd, Basingstoke, UK), 0.1% (W/V) sodium acetate trihydrate, and 0.37% acetate for isolation of *Lactobacillus*; and DHL agar (Nissui) for isolation of *Enterobacteriaceae*. TOS agar, Bacteroides agar, and LBS modified agar media were cultured anaerobically in an atmosphere of 7% H_2 and 5% CO_2 in N_2 at 37°C. DHL agar was cultured aerobically at 37°C. Bacterial numbers were expressed as log10 counts of viable bacteria/g wet weight of caecal contents.

Measurements of SCFAs and pH in caecal contents

SCFAs (acetic, propionic, and n-butyric acids) were measured using high performance liquid chromatography (Shimadzu, Kyoto, Japan) by the internal standard method, as described by Hoshi and colleagues.²⁹ Briefly, caecal contents were homogenised in DW, and the homogenate was centrifuged at 10 000 g . The suspension was passed through a 0.45 μm filter, and then protein precipitation was removed with Carrez I and II solutions. SCFAs were separated using an ion exclusion column and detected using a post column pH buffered electroconductivity detection method. SCFA concentration was expressed as mmol/kg of caecal contents. After homogenising and passing through a 0.45 μm filter, the pH level in caecal contents was measured with a pH meter (model F-21; Horiba, Tokyo, Japan).

Statistical analysis

Parametric data were statistically analysed by the Student's t test. Non-parametric data were statistically analysed using the Mann-Whitney U test. All results are expressed as means

(SEM) from 10 animals. A significant difference was defined as $p < 0.05$.

RESULTS

Survival rate and histological damage

There was a significant difference between survival rates in the DHNA prevention group and the DSS prevention control group on day 21. There was also a significant increase in survival rate in the DHNA therapy group on day 14 compared with that in the DSS therapy control group (fig 1). On H&E staining of colonic tissue sections, marked leucocyte infiltration, crypt distortion, and erosions were observed in the colonic mucosa in DSS treated groups (fig 2). However, in both the DHNA prevention and DHNA therapy groups, striking suppression of crypt distortion and erosion was observed. In addition to these ameliorations, cell infiltration to the lamina propria was significantly decreased in the DHNA treatment group in both protocols (fig 2). The crypt scoring system revealed that the damage score of the DSS therapy control was significantly greater than that of the DSS prevention control (fig 3A). These damage scores were significantly lower in the DHNA prevention and therapy groups than in the DSS alone control group. There was no significant difference between the extent of attenuation by DHNA at doses of 0.6 and 2 mg/kg in either protocol.

Effects of DHNA on MAdCAM-1 expression and $\beta 7$ integrin positive cells

Expression level of MAdCAM-1 was significantly higher in the DSS treated groups than in non-treated animals and was detected in the submucosal layer and in the lamina propria (figs 2, 3B). Upregulated expression of MAdCAM-1 induced by DSS administration was remarkably decreased, especially

Figure 4 mRNA expression of inflammatory cytokines in the proximal colon in dextran sodium sulphate (DSS) induced colitis and the attenuating effect of 1,4-dihydroxy-2-naphthoic acid (DHNA). Interleukin (IL)-1 β (A), IL-6 (B), and tumour necrosis factor α (TNF- α) (C) mRNA levels were determined by quantitative real time polymerase chain reaction using an ABI Prism 7000 Sequence Detection System. The columns represent the average ratio of cytokines and 18S rRNA from 10 mice. * $p < 0.05$ versus normal controls, † $p < 0.05$ versus DSS treated controls, ‡ $p < 0.05$ versus 0.6 mg/kg of DHNA, § $p < 0.05$ versus DHNA prevention protocol. DW, distilled water. Results are expressed as means (SEM).

in the lamina propria, both in the DHNA therapy and prevention groups. The concentration of DHNA did not affect the extent of reduction in MAdCAM-1 expression level (fig 3B).

In the DSS control group, numerous $\beta 7$ integrin positive cells were accumulated in the lamina propria and erosive area. In addition, some positive cells had infiltrated the submucosa. Both DHNA therapy and prevention reduced the increased $\beta 7$ integrin positive cell number (figs 2, 3C). There was no significant difference between the degrees of reduction in $\beta 7$ integrin positive cell number in the inflamed colon with the two different concentrations of DHNA (fig 3C).

Effect of DHNA on expression of proinflammatory cytokines

mRNA levels of various cytokines in the colonic tissue were determined by quantitative real time PCR (fig 4). In the DSS control group (in either prevention or therapy), mRNA expression levels of IL-1 β , IL-6, and TNF- α were significantly increased compared with those in non-treated mice, although the degree of cytokine upregulation was greater in DSS prevention controls than in therapy controls, especially IL-6. DHNA prevention and therapy, at both concentrations, significantly suppressed mRNA expressions of all cytokines.

Effect of DHNA on resident flora

Tables 1 and 2 show changes in the numbers of indigenous bacteria, *Bacteroides*, *Lactobacillus*, *Bifidobacterium*, and *Enterobacteriaceae*, in caecal contents. In the DHNA prevention group (table 1), the number of *Lactobacillus* was significantly decreased in the DSS control in addition to a reduction in *Enterobacteriaceae*. These reductions in numbers of bacteria were recovered by DHNA preventive treatment at either concentration. In the DHNA therapy group (table 2), the number of *Enterobacteriaceae* was significantly decreased by DSS administration alone, while this change was attenuated by both concentrations of DHNA. In addition to this recovery, the number of *Lactobacillus* was significantly increased by treatment with DHNA at 2.0 mg/kg. In both the DHNA therapy and prevention groups, the numbers of *Bacteroidaceae* and *Bifidobacteriaceae* in caecal contents were not affected by DSS administration. DHNA therapy and prevention also had no apparent effect on caecal contents of these bacteria.

Changes in SCFA concentration and luminal pH induced by DHNA

As shown in fig 5A, there was no significant difference between concentrations of acetic acid in DSS treated mice and non-treated mice. Only DHNA therapy and prevention at

Table 1 Changes in caecal bacterial flora induced by dextran sodium sulphate (DSS) treatment and effects of 1,4-dihydroxy-2-naphthoic acid (DHNA) administration in the DHNA prevention group

DHNA prevention protocol		Bacterial counts (log 10 cfu)		
Organism	Normal	DSS (prevention control)	DHNA (0.6 mg)-DSS	DHNA(2.0 mg)-DSS
<i>Bacteroides</i>	9.59 (0.21)	9.46 (0.29)	9.56 (0.28)	9.43 (0.23)
<i>Lactobacillus</i>	9.07 (0.33)	8.42* (0.21)	9.10† (0.45)	9.10† (0.24)
<i>Bifidobacterium</i>	8.33 (0.32)	8.38 (0.38)	8.09 (0.31)	8.52 (0.22)
<i>Enterobacteriaceae</i>	6.08 (0.44)	5.37* (0.27)	6.21† (0.22)	6.85† (0.35)

Results are expressed as log10 cfu, mean (SEM) of caecal contents for 10 mice.

Normal, non-treated control mice.

* $p < 0.05$ versus normal, † $p < 0.05$ versus DSS treated controls.

DHNA-DSS: DHNA administration followed by DSS treatment.

Table 2 Changes in caecal bacterial flora induced by dextran sodium sulphate (DSS) treatment and effects of 1,4-dihydroxy-2-naphthoic acid (DHNA) administration in the DHNA therapy group

DHNA therapy protocol		Bacterial counts (log 10 cfu)		
Organism	Normal	DSS (therapy control)	DSS-DHNA (0.6 mg)	DSS-DHNA (2.0 mg)
<i>Bacteroides</i>	9.59 (0.21)	9.38 (0.17)	9.74 (0.45)	9.39 (0.2)
<i>Lactobacillus</i>	9.07 (0.33)	8.01 (0.28)	9.02 (0.22)	9.90† (0.19)
<i>Bifidobacterium</i>	8.33 (0.32)	8.21 (0.34)	8.45 (0.41)	8.40 (0.44)
<i>Enterobacteriaceae</i>	6.08 (0.44)	4.55* (0.23)	6.30† (0.33)	5.78† (0.24)

Results are expressed as log10 cfu, mean (SEM) of caecal contents for 10 mice.

Normal, non-treated control mice.

* $p < 0.05$ versus normal, † $p < 0.05$ versus DSS treated controls.

DSS-DHNA: DSS treatment followed by DHNA administration.

a dose of 2.0 mg/kg induced a significant increase in acetic acid concentration. Butyric acid concentration was significantly decreased by DSS administration compared with that in non-treated animals. The decrease in butyric acid concentration induced by DSS recovered to control levels with DHNA prevention. In contrast, DHNA therapy was not effective for recovery of butyric concentration (fig 5B). The concentration of propionic acid was increased by DSS administration compared with that in non-treated animals; however, in both the DHNA prevention and therapy groups, these concentrations were significantly higher than that in the DSS treated groups (fig 5C). Figure 6 shows the changes in caecal luminal pH caused by DSS administration and the effect of DHNA treatment. The pH level was elevated to more than 7.5 by DSS administration. In contrast, in the DHNA prevention group, pH levels remained lower than 7, significantly lower than those in the DSS treated group. However, in the DHNA therapy group, pH levels remained higher than 7.0.

DISCUSSION

In this study, we demonstrated that DHNA was equally effective for both prevention and therapy of DSS induced colitis in mice. These results indicate that DHNA not only attenuates the development of colonic inflammation but also

has an inhibitory effect on established inflammation, suggesting the clinical usefulness of this agent for the treatment of IBD. DHNA is known as an intermediate metabolite of menaquinone biosynthesis.²⁴ We used two different doses of DHNA, and 0.6 mg/kg was chosen because this dose has been estimated to be effective for bone metabolism in mice (personal communication, 22nd Meeting of the Japanese Society for Bone and Mineral Research, 2004). On the other hand, 2.0 mg/kg was chosen as a maximum non-toxic dose for mice as doses of more than 10 mg/kg often showed a significant toxic effect in mice in our preliminary study. Nevertheless, in the present study, there was no difference between the effects of the two doses on DSS induced colitis, except for the inhibitory effect on production of cytokines and SCFAs.

DSS therapy control showed a higher histological damage score than the DSS prevention control. This may be due to the difference in time of assessment—namely, the DSS prevention protocol was assessed seven days after the start of DSS treatment while the DSS therapy protocol was assessed at 14 days (seven days after the end of DSS treatment). Because inflammation progresses even after stopping DSS treatment, the prevention protocol may show an earlier phase of colonic damage than the therapy protocol, corresponding to an aggravating phase of inflammation. As DHNA was shown to

Figure 5 Effect of 1,4-dihydroxy-2-naphthoic acid (DHNA) administration on concentrations of short chain fatty acid in dextran sodium sulphate (DSS) induced colitis. Concentrations of (A) acetate, (B) n-butyrate, and (C) propionate are represented as mmol/kg of caecal contents. * $p < 0.05$ versus normal controls, † $p < 0.05$ versus DSS treated controls, ‡ $p < 0.05$ versus 0.6 mg/kg DHNA. DW, distilled water. Results are expressed as means (SEM) ($n = 10$).

Figure 6 Effect of 1,4-dihydroxy-2-naphthoic acid (DHNA) administration on colonic intraluminal pH in dextran sodium sulphate (DSS) induced colitis. pH was determined by a pH meter in caecal contents. * $p < 0.05$ versus normal controls, † $p < 0.05$ versus DSS treated controls. DW, distilled water. Results are expressed as means (SEM) ($n = 10$).

be effective for both protocols, it is possible that DHNA has multiple targeting points in colonic inflammation during induction and development of mucosal damage.

It is becoming increasingly apparent that increased MAdCAM-1 in an inflammatory area is involved in the rolling and adhesion of leucocytes to vascular endothelial cells mediated by interaction with $\alpha 4 \beta 7$ integrin.³⁰ Furthermore, we previously showed that administration of anti-MAdCAM-1 significantly ameliorated colonic injury²² in DSS induced colitis through suppression of infiltration of $\beta 7$ integrin positive lymphocytes. In human IBD, colonic lamina propria of patients with CD and UC showed increased density of MAdCAM-1 and $\alpha 4 \beta 7$ integrin positive cells compared with that in IBS controls.³¹ These studies indicate that MAdCAM-1 plays an important role in inflammation in experimental colitis and in human IBD, and its suppression has been suggested to be effective for ameliorating IBD. In this study, expression of MAdCAM-1 and $\beta 7$ positive cells upregulated by DSS administration was significantly suppressed by DHNA therapy and prevention. Furthermore, the reduction in MAdCAM-1 and $\beta 7$ positive cells correlated well with survival rate and histological damage scores (figs 1, 3). By immunohistochemical examination, a reduction in the numbers of MAdCAM-1 and $\beta 7$ positive cells induced by DHNA treatment was also observed in the same specimens (fig. 2). Our data indicate that DHNA may have the ability to suppress inflammatory cell infiltration through modulation of cell migration mediated by MAdCAM-1- $\beta 7$ positive lymphocyte interaction.

It is well known that proinflammatory cytokines play an important role in inflammation of the intestinal mucosa.³² There is a close relation between the production of proinflammatory cytokines and expression of ECAMs, such as MAdCAM-1. Sikorski *et al* showed that increased expression of MAdCAM-1 on murine endothelial cells can be induced by stimulation with IL-1 and TNF- α .³³ Therefore, it is possible that DHNA induces a reduction in the level of MAdCAM-1 expression through suppression of IL-1 β and TNF- α . IL-6 also plays an important role in the initiation and persistence of

colitis.^{34, 35} Isaacs *et al* reported that the mRNA level of IL-6 was increased in UC and CD and that the magnitude of inflammation in UC correlated with the mRNA level of IL-6.³⁶ Recently, the clinical effectiveness of anti-IL-6 receptor antibody therapy in patients with active CD has been reported.³⁷ In the present study, we found significant increases in the levels of these proinflammatory cytokines, suggesting the involvement of these molecules in the early development of colitis. DHNA significantly attenuated the increase in mRNA levels of IL-1 β , IL-6, and TNF- α , concomitant with attenuation of mucosal damage. Roller *et al* showed that treatment with inulin enriched with oligofructose stimulated IL-10 and interferon γ production by Peyer's patch cells.¹⁵ Hoentjen *et al* reported that the combination of inulin and fructo-oligosaccharides significantly decreased gross and histological inflammation and caecal IL-1 β concentrations.³⁸ Taken together, the results of our study and previous studies suggest that treatment with prebiotics can directly induce immunomodulation in the intestine by directly affecting cytokine producing cells such as intestinal macrophages.

It is known that DHNA promotes proliferation of the genus *Bifidobacterium* in vitro,²⁴ but the in vivo effect of DHNA on intestinal bacterial flora remains unknown. *Lactobacillus* and *Enterobacteriaceae* are known intestinal bacterial flora in healthy humans and mice.^{39, 40} In contrast, IBD patients are known to have altered composition of commensal intestinal bacteria with increased *Bacteroides*, adherent/invasive *Escherichia coli*, *Enterococci*, and decreased *Lactobacillus* and *Bifidobacterium* species.⁴¹ Unexpectedly, we did not observe significant changes in *Bifidobacterium* after DSS administration, and DHNA treatment did not affect bacterial numbers in DSS mice. However, we observed a significant decrease in *Lactobacillus* in the DSS prevention control group and a decrease in *Enterobacteriaceae* in both the DSS prevention and therapy control groups, suggesting a decrease in *Lactobacillus* as an early change and a decrease in *Enterobacteriaceae* in an aggravating phase of inflammation. The numbers of *Lactobacillus* and *Enterobacteriaceae* were recovered by either DHNA prevention or therapy, suggesting that DHNA has the ability to improve the balance of intestinal bacterial flora in any phase of mucosal inflammation.

SCFAs, such as acetic acid, propionic acid, and butyric acid, are produced in the gut essentially by anaerobic bacterial fermentation of unabsorbed carbohydrates.^{42, 43} Our results revealed that the concentration of butyrate was decreased in DSS treated mice while the concentration was maintained at the physiological level by DHNA prevention. Concentrations of acetate and propionate were increased to levels higher than those under physiological conditions by DHNA prevention or therapy. In addition, in the DHNA prevention group, pH levels in caecal contents were lower than those under physiological conditions, while pH was higher than 7.8 in the case of DSS alone. Although the exact mechanism by which increased luminal SCFAs has an anti-inflammatory effect on intestinal mucosa remains unknown, it may be induced by decreasing luminal pH, improving epithelial barrier function, and inhibition of nuclear factor κB activity.^{12, 44, 45} However, increased butyrate production and downregulation of luminal pH may not be essential for DHNA therapy because these parameters were not significantly reversed in DHNA therapy.

In conclusion, DHNA improved survival rate and histological score in DSS induced colitis, partly through a decrease in expression of MAdCAM-1, $\beta 7$ positive cells, and mRNA levels of proinflammatory cytokines. Our findings demonstrate for the first time that oral administration of DHNA attenuated colonic inflammation not only in relation to the traditional functions of prebiotics, such as balancing intestinal bacterial flora and increasing SCFAs, but also by

suppression of lymphocyte infiltration through suppression of proinflammatory cytokines and adhesion molecules (for example, MAdCAM-1). The results of the present study suggest that DHNA is useful for preventing and treating human IBD.

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