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Nucleoside-nucleotide free diet protects rat colonic mucosa from damage induced by trinitrobenzene sulphonic acid

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Abstract

***Background*—Growing evidence suggests that intestinal recovery from injury induced by radiation, endotoxin, and protein deficiency is improved by the ingestion of nucleosides and nucleotides.**

***Aim*—This study examined the effect of dietary nucleosides and nucleotides supplementation on trinitrobenzene sulphonic acid induced colonic damage in experimental colitis.**

***Methods*—Sprague-Dawley rats were randomised into two groups and fed nucleic acid free 20% casein diet (control) or this diet supplemented with 0·5% nucleoside-nucleotide mixture for four weeks. On the second week, colonic inflammation was induced in rats by intracolonic administration of 0·25 ml of 50% ethanol containing 25 mg of trinitrobenzene sulphonic acid. Additionally, other sets of rats were treated with 0·25 ml of 50% ethanol, 25 mg of trinitrobenzene sulphonic acid in 0·25 ml saline, or 0·25 ml of 0·9% saline.**

***Results*—After two weeks, colon weight, macroscopic and microscopic damage scores, were significantly greater ($p<0·05$) in the nucleoside-nucleotide supplemented group compared with the non-supplemented control groups. The same variables seen in the trinitrobenzene sulphonic acid-ethanol group fed nucleoside-nucleotide free diet were greater ($p<0·05$) than in the rest of the groups fed nucleoside-nucleotide free diet and treated with ethanol, trinitrobenzene sulphonic acid in saline, or saline. Histologically, segmental ulceration and inflammation associated with significantly increased infiltration of polymorphonuclear leucocytes, macrophages, lymphocytes, fibroblasts were observed in the supplemented group compared with the controls. In the nucleoside-nucleotide supplemented group the epithelial damage, mucosal erosion, oedema, and coagulative necrosis of the muscularis propria was more extensive in comparison to the non-supplemented control groups.**

***Conclusions*—This study suggests that dietary nucleosides and nucleotides may aggravate colonic damage and inflammation in chemically induced experimental colitis in rats; and that nucleoside-nucleotide free diet combined with other pharmacological agents may offer a better response.**

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Keywords: nucleosides, nucleotides, rats, colitis.

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Over the past decade our laboratory, and many others, have been engaged in the search to identify the specific nutrient(s) necessary to maintain gut integrity and function after injury.[1–3] Dietary nucleosides and nucleotides were reported to prevent the intestinal mucosal atrophic changes in rat ileum induced by total parenteral nutrition,[4] increased the rate of maturation and growth in the young rat as determined by intestinal mass, RNA and DNA, protein concentrations and activity of brush border enzymes in the proximal and distal sites of the small intestines.[5] Nucleotide and nucleoside supplementation also restored crypt depth and villus height after induction of diarrhoea[6–8]; improved gut morphology and reduced the incidence of bacterial translocation in protein deficient mice.[9 10] Dietary nucleosides and nucleotides are also implicated in the maintenance of the immune system.[11–13]

Ulcerative colitis (UC) and Crohn's disease (CD) are major inflammatory bowel diseases (IBD) in humans. Animal models of colitis have been developed in rats, mice, guinea pigs, and rabbits,[14] and histopathological evaluations of lesions in these animals show disruption of the intestinal mucosa, crypt abscesses or loss of crypts, epithelial thinning, superficial ulcerations and polymorphonuclear cell infiltration of the lamina propria.[15 16] The aetiology of IBD, UC, and CD remains unknown. However, many theories including immunological, infective, psychological, and dietary have nevertheless been postulated. The current proposition is that dietary measures should be sought as a primary therapeutic procedure. Recent studies performed in animals suggest that diets high in linoleic acid increase inflammation in the colon.[17 18] In another development, it was observed that polymeric and elemental diets induced clinical remission in acute CD.[19 20] Previous studies from our laboratory showed that dietary supplementation with nucleosides and nucleotides may lead to repair of protein deficiency and endotoxin induced damage to the gut; and that dietary nucleosides and nucleotides may become conditionally essential nutrients for

maintenance of gut morphology and function under such circumstances.[10 11] Consequently, we examined the effect of nucleosides and nucleotides on trinitrobenzene sulphonic acid induced colitis in rats. We used this chemically induced colitis model reported by Wallace *et al*[21] because of its reproducibility and value in assessing the efficacy of therapeutic agents commonly used in colitis.[22 23]

Methods

Animals

Thirty four specific pathogen free Sprague-Dawley rats were obtained from Kyudo Breeding Laboratories (Kumamoto, Japan) and used for the experiment. Weight matched (150–200 g) female rats were kept in a constant temperature (25±2°C) and humidity (50–70%) room with a 12 hour light period from 0800 to 2000 hours. Animal care was in compliance with applicable guidelines from the Ryukyus University Policy on Animal Care and Use. The rats were kept for one week before the onset of the experiment to acclimatise to our laboratory conditions. The animals were housed in rack-mounted wire cages with a maximum of two animals per cage. During this period the rats received normal non-purified diet (Nihon Clear, Osaka, Japan). This standard rat diet contains 25·5% protein and 4·3% fat by weight.

Study protocol

After the period of acclimatisation, the rats were randomised into two groups according to the dietary treatment. Each diet was started two weeks before the instillation of 2,4,6-trinitrobenzene sulphonic acid (TNBS), and was continued until the rats were killed. After two weeks on the diet, each rat received an intracolonic instillation of TNBS (0·25 mg/0·25 ml) and killed two weeks later, and the damage to the colon was investigated.

TNBS

TNBS (Wako Chemical Co, Japan) was dissolved in 50% ethanol to a final concentration of 100 mg/ml; and then 0·25 ml was instilled intracolonically into each animal used for the experiment.

Diet

The control groups of rats were fed 20% nucleic acid free casein diet (NFD), and the remaining group of rats the same diet supplemented with 0·5% nucleoside-nucleotide mixture (NNM) per kg diet throughout the investigation. NNM (Ohtsuka Pharmaceutical Factory Inc, Tokushima, Japan) is a mixture of nucleosides and a nucleotide consisting of inosine (8 g/l), guanosine monophosphate (12·2 g/l), cytidine (7·3 g/l), uridine (5·5 g/l), and thymidine (1·85 g/l) at a molar ratio of 4:4:4:3:1. Table I shows the composition of the diets. The diets were made isonitrogenous and isocaloric by adding an appropriate amount of glycine. The nucleotide content of the control diet was negligible as determined by high performance liquid chromatography. Animals in all the groups were given free access to the food and water. From 10 to 11 am every morning, the animals were weighed and food and water renewed.

TABLE I *Composition of experimental diet*

	Nucleic acid free diet	*0·5% nucleic acid component diet*
	g/kg	g/kg
Casein*	200	200
Nucleic acid mixture†	0	5
Glycine	4·8	0
Carbohydrate‡	665·2	665
Soybean oil	50	50
Mineral mixture§	50	50
Vitamin mixture¶	10	10
Cellulose	20	20

*Casein (84·3% crude protein) was purchased from Oriental Yeast Co, Tokyo. †Inosine 230 g/l, cytidine 210 g/l, GMP 2Na 350 g/l, uridine 160 g/l, thymidine 50 g/l. ‡α-corn starch: sucrose, 2:1 ratio. §Obtained from Oriental Yeast Co, Tokyo. The composition was: (mg/kg) $CaHPO_4.2H_2O$, 7–280; KH_2PO_4, 12 860; NaH_2PO_4, 4–680; NaCl, 2–330; Ca-lactate, 17 550; Fe-citrate, 1–590; $MnSO_4$4–$6H_2O$, 60; $MgSO_4$, 3–950; $CuSO_45H_2O$, 15; KI, 5. ¶Obtained from Oriental Yeast Co, Tokyo. The composition was: (mg/kg) thiamin-HCl, 12; riboflavin, 40; pyridoxine-HCl, 8; vitamin B_{12}, 50; ascorbic acid 300; D-biotin, 0·2; folic acid, 2; calcium pantothenate, 50; ρ-aminobenzoic acid, 50; niacin, 60; inositol, 60; choline chloride, 2,000; dl-α tocopheryl acetate, 50; menadione, 52; and (in IU) retinyl acetate, 5000; ergocalciferol, 1–000.

Treatment groups

Animals were divided randomly into the following treatment groups. Group 1, NFD+saline administration (n=5). Group 2, NFD+50% ethanol administration (n=5). Group 3, NFD+TNBS in saline administration (n=8). Group 4, NFD+TNBS in 50% ethanol administration (n=8). Group 5, NNM+TNBS in 50% ethanol administration (n=8).

Induction of experimental colitis

After a 16 hour fast, rats in each group were sedated by an intraperitoneal administration of 50 mg/ml sodium pentobarbital (Wako Chemical Co, Japan; 0·1 ml/100 g body weight). A polypropylene catheter was lubricated with jelly and inserted 8 cm via the anal canal into the colon of the rat just proximal to the splenic flexure. TNBS (100 mg/ml) dissolved in 50% (vol/vol) ethanol was instilled into the colon (total volume of 0·25 ml per rat). After instillation the rats were supported in a supine position until recovery from anaesthesia to prevent immediate anal leakage of the instillate.

The control groups of normal rats received (administered as before) 0·25 ml of either 50% ethanol, 25 mg of TNBS in 0·9% saline, or 0·9% saline alone.

Assessment of colonic damage

Two weeks after the instillation, the rats in each group were killed by an injection of sodium pentobarbital intraperitoneally. The distal

TABLE II *Histopathological grading scale of chemically induced colitis*

Grade of colitis	*Microscopic findings*
0	Histological findings identical to control.
1	Mild mucosal and/or submucosal inflammatory infiltrate (admixture of neutrophils, mononuclear cells, and occasional eosinophils) and oedema. Punctate mucosal erosions often associated with capillary proliferation. Muscularis mucosae intact.
2	Grade 1 changes involving ≧50% of the specimen.
3	Prominent inflammatory infiltrate and oedema (neutrophils usually predominating) frequently with deeper areas of ulceration extending through the muscularis mucosae into the submucosa. Rare inflammatory cells into the muscularis propriae but without muscle necrosis.
4	Grade 3 changes involving ≧50% of the specimen.
5	Extensive ulceration with coagulative necrosis bordered inferiorly by numerous neutrophils and lesser numbers of mononuclear cells. Necrosis extends deeply into the muscularis propria.
6	Grade 5 changes involving ≧50% of the specimen.

colon was removed, opened longitudinally, and cleared of faecal material with a gentle spray of 0·9% saline. The freshly opened colonic segments were pinned out on a wax block and examined under a stereomicroscope by two independent observers blinded to the treatment. The extent of the mucosal damage was assessed using the colon macroscopic scoring system of Wallace *et al.*[21] After scoring, the distal 8 cm segment of the colon was blotted dry and weighed.

Colon histology

Samples of the inflamed and non-inflamed tissues were removed for histological analysis. The tissues were fixed in 37–40% phosphate buffered formalin (37–40% formaldehyde, 10 ml; sodium phosphate monobasic, 1·86 g; sodium hydroxide, 0·42 g; distilled water, 90 ml) at room temperature overnight. The tissues were sliced into 4–6 mm pieces, dehydrated in ethanol, embedded in paraffin wax, sectioned, and stained with haematoxylin and eosin. The microscopic slides were reviewed by two of us (TM and AK) blinded to the experimental groups, and the extent of damage and colonic inflammation assessed using a modification of the histopathological grading system of Macpherson and Pfeiffer[24] (Table II). The sections were examined and photographed with the use of an Olympus microscope (Olympus Kogyo Co, Tokyo, Japan).

Statistical analysis

Statistical analysis of the data was performed by analysis of variance (ANOVA) and the Mann-Whitney U test for damage score. Duncan's multiple range tests was used to determine significant differences among means. A p value <0·05 was considered significant.

Results

General findings

Prior to the induction of colitis, weight gain was similar in all the dietary groups. After induction of colitis, a slight decrease in total body weight was seen for one week in all the groups except Group 1. The growth rates returned to normal two weeks after induction of colitis in Groups 1 and 2 (Table III). In Groups 3–5, the growth rates two weeks after induction were slightly lower relative to the weights before induction. Diarrhoea was observed in >40% of the TNBS/ethanol administered rats (Groups 4 and 5) but never seen in the other control groups. There was bowel obstruction in the TNBS/ethanol administered groups (Groups 4 and 5) when the rats were killed. There was no death in any of the groups during the period of investigation.

TABLE III *Body weight changes before and after induction of chemically induced colitis*

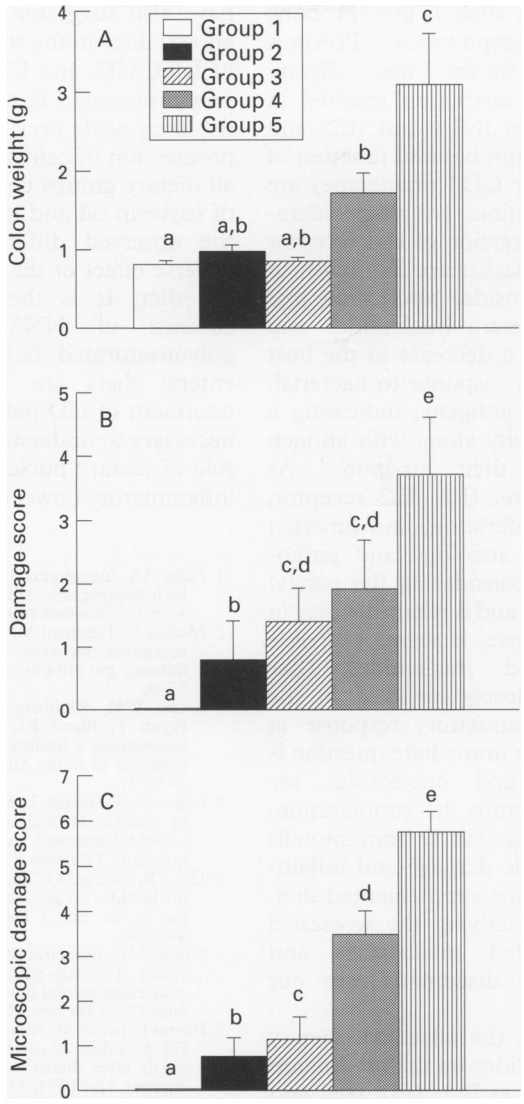
	Body weight		
Experimental groups	*Before induction of colitis (g)*	*1 week after induction of colitis (g)*	*2 weeks after induction of colitis (g)*
Group 1	296 (6·8)[a]	294 (14·3)[a]	301 (16·2)[a]
Group 2	300 (8·2)[a]	289 (14·8)[a]	301 (14·2)[a]
Group 3	297 (4·4)[a]	280 (14·7)[a]	258 (39·3)[a,b]
Group 4	301 (7·2)[a]	268 (20·2)[a]	262 (56·4)[a,b]
Group 5	298 (7·1)[a]	257 (24·1)[a]	247 (72·4)[b]

Values are means (SD). n=5 in groups 1, 2 and n=8 in groups 3–5. Within a column, values with different letter(s) indicates significant difference, p<0·05.

Colonic inflammation

Macroscopic and microscopic examinations showed varying degrees of colitis in all the animals administered TNBS in 50% ethanol (Groups 4 and 5), TNBS in saline (Group 3), and 50% ethanol (Group 2) whereas inflammatory changes were absent in the saline administered group (Group 1). After two weeks, colon weight and macroscopic damage score, in the rats administered TNBS/ethanol and fed NNM (Group 5) were greater (p<0·05) compared with the rest of the groups (Groups 1–4) (Figure A, B). The damage and inflammation observed in the TNBS/ethanol group fed NFD (Group 4) was also greater (p<0·05) compared with the other control groups (Groups 1–3). Histologically, the rats fed the supplemented diet (Group 5) developed severe bowel thickening, inflammation, and ulcers compared with the non-supplemented diet groups (Groups 1–4, data not shown). Moreover, in Groups 4 and 5 respectively, the mucosal ulcers were broad based with a surface layer of necrotic slough, accumulations of mesenteric fats, fibrinous adhesions to the bowel, and epitheloid granulomas compared with Groups 1–3. In the NFD control groups treated with 50% ethanol (Group 2), TNBS/saline (Group 3), TNBS/ethanol (Group 4), acute colonic damage with haemorrhage and bowel wall thickening were also seen; however the damage was significant in the TNBS/ethanol group (Group 5, data not shown).

Microscopic examination showed that the inflammatory infiltrate consisted of neutrophils, lymphocytes, macrophages, fibroblasts,



Colon weight (A), macroscopic (B), and microscopic (C) damage scores of rats killed two weeks after intracolonic administration of 0·25 ml TNBS/ethanol (100 mg/ml), TNBS/saline (100 mg/ml), 50% ethanol, or 0·9% saline. Rats were fed nucleic acid free 20% casein diet (NFD) or this diet supplemented with 0·5% nucleoside-nucleotide mixture (NNM). Values are means (SEM); n=8 for TNBS/ethanol (NNM; Group 5), TNBS/ethanol (NFD; Group 4), TNBS/saline (Group 3), and n=5 for 50% ethanol (Group 2), Saline (Group 1), respectively. Bars with different letter(s) denotes significant difference, p<0·05).

and connective tissue mast cells, with extensive crypt distortion and transmural necrosis, which was more extensive in the supplemented group (Group 5, data not shown). The median grade of colitis recorded in the colonic segments of the supplemented group (Group 5) was significantly higher ($p<0·05$) compared with the rest of the groups (Groups 1–4, Figure C). The histological grade of colitis in the TNBS/ethanol (Group 4) was also higher ($p<0·05$) than that of Groups 1–3.

Discussion

The aetiologies of IBD, CD, or UC remain unclear. Several theories have been advocated, including immunological, infectious, genetic, allergic, and environmental causes. It has been shown that the gut lacks an active pathway of de novo purine and pyrimidine biosynthesis.[25] Suppression of the immune function has also been shown when exogenous nucleosides and nucleotides are eliminated from the diet.[13] In the presence of IBD, CD, or UC the supply of purines and pyrimidines and their precursors may be a limiting factor for both disease activity and healing. In this study, an animal model was used by feeding rats adequate amounts of diets essential to support normal growth and development. Subsequently, animals fed each of the diets were then subjected to a TNBS/ethanol challenge to determine if structural improvements produced by nucleosides and nucleotides supplementation can be translated into functional benefits. The results of our studies suggest that dietary nucleosides and nucleotides supplementation may aggravate the severity of colitis. Provision of nucleosides and nucleotides resulted in a significant increase of both microscopic and macroscopic inflammation damage when compared with rats fed the NFD (Figure). Histologically, the degree of mucosal ulceration was severe with extensive crypt distortion, and increased infiltration of polymorphonuclear leucocytes, macrophages, fibroblasts, connective tissue mast cells, and eosinophils (data not shown).

One of the main histological features of intestinal tissue affected by IBD, CD, and UC is the presence of chronic inflammatory infiltrates consisting mostly of polymorphonuclear leucocytes, macrophages, fibroblasts, and connective mast cells. These immune cells release a number of biochemical mediators of inflammation called cytokines, which have been implicated in the pathogenesis of tissue injury in IBD, CD, and UC.[26–28] It has been shown that mucosal biopsy specimens from patients with CD and UC contained significantly increased amounts of interleukin 1 (IL1), IL2, IL2 mRNA, and IL8, and that the highest amounts were found in the most inflamed specimens.[29–31] Gross *et al*[32] observed increased concentrations of serum IL6 and IL8 in active CD patients. In other related studies, Neilly *et al*[33 34] reported that increased administration of L-arginine aggravated mucosal damage in experimental colitis. They postulated that L-arginine caused nitrous oxide release by gut immune cells resulting in endotoxin and cytokines (IL1, tumour necrosis factor, interferon γ (INFγ)) contributing to the cytotoxic capability of the cells and thereby increasing mucosal inflammatory response. L-arginine has similarly been shown to have immunopotentiating activity similar to NNM.[35]

Many foods like chicken, beef, and pork contain high amounts of nucleotides mainly in the form of nucleoproteins from which the nucleic acids are liberated in the gut as free nucleotides. Vegetables, cheese, and potatoes contain low amounts. Nucleotides are partly absorbed in the gut as nucleosides through different mechanisms and are incorporated into body tissues mainly the liver, gut, bone marrow, and spleen.[36 37] Dietary nucleotides and nucleosides are of special importance in the development and proliferation of tissues

with rapid cell turnover such as gut and bone marrow and also lymphocytes. Previous investigations have shown that dietary nucleotide, nucleoside restriction resulted in decreased production of INFγ and IL2; and that the effects on the number and function of T helper cells (Lyt1 or CD4 phenotype) are significant.[38 39] In addition, lymphoproliferative responses, and expression of IL2 receptor and Lyt1 cell surface marker are also lower in animals fed the nucleoside, nucleotide free diet.[40] Absence of dietary nucleotides and nucleosides resulted in a decrease in the host delayed hypersensitivity response to bacterial, chemical, and cellular antigens, indicating a decrease of T cell activity along with antigen processing cells and their function.[41] As increased T lymphocyte, IL2, IL2 receptor, INFγ production, proliferation, and function are implicated in the aetiology and pathogenesis of colitis and considering the part(s) that dietary nucleosides and nucleotides play in the gut immune response, it seems probable that nucleosides and nucleotides (like L-arginine) may be closely involved in the expression of the inflammatory response in CD, IBD, and UC. Our immediate question is whether nucleosides and nucleotides are participants in augmenting the proliferation, production, and function of these immune cells leading to severe colonic damage and inflammation in the rats fed the supplemented diet. The mechanism(s) underlying the worsened conditions of rats fed nucleosides and nucleotides cannot be discerned from our study.

Published reports on the effects of dietary nucleosides and nucleotides on the production of other cytokines such as IL1, IL6, IL8, and TNF are scanty. To our knowledge, our study is the first report that has assesed the effect(s) of nucleosides and nucleotides on experimental colitis in rats. It seems probable that the effects of diet on IBD are multifactorial. Although the precise mechanisms are not well understood, both enteral and parenteral nutritional support has been reported to be efficacious for the symptomatic treatment and management of CD.[23 41] Comparison between enteral diets having different sources of nitrogen in the treatment and management of CD, IBD, and UC has yielded inconclusive results.[43 44] It has been suggested that the efficacy of such elemental diets in the treatment of CD is probably because they contain no purines or pyrimidines.[45 46] Determination of the role of diet in the aetiology and pathogenesis of colitis is important to formulate a logical approach to treatment as well as determine which components of the diet might be the precipitating factor(s) in these conditions.

In conclusion, this study suggests that dietary nucleosides and nucleotides may aggravate colonic damage and inflammation in experimental colitis induced in rats; and that it is probable that their absence in enteral diets used for the treatment and management of IBD, UC, and CD contributes to the observed clinical remission in patients. Some studies have also suggested that the success of such enteral diets in the treatment and management of IBD, UC, and CD is due to their low n-6 polyunsaturated fatty acid content,[23] and that n-3 fatty acids exert a beneficial effect on the progression of colitis.[47] However, in our study, all dietary groups contained the same amount of soybean oil and it is therefore unlikely that the observed differences were due to the adverse effect of the fatty acid composition of the diet. It is therefore possible that the absence of NNM and the low n-6 polyunsaturated fatty acid content of such enteral diets are both beneficial for the treatment of CD patients. Further studies are necessary to understand and define exactly the role of dietary nucleosides and nucleotides in inflammatory bowel diseases.

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