

Proteus mirabilis sp. intestinal microflora grow in a dextran sulfate sodium-rich environment

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Abstract. The pathogenic mechanisms responsible for inflammatory bowel disease, especially ulcerative colitis (UC), are poorly understood. As an animal model, the oral administration of dextran sulfate sodium (DSS) induces colitis, which exhibits several clinical and histological features similar to UC. However, the pathogenic factors responsible for DSS-induced colitis and above all, the intestinal microflora in this colitis remain unclear. Therefore, we investigated the relationships between DSS and the intestinal microflora in this study. First, the depolymerization of DSS in mouse feces was analyzed using a pyridylamino-labeling (PA-DSS) and HPLC system. Next, a bacteriological study of the fecal contents using DSS-rich media and subsequently a classification using 16S rRNA were performed. Surprisingly, DSS was depolymerized in mouse feces under aerobic conditions, not under anaerobic conditions. Several kinds of microflora were suggested to be involved in this depolymerization. In particular, *Proteus mirabilis* can grow in DSS-rich media and has an ability to desulfonate and depolymerize DSS. Then, we produced chemically-modified *Mr* 2500 DSS from native *Mr* 5000 DSS. This depolymerized *Mr* 2500 DSS was administered orally to mice and the colitis was evaluated histologically. The cytotoxicity of *Mr* 2500 DSS on Caco-2 cells was also investigated. *Mr* 2500 DSS induced weaker colitis in mice and weak cytotoxicity on Caco-2 cells as compared to *Mr* 5000 DSS. These findings give insight into the mechanisms responsible for DSS-induced colitis, especially with respect to the molecular mass of DSS.

Introduction

Dextran sulfate sodium (DSS) has been used in animals to induce experimental colitis (1). The DSS-induced colitis model

is favored because of the high reproducibility of the colitis lesions and because these lesions are induced mainly in the left colon in a fashion similar to human ulcerative colitis (UC) (1). Therefore, DSS is widely used in investigations on the pathogenesis of UC and the efficacy of therapeutic agents. DSS is a heparin-like sulfated polysaccharide containing ~17% sulfur, with up to three sulfates per glucose molecule (Fig. 1) (2). With respect to the pathogenic factors in this colitis model, previous studies have postulated the importance of various factors such as local immunological disturbances (3), the activation of mucosal macrophages (4), effects related to the strong negative-charge of DSS (5), obliteration of the crypt lumina (6) and changes in the intestinal microflora (7). Our previous studies also have postulated the importance of various factors such as the local involvement of reactive oxygen species (8,9), short chain fatty acids (SCFA) (10) and cell cycle arrest (11). However, the precise etiology of this model remains unclear.

We previously reported that the microflora in the fecal contents was altered in DSS-induced colitis. In that study, *Eubacterium*, *Bifidobacterium* and total anaerobes were all significantly decreased, although *Lactobacillus* is significantly increased in DSS-induced colitis (12). In addition, even certain dietary fiber (germinated barley foodstuff) can alter the intestinal microflora, produce SCFA, subsequently colitis improved in human and DSS-induced colitis (13-16). Probiotics have been reported to have therapeutic effects for both diseases (10,17). These results strongly suggest that the intestinal microflora play a certain role in intestinal inflammation.

First, it remains unknown whether some microflora can grow in a DSS-rich environment. If some microflora can grow in a DSS-rich environment, how do these microflora influence the luminal environment? This question seems very important to the pathogenic mechanisms of DSS-induced colitis. Therefore, we planned the present experiments in order to answer this question.

Materials and methods

Chemicals. DSS (*Mr* 5000, total sulfur 15.0-20.0%), 2-amino-pyridine, phthalic acid and tetra-n-butylammonium hydroxide were obtained from Wako Pure Chemical (Osaka, Japan). Sodium cyanoborohydride were obtained from Nacalai Tesque

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Inc. (Kyoto, Japan). DSS (*Mr* 8000 or 10000) and D-glucose 3-sulfate was obtained from Sigma Chemical Co. (St. Louis, USA).

Animals. We used the fecal contents of specific pathogen-free male BALB/cA Jcl mouse, 6 weeks old, which were purchased from Nippon Clea Inc. (Tokyo, Japan). They were housed in a room with controlled temperature (20–22°C), humidity (50–60%) and a preset light-dark cycle (12 h: 12 h). The experimental protocol was approved by the Animal Care and Use Committee of the Shiga University of Medical Science.

Labeling of DSS using 2-aminopyridine. The pyridylation of the reducing termini of sugar chains has been useful for structural analysis and metabolic studies of *N*- or *O*-glycosidically linked sugar chains (18). We used a labeling system of DSS in order to investigate the metabolism of DSS. Labeling of DSS using 2-aminopyridine was carried out according to our previous study (19). Briefly, 1.0 g of DSS was dissolved in 25 ml of a 2-aminopyridine solution (prepared by mixing 5 g of 2-aminopyridine, 3.8 ml of conc. HCl and 11 ml of distilled water, with a final pH of 6.7) and coupled with 2-aminopyridine at 100°C for 13 min. After the addition of 1.5 ml of a reducing reagent (prepared by mixing 600 mg of sodium cyanoborohydride and 1.5 ml of distilled water just before use), the reaction mixture was heated at 90°C for 20 h for reductive amination. Four volumes of NaCl-saturated ethanol were then added to the reaction mixture. The resulting precipitate was recovered by centrifugation at 3,000 rpm for 5 min and washed with ethanol 10 times to remove excess reagents. D-glucose 3-sulfate was also labeled using 2-amino-pyridine as a monomer of sulfated polysaccharide.

HPLC conditions. An HPLC apparatus LC6A (Shimadzu, Kyoto, Japan) was used. The elution time of DSS was determined by gel filtration chromatography on a Cosmosil 5Diol-120 Packed column (7.5x300 mm; Nacalai Tesque Inc). Since DSS is strongly negatively-charged polysaccharide in water, the interaction between DSS and column is suggested on this HPLC system. Therefore, 0.2 M phosphate buffer (pH 3.0) was used as the mobile phase (19). The mobile phases were delivered isocratically at a flow rate of 1.0 ml/min. The column temperature was maintained at 60°C throughout this experiment. For the detection of pyridyl-amino-DSS (PA-DSS), an RF-535 fluorescence detector (Shimadzu) at excitation and emission wavelengths of 320 and 400 nm, respectively, was used.

DSS depolymerization in the feces. Mouse fecal samples were immediately collected after defecation. A total of 2 ml of distilled 5% PA-DSS solution was added to 1 g of the sample. Under aerobic or anaerobic conditions, the diluted samples were incubated for 48 h. After the incubation period, the samples were centrifuged at 10,000 rpm for 10 min. The supernatant was then filtered through a membrane filter (pore size 0.45 µm) and was injected into the HPLC columns. The PA-DSS in the samples was monitored using a fluorescence detector.

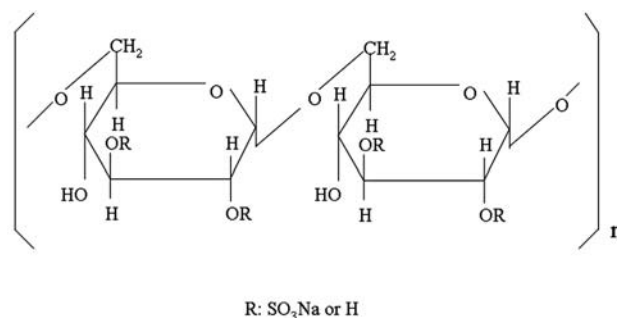


Figure 1. Structure of dextran sulfate sodium (DSS).

Bacteriological study of the fecal contents. We produced a novel DSS-rich solid medium composed of 4% DSS, 0.5% polypepton and 1.5% agar. The fecal contents were collected and then incubated in an aerobic or anaerobic environment. The anaerobic strains of microflora were investigated using a slight modification of previous methods (20,21). Briefly, samples were serially diluted with a dilution solution under a CO₂ environment. The plated microflora were then incubated in an anaerobic environment (AnaeroPack, Mitsubishi Gas Chemical Co., Tokyo, Japan) at 37°C for 72 h. Differentiation of the microflora was performed by: i) an investigation of the colony morphology; ii) gram staining; iii) an investigation of the growth under aerobic conditions; and iv) an investigation of reactions in media consisting of p-nitrophenyl sulfate.

Classification using 16S rRNA. We obtained some microflora from DSS-rich solid media and then performed PCR to amplify a portion of the 16S rRNA gene using specific primers. DNA was extracted from the microflora by using the SOIL for Beads Beating kit (Nippon Gene Co., Ltd., Tokyo, Japan). For amplification of the 16S rRNA, DNA was amplified using two primers: 10F: 5'-GTTTGATCCTGGC TCA-3', 800R: 5'-TACCAGGGTATCTAATCC-3'. The first-round amplification included 30 cycles of denaturation at 94°C for 30 sec, annealing at 72°C for 1 min and chain extension at 72°C for 3 min. The amplification products were then analyzed by agarose gel electrophoresis. The RNA obtained was purified using a commercial kit and sequenced twice with an ABI 3100 genetic analyzer (Model 3100; Applied Biosystems, Foster City, CA, USA). The 16S rRNA sequences obtained were compared by BLAST (www.ncbi.nlm.nih.gov/BLAST/) with sequences available at GenBank. Calculation of the distance matrices and construction of a phylogenetic tree were performed with MEGA 3.1 software (www.megasoftware.net). A phylogenetic tree was constructed by the neighbor-joining method and the distance matrices for the aligned sequences were calculated by the Kimura 2-parameter method.

Capability of microflora obtained from DSS-rich solid media to depolymerize PA-DSS. We investigated the capability of microflora obtained from DSS-rich solid media to depolymerize PA-DSS. The microflora were incubated in a PA-DSS-rich liquid medium composed of 5% PA-DSS, 0.5% polypepton and 0.05% sodium dihydrogenphosphate for 48 h under aerobic conditions. After incubation, the supernatant was analyzed by HPLC.

Chemically depolymerized Mr 2500 DSS. We previously reported a method to depolymerize chemically Mr 5000 DSS (22). Mr 5000 DSS was depolymerized to a Mr 2500 moiety under 1 N acidic conditions. In addition, a small amount of sulfate was depleted from DSS under these conditions (<10%).

Mouse Mr 2500 or 5000 DSS-induced colitis. The mice were allowed standard rodent food (CE-2, Nippon Clea Inc.) and drinking water *ad libitum*. At the beginning of the experiment, the mice were fed the standard diets (MF, Oriental Yeast Co., Lt, Tokyo, Japan) containing 5% (w/w of diet) Mr 25000 or 5000 DSS for 8 days to compare the colonic damage (n=5). During the experimental period, food intake and body weight were measured every other day. On the final day of the experiment, the rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (40 mg/kg) and were sacrificed by cervical dislocation. After a laparotomy was performed, the large intestine was resected, cut along the anti-mesenteric border and irrigated with chilled saline. A specimen (5x5 mm) at 2 cm distance from the anal margin (middle colon) was removed, frozen and cut into 5 μ m sections. Finally, the sections were stained with hematoxylin and eosin (HE) and observed under a microscope. The mucosal damage was determined according to a previously described method (23). Briefly, the following three parameters were used: surface epithelium loss, crypt destruction and inflammatory cell infiltration to the mucosa. A score of 0-4 was assigned to each of three parameters according to the extent and severity of change: 0 = no change; 1 = localized and mild; 2 = localized and moderate; 3 = extensive and moderate; 4 = extensive and severe. The sum of the scores of the three parameters represented the mucosal damage score in each animal.

Cell culture. Caco-2 (a human colon cancer cell line) cells were purchased from the American Type Culture Collection (Rockville, USA). Passages 15-21 were used in this experiment. The Caco-2 cells were cultured in Dulbecco's modified Eagle's minimum essential medium (DMEM, pH 7.4) supplemented with 25 mM glucose, 10% inactivated fetal bovine serum (FBS), 1% penicillin-streptomycin and 1% non-essential amino acid solution. The cells were maintained at 37°C in a humidified 5% CO₂ atmosphere.

Cytotoxicity (viability) tests. Cell viability assays were performed according to our previous method (11). Briefly, cells were inoculated in 96-multiwell plates (Costar, Corning, NY, USA) at a cell density of 1.2×10^5 cells/well. After cells reached confluence, cells were incubated with serially diluted and filter-sterilized Mr 2500 or 5000 DSS in a growth medium for pre-determined time periods. Viability was assayed by a commercially available kit (Cell Titer 96™ AQueous, Promega, Madison, USA), which depends on the physiologic reduction of MTS to formazan. Analyses were performed in triplicate. The most important thing here is that the Mr 5000 PA-DSS is easily depolymerized to Mr 1800 and ~70% of the sulfate groups are depleted from one DSS molecule following autoclave treatment (at 115°C for 15 min, 1.7 atm). Therefore, autoclave treatment should be avoided for sterilization and filter treatment is recommended (22).

Statistical analysis. The results are presented as means \pm SEM. The variance was analyzed by the F test. Subsequently, Student's t-test for unpaired values was performed to compare the means of normally distributed data. The Mann-Whitney U test was also performed to compare the means of non-parametric or abnormally distributed data. Differences were regarded as statistically significant at P-values <0.05.

Results

DSS depolymerization in the feces. Under aerobic conditions, native Mr 5000 PA-DSS in the feces was depolymerized to a certain degree and two new peaks appeared in the chromatogram (Fig. 2A). The molecular masses of these peaks were speculated to be ~Mr 1200 and 750, respectively, according to the relationship between the elution time and the molecular mass of each PA-DSS and PA-D-glucose 3-sulfate moiety. In addition, the Mr 1200 and 750 DSS were calculated to comprise ~3.4 and 6.0% of the depolymerized products from the total PA-DSS amount, respectively. Therefore, a total of 9.4% of the PA-DSS was depolymerized under these conditions. In contrast, under anaerobic conditions, this depolymerization was far weaker (Fig. 2B).

Bacteriological study of the fecal contents. Some microflora grew on the DSS-rich media. These microflora were gram negative. On the other hand, obvious microflora were not recognized in the anaerobic environment. These microflora obtained in the aerobic environment formed clear films on the growth media (or 'swarming motility') in GAM media, which indicated that these microflora were of *Proteus* sp. In addition, these flora had desulfonating activities toward p-nitrophenyl sulfate, which indicated that these microflora had arylsulfatase activity.

Classification using 16S rRNA. The sequences obtained were similar to those of *Proteus mirabilis*. Fig. 3 shows a phylogenetic tree.

Capability of *Proteus mirabilis* to degrade PA-DSS. Fig. 2C shows a chromatogram of the supernatant from PA-DSS-rich liquid media, which showed a small peak after a PA-DSS peak. This result indicated that *Proteus mirabilis* had some capability to depolymerize PA-DSS.

Mouse Mr 2500 or 5000 DSS-induced colitis. After an oral administration of Mr 5000 DSS, diarrhea occurred on days 3-4. Macroscopic examination of the colon revealed hyperemia, erosion and occasional tiny blood clots in the distal colon. Using HE staining, there was obvious evidence of inflammatory cell infiltration into the mucosa and submucosa in the distal portions on day 8 after the DSS administration (Fig. 4C). Entire crypt loss, surface epithelial loss and mucosal edema were also evident as compared to the control mice (Fig. 4A). On the other hand, after an oral administration of Mr 2500 DSS, the appearance of diarrhea was delayed until days 6-7. Macroscopic and microscopic examinations of the colon revealed that colitis in mice fed Mr 2500 DSS was milder than in mice fed Mr 5000 DSS (Fig. 4B). The mucosal damage quantified by a scoring system is shown in Fig. 4D.

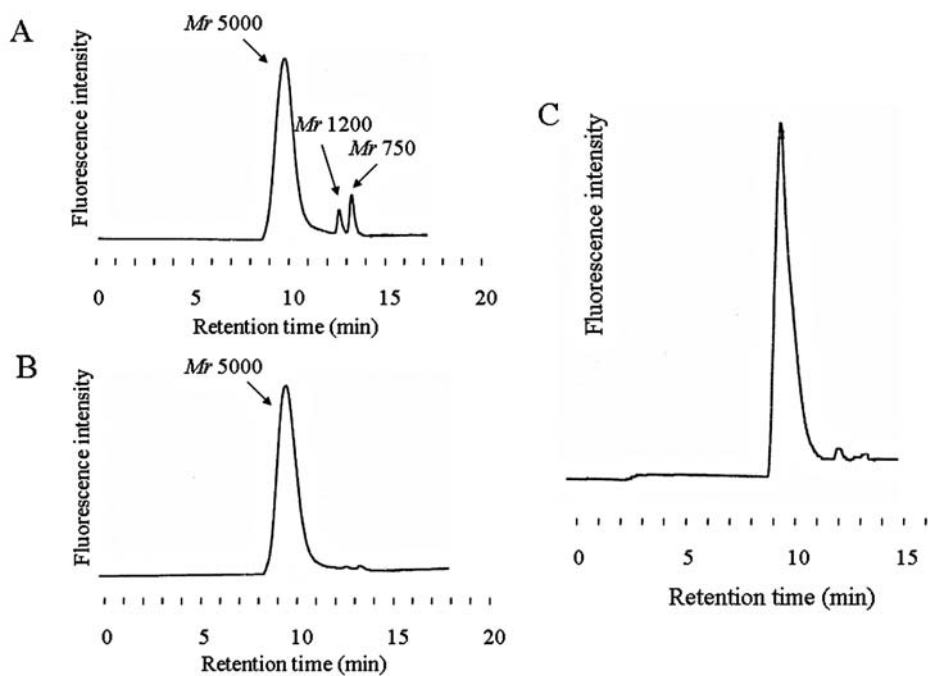


Figure 2. Gel filtration chromatograms of pyridylamino-DSS (PA-DSS). (A) Depolymerization of PA-DSS by mouse feces under aerobic conditions. (B) Depolymerization of PA-DSS by mouse feces under anaerobic conditions. (C) A chromatogram of supernatant obtained from PA-DSS-rich liquid media. PA-DSS was depolymerized by microflora. The HPLC conditions are described in Materials and methods.

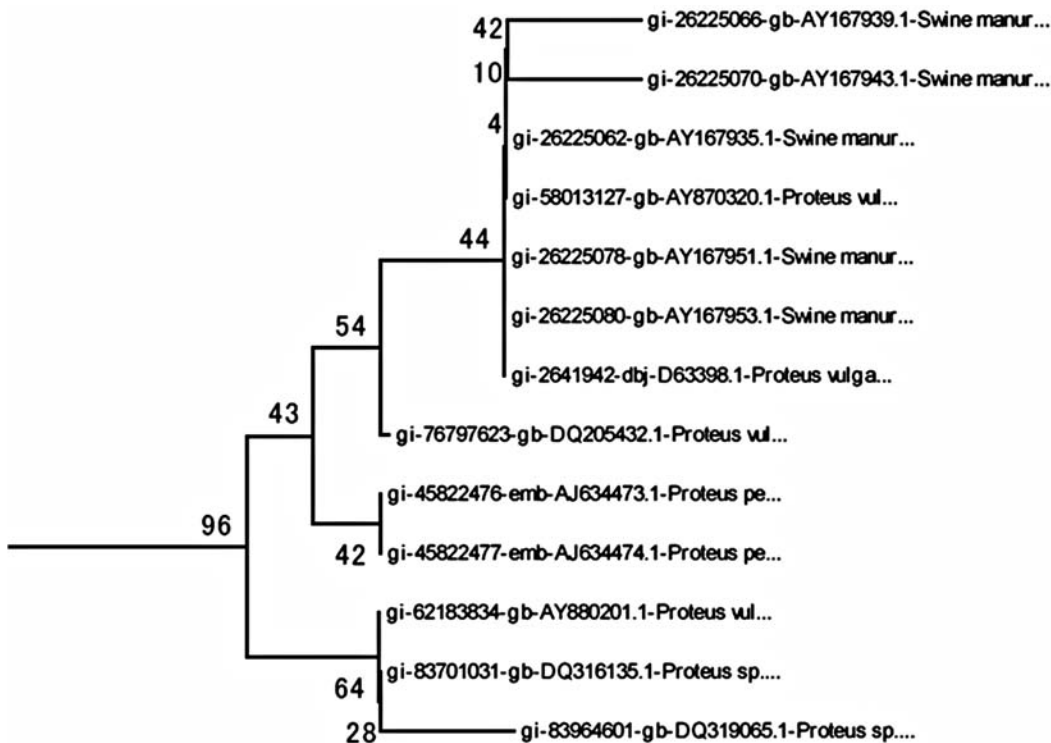


Figure 3. A maximum likelihood phylogenetic tree of the microflora, which grew on DSS-rich solid media. Analytical procedures are described in Materials and methods.

The difference between *Mr* 5000 and 2500 was statistically significant. These results indicated that *Mr* 2500 DSS induced far milder colitis than *Mr* 5000 DSS.

Cytotoxicity (viability) tests. Fig. 5 shows Caco-2 survival curves for cells exposed to *Mr* 2500 or 5000 DSS. The percent

survival was expressed as a percentage of the growth compared to the corresponding untreated control cells at various incubation periods. The survival curves of cells exposed to *Mr* 5000 DSS at 0.1% were comparable to the controls, but cytotoxic effects became clear at 1-5%. With respect to *Mr* 2500 DSS, the cytotoxic effects were not detectable even at 5%.

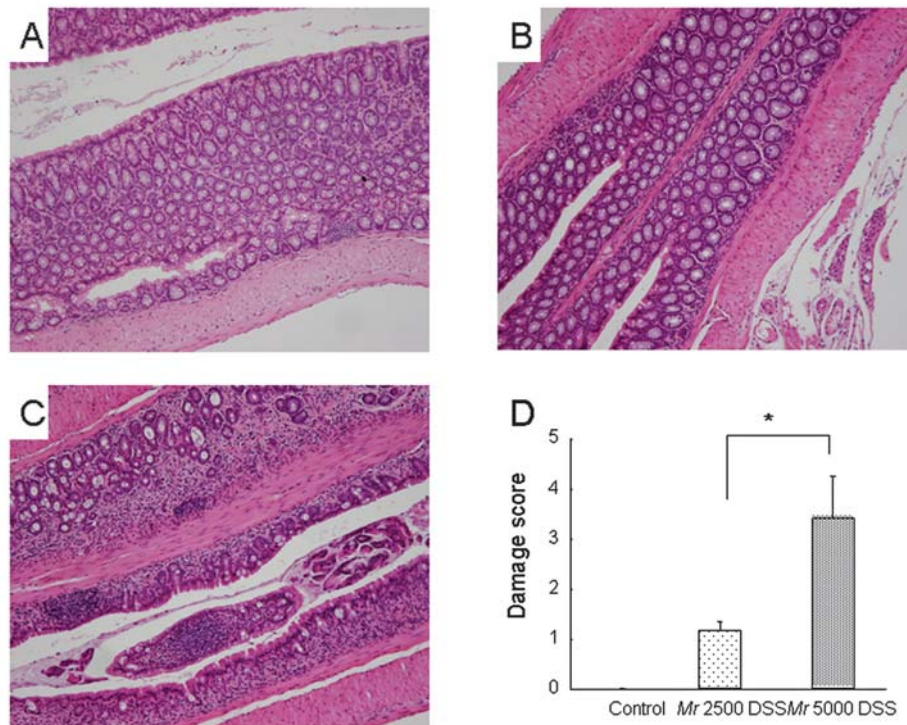


Figure 4. *Mr* 2500 or 5000 DSS-induced colitis. (A) HE staining of the colon in a control mouse. (B) HE staining of the colon in a *Mr* 2500 DSS-induced colitis mouse. (C) HE staining of the colon in a *Mr* 5000 DSS-induced colitis mouse. (A-C) Magnification x100. (D) The mucosal damage quantified by a scoring system according to (23). All values are expressed as means \pm SEM. * $P < 0.05$.

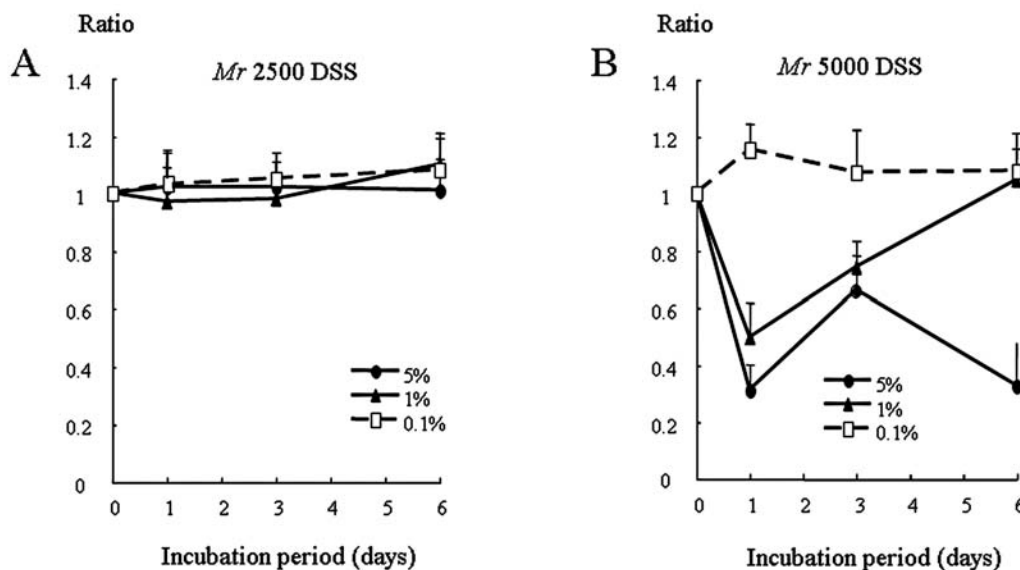


Figure 5. Cytotoxicity (viability) tests of *Mr* 2500 or 5000 DSS on Caco-2 cells. The relative numbers of viable cells were determined using an MTS assay. The percent survival was expressed as a percentage of the growth compared to the corresponding untreated control cells at various incubation periods. Each point represents the mean of three determinations. (A) Survival curves of cells exposed to *Mr* 2500 DSS at each DSS concentration. (B) Survival curves of cells exposed to *Mr* 5000 DSS at each DSS concentration. All values are expressed as means \pm SEM. * $P < 0.05$.

Therefore, the cytotoxicity of depolymerized *Mr* 2500 DSS on Caco-2 cells was very weak.

Discussion

The intestinal microflora have been suggested to play important roles in intestinal inflammation (24). In particular, the microflora in the fecal contents were altered in DSS-

induced colitis (12). The precise meaning of these alterations, however, remains unknown. In other words, are these alterations a causative factor or just an indirect result of the colitis?

In this study, we discovered the following facts for the first time. i) Some microflora present in the feces can depolymerized DSS, especially under aerobic conditions. ii) *Proteus mirabilis* can grow in a DSS-rich environment and

may be one of the microflora responsible for the depolymerized DSS in the feces. Moreover, *Proteus mirabilis* had arylsulfatase activity and could desulfonate DSS.

In general, *Proteus mirabilis* is a gram negative and facultatively anaerobic bacterium. It shows swarming motility and urease activity. *Proteus mirabilis* belongs to the *Enterobacteriales*, *Gamma Proteobacteria*. *Proteus mirabilis* is known to form crystallites in urine. Moreover, it has been reported that 1 out of 4 people have *Proteus mirabilis* in their gut lumen (25). However, it has not been reported that *Proteus mirabilis* can cause colitis.

Also in this study, iii) depolymerized *Mr* 2500 DSS could not induce strong colitis any more. In addition, it was evident that *Mr* 2500 DSS is less toxic on Caco-2 cells than *Mr* 5000 DSS in the cytotoxicity test.

We previously reported that *Mr* 50 k DSS exhibited stronger cytotoxicity than *Mr* 5000 DSS (11). Kitajima *et al* also reported in detail the relationship between the molecular mass of DSS and the features of colitis in mice. According to their study, the degree of colitis was stronger in the order of *Mr* 40 k DSS > *Mr* 5000 DSS. *Mr* 500 k DSS could not induce colitis any more (26). These results suggest that a certain range of molecular mass (*Mr* 5000-50 k) may be required to induce colitis *in vivo*.

Taken together, it is strongly suggested that there are some kinds of microflora, for example *Proteus mirabilis* influencing the molecular mass of *Mr* 5000 DSS in the feces. The depolymerized *Mr* 2500 DSS can not induce strong colitis any more. Therefore, it is possible that *Proteus mirabilis* depolymerizes DSS and detoxifies DSS. In addition, the biological materials derived from *Proteus mirabilis* may be useful therapeutic agents for DSS-induced colitis.

In conclusion, the molecular mass of DSS is a very important factor in the induction and progression of DSS-induced colitis. Certain types of microflora including *Proteus mirabilis* sp. are suggested to influence the distribution of DSS molecular mass and progression of DSS-induced colitis.

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