Alterations in Muc2 biosynthesis and secretion during dextran sulfate sodium-induced colitis

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DSS-induced colitis model gives the opportunity to study the dynamic disease process in different regions of the colon from the onset of disease to complete remission. In humans, such a study is not possible, especially because the disease is usually diagnosed during advanced stages. In addition, the DSS model can be used to develop new therapeutic strategies. Previous studies demonstrated that DSS is directly cytotoxic to the colonic epithelium, inducing crypt damage, crypt loss, and massive erosions (4, 11, 12) leading to an overall decrease in the number of goblet cells. Analogue to UC, the DSS-induced goblet cell loss appears to be the predominant secretory mucin in the healthy colon of the human, rat, and mouse (21, 22, 27).

Because mucins are the structural components of the mucus layer (7, 26), changes in mucin quantity, secretion, and structure could lead to diminished protection of the colonic epithelium. Indeed, in humans with ulcerative colitis (UC) changes in the number of goblet cells, thickness of the mucus layer, and Muc2 synthesis, secretion, and sulfation were reported. Specifically, goblet cells contain less mucin and are reduced in number in active UC (9, 10). The mucus layer in UC patients is thinner than in controls (16). Mucin sialylation appears to be increased in patients with inactive UC (15). Muc2 precursor synthesis and total Muc2 levels in active UC are significantly decreased compared with controls and UC in remission (24). Moreover, because less Muc2 is synthesized in active UC, Muc2 secretion is decreased in this stage of the disease (28). Furthermore, sulfation of Muc2 appeared to be decreased in the rectum and sigmoid colon of patients with UC (17, 28).

To gain insight into the mechanisms underlying the pathology of colitis, several experimental colitis models are currently used as models for human inflammatory bowel disease. One thoroughly described colitis model regarding clinical symptoms, histopathological changes, and the application of therapeutic drugs is the dextran sulfate sodium (DSS)-induced colitis model (1–4, 11). The DSS-induced colitis model gives the opportunity to study the dynamic disease process in different regions of the colon from the onset of disease to complete remission. In humans, such a study is not possible, especially because the disease is usually diagnosed during advanced stages. In addition, the DSS model can be used to develop new therapeutic strategies.

COLONIC EPITHELIUM is covered by a mucus layer that protects the epithelium against mechanical stress, luminal substances, and pathogens (7, 26). The mucin
might also have consequences for the thickness and constitution of the mucus layer and specifically for Muc2 synthesis and secretion. Therefore, it is of relevance to analyze Muc2 synthesis both quantitatively and qualitatively in the DSS-induced colitis model.

In this study, we investigated changes in numbers of goblet cells, Muc2 precursor synthesis, total Muc2, Muc2 secretion, and sulfation in a rat DSS-induced colitis model. These aspects were studied in the proximal as well as distal colon from onset of disease to the regenerative phase of disease. Collectively, these data were used to establish how the mucin production in the colonic epithelium adapted to the damage induced during DSS colitis.

**METHODS**

**Animals.** Eight-week-old, specified pathogen-free, male Wistar rats (Broekman, Utrecht, The Netherlands) were housed at constant temperature and humidity on a 12-h light/dark cycle. One week before and during the experiment, the rats were housed individually. The rats had free access to a standard pelleted diet (Hope Farms, Woerden, The Netherlands) and sterilized tap water (controls) or sterilized tap water supplemented with DSS. All the experiments were performed with the approval of the Animal Studies Ethics Committee of our university.

**Experimental design.** Rats were given 7% DSS (37–40 kDa; TdB Consultancy, Uppsala, Sweden) in their drinking water for 7 days, followed by a 7-day recovery period during which DSS was omitted from the drinking water. Fresh DSS solutions were prepared daily. On days 0 (control), 2, 7, and 14, five animals per time point were killed. Segments of the proximal and distal colon were dissected and prepared for light microscopy or snap-frozen in liquid nitrogen and stored at −70°C until RNA isolation. In addition, tissue explants (10 mm3) of each colonic segment were metabolically labeled, as described below, to study mucin biosynthesis.

**Histology and immunohistochemistry.** Colonic segments were fixed in 4% (wt/vol) paraformaldehyde immediately after excision, embedded, and prepared for light microscopy. Sections were stained with hematoxylin and eosin to study DSS-induced crypt loss and ulcerations. The area of crypt loss and ulcerations was measured using a micrometer in three tissue sections per segment and the area involved was expressed as the percentage of the total mucosal surface area. The score of crypt loss and ulceration was averaged per segment per animal. Thereafter, the mean score per segment per time point (± SE) was calculated.

Sections (5 μm thick) were cut and prepared for immunohistochemistry as described previously (29). To identify goblet cells, sections were incubated with a 1:500 dilution of a Muc2-specific monoclonal antibody (WE9) that recognizes the non-α-glycosylated unique termini of Muc2 (25). Immunoreaction was detected using the Vectastain ABC Elite kit (Vector Laboratories, Burlingame, England), and staining was developed using 3,3'diaminobenzidine. Finally, sections were counterstained with hematoxylin.

**Metabolic labeling of tissue.** Metabolic labeling of colonic tissue in vitro was performed as described previously (6, 24, 25, 28). Briefly, Muc2 biosynthesis was studied by metabolic labeling with 35S-labeled amino acids ([35S]methionine/cysteine, Tran-35S-label; ICN Radiochemical, Zoetermeer, The Netherlands), to label polypeptides, or with [35S]sulfate (ICN Radiochemical) to label mature mucins. Two tissue explants (10 mm3) of the proximal colon and three explants of the distal colon were cultured and pulse-labeled with either Tran-35S-label or [35S]sulfate for 30 min, using 100 μCi of each label per 100 μl medium per tissue explant. In case of pulse labeling with [35S]sulfate, the pulse labeling was followed by a chase incubation of 4 h in the absence of radiolabeled sulfate. Thereafter, the tissue as well as the culture medium was collected. After the respective pulse or pulse-chase experiments, explants were homogenized in, or culture medium was mixed with, a Tris buffer containing 1% SDS and protease inhibitors.

**Measurement of protein synthesis.** Protein concentration of each homogenate was measured using a bichinchoninic acid protein assay reagent kit (Pierce, Rockford, IL). Total incorporation of radioactivity in proteins was determined after trichloroacetic acid (TCA; Merck, Darmstadt, Germany) precipitation by autoradiography using a PhosphorImager, as described previously (24). The protein synthesis within the tissue was calculated as the amount of incorporated radioactivity ([35S]-labeled amino acids) in the TCA precipitate divided by the total protein content of the homogenate. Average metabolism (radioactivity of TCA precipitate/g of tissue) was calculated per segment per rat, followed by calculation of the mean protein synthesis (± SE) per segment for each phase of disease.

**Quantitation of radiolabeled Muc2.** An aliquot of each 35S-labeled amino acid-labeled and [35S]sulfate-labeled homogenate was analyzed on reducing SDS-PAGE (13% stacking and 4% running gel) as described (6). To visualize and quantify mucin-bands, the gels were fixed with 10% methanol/10% acetic acid, periodic acid-Schiff (PAS)-stained and analyzed by fluorography and autoradiography using a PhosphorImager. For reference to very high molecular mass molecules, metabolically labeled, unbridged rat gastric mucin precursors were used (molecular mass of monomer and dimer, 300 kDa and 600 kDa, respectively) (5). To determine the Muc2 precursor biosynthesis, the amount of 35S-labeled amino acid-labeled Muc2 in the tissue was divided by the amount of incorporated radioactivity ([35S]-labeled amino acids) in the TCA-precipitate. Sulfate incorporation into Muc2 was determined as the sum of [35S]sulfate-labeled Muc2 in tissue and media expressed relative to 35S-labeled amino acids-labeled Muc2 in tissue as determined in separate duplicate/triplicate explants. For each segment, the [35S]sulfate incorporation into Muc2 was corrected for differences in protein contents among the individual explants. The percentage of [35S]sulfate-labeled secreted Muc2 was calculated as the amount of [35S]sulfate-labeled Muc2 in the medium divided by the sum of the amount of [35S]sulfate-labeled Muc2 in the tissue and in the medium. This was multiplied by 100 to give the percentage of [35S]sulfate-labeled secreted Muc2. For the Muc2 precursor synthesis, sulfate incorporation into Muc2 and secretion of [35S]sulfate-labeled Muc2 average values were calculated per segment per rat, followed by mean values (± SE) for each of the disease phases studied.

**Quantitation of total Muc2 and total Muc2 secretion.** Quantitation of total Muc2 and total Muc2 secretion was described previously (6). To analyze the concentrations of total Muc2 and total Muc2 secretion, each 35S-labeled amino acid-labeled homogenate, [35S]sulfate-labeled homogenate, and medium was dot-blotted on nitrocellulose (Nitin, Schleier & Schuell, Dassel, Germany). Briefly, the blots were blocked for 1 h with blocking buffer containing 50 mM Tris-HCl, pH 7.8, 5% (wt/vol) nonfat dry milk powder (Lyemf, Kampen, The Netherlands), 2 mM CaCl2, 0.05% Nonidet P40 (BDH), and 0.01% antifoam (Sigma). The blots were incubated 18 h with the Muc2-specific antibody WE9 or anti-rat colonic mucin (RCM) (21, 23). After being washed in blocking buffer, the
blot was incubated with 125I-labeled protein A (specific activity 33.8 mCi/mg; Amersham, Bucks, UK,) for 2 h. The blot was covered by two sheets of 3 mm Whatman filter paper, to eliminate background radiation of the 35S-label, and binding of 125I-labeled protein A to the Muc2-specific antibodies was detected using a PhosphorImager. The elicited signal was quantified, and total Muc2 was expressed per μg protein of the tissue to determine total Muc2 levels. The percentage of total Muc2 secretion was calculated as the amount of Muc2 in the medium divided by the sum of the amount of Muc2 in tissue and in the medium. This was multiplied by 100 to give the percentage of total Muc2 secretion. Averages of total Muc2 and total Muc2 secretion were calculated per segment per rat, followed by calculation of the mean total Muc2 secretion (± SE) for each of the disease phases studied.

Quantitation of Muc2 mRNA. Total RNA was isolated from proximal and distal colon using Trizol following the manufacturers protocol (GIBCO-BRL, Gaithersburg MD). Integrity of the RNA was assessed by analysis of the 28S and 18S ribosomal RNAs after electrophoresis and staining with ethidium bromide. Subsequently, 1 μg of total RNA from each segment was dot-blotted on Hybond-N+ (Amersham). The blot was hybridized to a 32P-labeled rat Muc2 cDNA probe, as described (29). Hybridization of the probe to Muc2 mRNA was detected and quantified using a PhosphorImager. Hybridized signals were corrected for GAPDH mRNA to correct for the amount of loading by using 1.4 kb GAPDH cDNA as a probe (29). Average Muc2 mRNA levels were calculated per segment per rat, followed by calculation of the mean Muc2 mRNA expression levels (± SE) for each of the disease phases studied.

Statistical analysis. To compare two groups, an unpaired t-test was used and to compare three or more groups; ANOVA was performed followed by an unpaired t-test. Differences were considered significant at P < 0.05. Data were represented as the mean ± SE.

RESULTS

Evaluation of DSS-induced damage. DSS-treated rats were assigned, according to time of DSS treatment, to the following groups: onset of disease (day 2), active disease (day 7), regenerative phase of disease (day 14), and a control group (no DSS). Representative immunohistochemical stainings of Muc2 in the distal colon of each group are given in Fig. 1. Additionally, crypt loss and ulcerations were scored, and the mucosal area involved was expressed as the percentage of the total mucosal surface area (Table 1). The first signs of DSS-induced damage consisted of crypt loss (6.3%) appearing in the distal colon during the onset of disease (Fig. 1B). During active disease, crypt loss and ulcerations were observed in the proximal colon and
Table 1. Crypt loss and ulcerations in the proximal and distal colon during different phases of DSS-induced colitis and in controls

<table>
<thead>
<tr>
<th>Day of DSS Treatment, Disease Phase</th>
<th>Proximal Colon, crypt loss/ulceration</th>
<th>Distal Colon, crypt loss/ulceration</th>
</tr>
</thead>
<tbody>
<tr>
<td>0, control</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>2, onset of disease</td>
<td>6.0 ± 0.0**</td>
<td>6.3 ± 2.1</td>
</tr>
<tr>
<td>7, active disease</td>
<td>12.5 ± 4.0**</td>
<td>40.1 ± 4.9**</td>
</tr>
<tr>
<td>14, regenerative phase</td>
<td>6.6 ± 2.4*</td>
<td>20.6 ± 3.5**</td>
</tr>
</tbody>
</table>

Mean score of crypt loss and ulcerations per segment per day (± SE) are expressed. Statistically significant differences were seen between proximal and distal colon during days 2, 7, and 14 (*P < 0.02). In the proximal colon, significant differences were seen between days 0 and 7 and days 2 and 7 (**P < 0.01). Significant differences in the distal colon were observed between days 0 and 7 and days 2 and 7 (***P < 0.001); days 0 and 14 and days 7 and 14 (**P < 0.01); and days 2 and 14 (†P < 0.05). DSS, dextran sulfate sodium.

distal colon (Fig. 1C). Damage was most pronounced in the distal colon where it involved 40.1% of the total mucosal surface versus 12.5% of the proximal colon. During the regenerative phase, crypt loss and ulcerations were still observed in the proximal (6.6%) and distal colon (20.6%) (Fig. 1D).

Total protein synthesis. In the proximal colon, total protein synthesis progressively increased during onset and active disease (Fig. 2). During the latter disease phase, total protein synthesis was significantly increased compared with the other groups. In the distal colon total protein synthesis levels were slightly, but not significantly, increased during onset and active disease.

Identification and quantitation of Muc2 precursor synthesis. We previously demonstrated that Muc2 is the major colonic mucin in rat (21). It is synthesized as a precursor protein with an apparent molecular mass of about 600 kDa, after pulse-labeling with [35S]sulfate followed by immunoprecipitations (21). This 600-kDa band can only be metabolically labeled with amino acids, cannot be stained by PAS, and cannot be metabolically labeled with [35S]sulfate. In the present study, we could easily identify the Muc2 precursor band, according to the biochemical criteria as described above, in tissue homogenates after pulse labeling with [35S]labeled amino acids for 30 min (Fig. 3A). After identification, the specific radioactivity present in the Muc2 precursor band was quantified and the Muc2 precursor synthesis was calculated. Muc2 precursor synthesis was significantly higher in the proximal colon compared with distal colon during each phase of the disease (Fig. 3). In the proximal colon, Muc2 precursor increased in the course of DSS treatment. In active disease, the increase was twofold and significantly different from control levels. During the regenerative phase, Muc2 precursor synthesis decreased but still tended to be elevated compared with control levels. In the distal colon, Muc2 precursor synthesis was maintained during the onset of disease and active disease. However, during the regenerative phase, Muc2 precursor synthesis increased significantly compared with controls and the previous phases of disease.

Quantitation of total Muc2. The regenerative phase excluded, total Muc2 in each group was slightly higher in the proximal colon than in the distal colon (Fig. 4). Although not statistically significant, total Muc2 levels in the proximal colon increased during the onset of disease and active disease but normalized during the regenerative phase. In contrast, in the distal colon, total Muc2 was unaltered during onset of disease and active disease but significantly increased during the regenerative phase. Comparison of total Muc2 levels determined with either WE9 or anti-RCM revealed similar expression patterns (not shown).

Quantitation of total Muc2 secretion. Total Muc2 secretion in the proximal colon was significantly lower in controls and during the regenerative phase than in the distal colon (Fig. 5). Compared with controls, total Muc2 secretion in the proximal and distal colon was unchanged during each disease phase. Comparison of total Muc2 secretion levels determined with either WE9 or anti-RCM revealed similar secretion patterns (not shown).

Identification of mature Muc2 and quantitation of [35S]sulfate incorporation into Muc2. By performing pulse-chase experiments with [35S]sulfate followed by immunoprecipitations, our laboratory previously demonstrated that mature rat Muc2, with an apparent molecular mass of 650 kDa on SDS-PAGE, was detectable after 30-min pulse labeling and 4-h chase incubation in tissue and medium (21). The “650-kDa” band was PAS-stainable and, after Western blotting, was recognized by anti-Muc2 antibodies (20). In the present study, mature Muc2 could also be easily identified by PAS-staining on SDS-PAGE. After pulse labeling with [35S]sulfate, 4-h chase incubation and homogenization, [35S]sulfate-labeled mature Muc2 was detected on SDS-PAGE at an identical position (about 650 kDa) as

![Fig. 2. Total protein synthesis of proximal (open bars) and distal (hatched bars) colonic explants of control and DSS-treated rats. Mean protein synthesis (± SE) was calculated per segment for each disease phase: day 0, control; day 2, onset of disease; day 7, active disease; day 14, regenerative phase. Statistical significant differences were seen in the proximal colon among day 7 and the other groups (***P < 0.05). a.u., Arbitrary units.](http://ajpgi.physiology.org/)
the PAS-stained band (Fig. 3A). After identification and quantitation, the amount of $^{35}$S-sulfate incorporation into Muc2 was determined.

Comparison of proximal with distal colon revealed that the amount of $^{35}$S-sulfate incorporation into Muc2 was significantly lower in the proximal colon in each group investigated (Fig. 6). In the proximal colon, $^{35}$S-sulfate-incorporation into Muc2 was significantly reduced in each disease phase compared with controls. In the distal colon, no significant alterations in sulfate incorporation were observed among any of the disease phases and the control group.

Quantitation of $^{35}$S-sulfate-labeled Muc2 secretion. Analysis of $^{35}$S-sulfate-labeled Muc2 secretion revealed differences between the proximal and distal colon (Fig. 7). Specifically, in the proximal colon, the amount of secreted $^{35}$S-sulfate-labeled Muc2 was increased in active disease and the regenerative phase. In contrast, in the distal colon, $^{35}$S-sulfate-labeled Muc2 secretion appeared unaltered during the various disease phases. Comparison of the proximal colon with...
distal colon revealed that the secretion of $[^{35}\text{S}]$sulfate-labeled Muc2 in the distal colon was much higher in each group.

Quantitation of Muc2 mRNA. Muc2 mRNA levels in the proximal colon were significantly higher than in the distal colon in controls and each disease phase (Fig. 8). Both in proximal colon as well as distal colon, Muc2 mRNA levels were maintained during the onset of disease and active disease. In contrast, in the regenerative phase, Muc2 mRNA levels were significantly decreased compared with control values in both colonic segments.

**DISCUSSION**

In the present study, we investigated changes in the number of goblet cells, Muc2 biosynthesis, secretion, and sulfation in the proximal and distal colon of DSS-treated rats. Morphological analysis revealed that the DSS-induced damage (i.e., crypt loss and ulcerations) started and was most pronounced in the distal colon, analogous to DSS-induced damage in mice and UC in humans (8, 14, 18). Moreover, as a consequence of the DSS-induced damage, the overall number of goblet cells decreased in the proximal and distal colon. Similarly, reduced numbers of goblet cells were also reported in the colon of humans with active UC (9).

Total protein synthesis of the explants was significantly increased during active disease in the proximal colon. This increase can be, at least partly, attributed to the twofold increase in Muc2 precursor synthesis in this colonic segment. No differences were seen among controls, onset of disease, and the regenerative phase. Although not significant, protein synthesis of the distal colon seemed to be increased as well during onset of disease and active disease. Because during these phases Muc2 precursor synthesis was unaltered, synthesis of other proteins must be increased.

Detailed analysis of the Muc2 precursor synthesis demonstrated a progressive increase in Muc2 precursor synthesis in the proximal colon during the onset of disease and active disease, followed by normalization of Muc2 precursor synthesis levels during the regener-
Muc2 precursor synthesis was maintained during the onset of disease and active disease but increased significantly during the regenerative phase. Although differences were less pronounced, the alterations in total Muc2 protein were also similar to alterations seen in Muc2 precursor synthesis. Moreover, total Muc2 secretion was maintained during each disease phase studied. These data in conjunction demonstrate that in the rat the DSS-induced damage, and thus loss of goblet cells is accompanied by an increase, or at least a maintenance of Muc2 precursor biosynthesis, total Muc2 levels, and total Muc2 secretion. This implies an increase in Muc2 synthesis per goblet cell, and more importantly these data suggest that the thickness of the mucus layer is at least maintained or even increased, offering optimal protection to the colonic epithelium during the different phases of the disease. Yet, these data are in contrast with changes as seen in humans with active UC. Namely, in humans, Muc2 precursor synthesis and total Muc2 levels were significantly decreased in active UC compared with controls and UC in remission (24, 28). In part, these differences might be explained by the difference in type of colitis between DSS-induced colitis in rat and UC in human. The DSS-induced colitis is a relatively acute model, whereas UC in patients is chronic.

Sulfate is incorporated in the last steps of mucin-biosynthesis (20). Changes in sulfate incorporation into Muc2 and the secretion of sulfate-labeled Muc2 could reflect changes in the structure of Muc2 and indicate qualitative changes in the mucus layer. Therefore, we performed metabolic labeling studies with $^{35}$S-sulfate in the course of DSS-induced disease. In the proximal colon, $^{35}$S-sulfate incorporation was decreased, whereas $^{35}$S-sulfate-labeled Muc2 secretion was increased, during active disease and the regenerative phase. Thus in the proximal colon $^{35}$S-sulfate-labeled Muc2 was preferentially secreted during active colitis and the regenerative colitis. As a result of decreased incorporation of sulfate into Muc2, combined with the preferential secretion of sulfated Muc2, the sulfate content of the secreted Muc2 in the proximal colon may be normal during active disease. Previously, Van Klinken et al. (28) reported similar results for the distal colon in humans with active UC. However, in the distal colon of rats, no alterations in $^{35}$S-sulfate incorporation into Muc2 and $^{35}$S-sulfate-labeled Muc2 secretion were observed in active disease or any other disease phase. When the proximal colon is compared with the distal colon, $^{35}$S-sulfate incorporation into Muc2 and secretion of $^{35}$S-sulfate-labeled Muc2 were significantly lower in the proximal colon in controls and during each disease phase studied. Previously, we (21) demonstrated that degree of Muc2 sulfation is increased from the proximal to distal colon in rat healthy colon. Because sulfate is thought to confer resistance to enzymatic degradation of the mucus layer (13), Muc2 synthesized in the proximal colon may be more sensitive to enzymatic degradation than Muc2 produced in the distal colon. Therefore, the higher Muc2 precursor synthesis levels and higher total Muc2 levels in the proximal colon might constitute a mechanism within the proximal colon to compensate for the increased sensitivity of Muc2 to enzymatic degradation.

In the distal colon, where Muc2 precursor synthesis and total Muc2 levels were lower and the $^{35}$S-sulfate incorporation into Muc2 and the secretion of $^{35}$S-sulfate-labeled Muc2 were both higher compared with proximal colon, the high sulfate content confers optimal resistance to enzymatic degradation. On the other hand, a high sulfate content could also be disadvantageous, because more sulfate residues would be available to sulfate-reducing bacteria, which produce sulfides highly toxic to the colonic mucosa. In the colon of UC patients, these bacteria are indeed overrepresented (19). If, in DSS-treated rats, sulfate-reducing bacteria would be overrepresented during DSS treatment, then the higher degree of sulfation in the distal colon might be one of the reasons why the distal colon is more severely damaged by DSS than the proximal colon. However, to assess the effects of alterations in Muc2 sulfation in relation to enzymatic degradation and sulfate-reducing bacteria during DSS-induced colitis, further studies are necessary.

When we focused on Muc2 mRNA, a significant downregulation of Muc2 mRNA levels was observed in proximal and distal colon during the regenerative phase. Taking into account that the Muc2 precursor synthesis and total Muc2 levels in both colonic segments were maintained or increased during the regenerative phase, these data indicate that the Muc2 translation efficiency was increased during the latter disease phase. Once more, these data emphasize the importance of Muc2 production and the mucus layer in protecting the colonic surface epithelium.

In summary, DSS induced a decrease in the number of goblet cells in the proximal and distal colon. This is accompanied by the maintenance, or even an increase, of 1) Muc2 precursor biosynthesis, 2) total Muc2 levels, and 3) total Muc2 secretion. These quantitative data suggest a maintained or even elevated barrier function of the mucus layer during DSS-induced disease. During active disease and the regenerative phase, Muc2 becomes undersulfated in the proximal colon, whereas sulfated Muc2 is preferentially secreted. However, due to the decreased incorporation of sulfate into Muc2, the sulfate content of luminal Muc2 will likely be unaltered. As Muc2 mRNA decreased and total Muc2 levels were maintained or elevated during the regenerative phase, Muc2 translation efficiency is specifically increased during this phase. Collectively, these data emphasize the importance of the protective mucus layer, and suggest enhanced protective capacities through the mucus layer during the different phases of DSS-induced colitis.

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REFERENCES


