

Intestinal permeability and faecal Granulocyte Marker Protein in Dextran Sulphate Sodium - induced colitis in rats

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Summary

The aims of this preliminary study were to foster the development of an animal model of inflammatory bowel disease (IBD). We hereby studied the effects of dextran sulphate sodium (DSS) in drinking water on (a) intestinal permeability and (b) faecal levels of granulocyte marker protein (GMP) in rats. Methods were adopted to keep stress at a minimum. The animals had free access to DSS for 36 or 96 hrs. Controls received only water. Oral administration of ⁵¹Cr-labelled ethylenediaminetetraacetic acid (⁵¹CrEDTA) is used for clinical evaluation of intestinal permeability in IBD. ⁵¹CrEDTA was administered by training the animals to drink a given quantity of fluid within 5 minutes in a specific environment. On the test day, they voluntarily consumed 1 ml water containing 2 µCi ⁵¹CrEDTA. Urine samples were taken from filter paper in their home-cages for 6 hrs and radioactivity measured in a gamma counter. Faecal samples were collected after 36 and 96 hrs on DSS or water for GMP analysis. RESULTS: GMP was elevated in the DSS-induced animals after both 36 hrs (p<.05) and 96 hrs (p<.01), the elevation being greater after 96 hrs. Consumption of DSS increased the urinary excretion of ⁵¹CrEDTA after 36 hrs, but not after 96 hrs. Only DSS consumption for 96 hrs resulted in visible colonic erosions in either the proximal, mid or distal part of the colon. CONCLUSION: DSS has an acute effect upon urinary excretion of ⁵¹CrEDTA, and 96 hrs of access to DSS is sufficient to induce elevated GMP levels and colonic erosions in adult rats.

Introduction

Our main interest is in interactions between stress and inflammatory bowel disorders (IBD). Thus, one of our primary motivations is to develop measurement methods for markers of colonic inflammation by using an animal model which involves as little discomfort and stress as possible to the rats.

In recent years, several human studies of intestinal permeability in patients with ulcerative colitis (UC)

or Crohn's disease (CD) have addressed whether these patients have a defect in the mucosal barrier function (Thomas, 1997). Studies have differed with respect to methods of measuring permeability, both with regard to administration procedures and outcome measures (Jenkins *et al.*, 1988). In the present study, we have examined both intestinal permeability and inflammation in adult rats induced by dextran sulphate sodium (DSS).

Chemically induced intestinal inflammation in rodents has been reported to compare with symptomatology in human IBD. Transmural inflammation in the intestinal mucosa can be induced by rectal administration of the hapten trinitrobenzene sulfonic acid (TNBS) dissolved in ethanol.

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Symptomatology corresponds histologically and macroscopically with the course of CD (Wallace & Keenan, 1990). Alternatively, oral administration of DSS in drinking water produces effects more comparable to UC. The extent of the damage is dependent upon length of exposure, but the literature reports damage being limited to the large intestine and rectum without affecting muscularis externa (Gaudio *et al.*, 1999). Colonic permeability is reported to be elevated after 1 day of DSS administration when *in vitro* studies of colonic tissue from rats were performed (Venkatraman *et al.*, 2000). Oral administration of DSS does not require anaesthesia compared to the TNBS procedure. Consequently, stress associated with its administration is reduced, although it is more time consuming.

One way of assessing epithelial barrier function *in vivo* is by analysing urinary excretion of ⁵¹Cr-labelled ethylenediaminetetraacetic acid (⁵¹CrEDTA) following oral administration. This is a water-soluble marker known to move with the liquid phase of digestion and with the fine particles associated with solid phase. It is used clinically to assess intestinal permeability and has been found to precede disease recurrence (Tibble *et al.*, 2000). Studies have shown that orally ingested ⁵¹CrEDTA in patients with IBD is significantly elevated in urine, particularly in UC patients (Arslan *et al.*, 2001). In animal studies, ⁵¹CrEDTA is normally delivered to the animal by gavage (Willoughby *et al.*, 1996), but we believe that even in the hands of the most experienced investigators this procedure is associated with discomfort for the animals. In the present study, we attempted to avoid this potential problem by daily training animals to drink a constant amount of water in a given context, and on the test day the water also contained ⁵¹CrEDTA solution.

Another clinical marker of intestinal inflammation is elevated levels of faecal calprotectin, derived from neutrophils and monocytes. A dysfunction in the tight junction function allows penetration of granulocytes into the gut lumen, and a correlation between permeability and calprotectin has been found in IBD patients (Berstad, *et al.*, 2000). Ele-

vated faecal granulocyte marker protein (GMP) has been reported with the pathophysiological changes due to IBD (Røseth *et al.*, 1999). The animal counterpart of calprotectin is GMP. Simply collecting stools from the animal's home-cages involves minimal disturbance to the animal and may be performed over longer time periods.

Materials and Methods

Subjects

20 adult male outbred Wistar rats (Møllegaard, Denmark) were used, weighing 335-435 gram (g) at delivery. They were singly housed in transparent cages (Macrolon III 37 x 21 x 15 cm) with chopped wood bedding. A standard diet (Beekey Feeds, Expandent Rodent, Low protein diet) and tap water was given *ad libitum*. The animal quarters were maintained at a temperature between 20-22°C with a 12:12 hour light cycle (light on at 6 am.).

The animals were randomly assigned into four equal sized groups (n's=5): DSS for 36 hrs (DSS-36), DSS for 96 hrs (DSS-96) and two associated control groups (Water-36 and Water-96) given normal tap-water and tested for permeability and granulocyte marker protein (GMP) at the same times as the experimental groups.

The procedures described in this article have been approved by the local responsible laboratory animal science specialist under the surveillance of the Norwegian Animal Research Authority (NARA) and registered by the Authority.

Drinking training

One week prior to testing, all animals were habituated daily to transport routines between the animal rooms and the laboratory facilities. They were deprived of water from 0800 to 1300, a total period of 5 hrs for each day during this training period. The animals were then individually placed in chambers (45 x 20 x 24 cm) with a transparent front and top. A white plastic cup (4 x 4 x 1 cm) filled with 1 ml distilled water was fastened at the front of each chamber with glue-tape. After mouth contact, they drank the water either immediately or

after several trials. After the water had been consumed, the animals were immediately returned to their home-cages. By the 6th day of training, all animals voluntarily drank the 1 ml water in the chamber within 5 min.

Induction of colitis

DSS with an approximate molecular weight of 45000, 17.2% sulphur substitution, and pH 7.0 (TdB Consultancy, Sweden) was added to distilled water to a fixed concentration of 4 %. 160 ml was provided for each animal in their normal drinking bottle, to which they had free access except for during the periods of fluid deprivation necessary for drinking training.

Granulocyte Marker Protein and ⁵¹CrEDTA

Prior to testing, faeces were collected from each home-cage for analysis of basal levels of granulocyte marker protein (GMP). Faeces were again collected from the animals after 36 hrs on DSS (DSS-36) or water (Water-36), and from animals exposed to either DSS for 96 hrs (DSS-96) or water (Water-96). Prior to the administration of the ⁵¹CrEDTA (Amersham Int., England), they were food-deprived overnight. At 0800 the next day, bottles were removed. The animals were transported to testing facilities at 1300 and placed in the test chambers. 1 ml distilled water containing 2 µCi

⁵¹CrEDTA was administrated from a fixed solution so that all animals were given the same dosage of ⁵¹CrEDTA. The animals drank the 1 ml solution from the plastic-cup within 5 min and were immediately placed in their home-cages with grid floor inserts. The bottom of each cage was covered with filter paper for urine collection. After 1h, all animals were given tap water, and normal food was given after 2 hrs. At hourly intervals, the filter paper was removed from the cages and replaced with clean paper. From the soiled paper, urine spots were cut out and placed in separate containers for later analysis. All equipment used for cutting paper was cleaned in water after each animal. The total time of urine collection was 6 hrs after ⁵¹CrEDTA consumption.

Macroscopic analysis of colon

After the last urine sampling, the animals were sacrificed by CO₂ followed by cervical dislocation. A laparotomy was performed and the large intestine from rectum to caecocolonic junction was removed, and cut along the mesenteric side. Each colon was gently cleaned for luminal contents, and exposed inside out by cutting longitudinally. Without stretching, it was pinned out on a board for scoring macroscopic damage under a 4x-magnifying lamp. Criteria for macroscopic assessment are listed in Table 1.

Animal No.	Number of colonic erosions	MM length of colonic erosions	Oedema	Vasodilation	Focal hyperemia, no erosions	GMP level after 96 hrs on DSS
1	1	1	yes	yes	no	166,75
2	1	2	yes	yes	no	46,1
3	1	0,5	no	yes	no	55,25
4	1	2	yes	yes	no	73,25
5	0	0	no	yes	yes	24,25

Table 1. Criteria for macroscopical assessment shown by the group given DSS for 96 hrs. MM = millimeter, GMP = granulocyte marker protein (mg/l) DSS = dextran sulphate sodium

Analysis of urine

The tubes containing the soiled filter papers were filled with 2 ml water and analysed in a gamma counter. A standard row with 2 µCi in 2 ml distilled water was used to calculate nanocurie ⁵¹CrEDTA levels in the samples. For analyses, the following formula was used:

$$R = a / (c - k)$$

R = recovery, a = urine value in nanocurie from paper, c = value from 2 µCi control vial, k = value from pure distilled water alone.

Recovery expresses the amount of ⁵¹CrEDTA that was passed into the urine and captured on paper.

Analysis of Granulocyte Marker Protein

Stools were stored frozen at -20C until further analysis. In short, 1 gr. faeces were diluted in 4 ml extraction buffer and thoroughly homogenised using an Ultra Turrax (20000 rpm) before centrifugation at 45000 g for 20 minutes. The upper halves of the supernatants were carefully harvested and GMP analysed by ELISA (Mahmud *et al.*, 1998).

Statistical analysis

The Statistica 6.0 package was used for analysing the data. Between-group differences in body weight were analysed with a two-way analysis of variance (Liquid by period of time). With respect to GMP data at study completion, the four groups showed significant heterogeneity of variance (Levene's test), $F(1,18)=7,0377$, $p=.01$, and data were therefore log10 transformed. Two-way analysis were then performed (Liquid by period of time). One-way analysis of variance was used for analysing basal levels of GMP. Permeability measures (⁵¹CrEDTA recovery) were analysed with a two-way analysis of variance (Liquid by period of time) followed by planned comparisons and Bonferroni post hoc test. In all comparisons $p<.05$ was regarded as statistically significant.

Results

Body weights

A two-way analysis of variance (liquid x period) revealed no main effect of liquid, $F(1,16)<1$, period,

$F(1,16)<1$, nor any significant interaction, $F(1,16)<1$ at study completion for the four groups. Similar results appeared for differences in body weight from arrival to study completion (data not shown).

Intestinal permeability

A two-way (liquid x period) analysis of variance of ⁵¹CrEDTA recovery yield over the 6 hour measurement period revealed a trend towards a significance of liquid, $F(1,16)=3,92$, $p=.06$, and a trend towards a significant effect of period, $F(1,16)=4,18$, $p=.058$. The interaction was significant, $F(1,16)=5.78$, $p<.03$. Planned comparisons showed that the source of this interaction was due to elevated urine secretion of ⁵¹CrEDTA in the DSS-36 animals compared to the Water-36 animals, $F(1,16)=9,6$, $p<.01$ where a Bonferroni test showed $p<.05$, but not between the DSS-96 and Water-96 animals, $F(1,16)<1$. DSS-36 animals also showed a higher urine excretion of ⁵¹CrEDTA than the DSS-96, $F(1,16)=9,9$, $p<.01$, where a Bonferroni test showed $p<.05$. (Figure 1).

Levels of Granulocyte Marker Protein (GMP)

One-way analysis of variance showed no significant effect on basal GMP levels between the four groups, $F(1,17)<1$.

Two-way analysis of variance (liquid x period) showed a significant main effect of liquid, $F(1,16)=27,7$, $p<.001$, and period, $F(1,16)=9,56$, $p<.01$. The interaction was not significant, $F(1,16)=1,39<1$. Planned comparisons showed that the DSS-36 animals had significant elevated GMP levels compared to the Water-36 animals, $F(1,16)=8,33$, $p=.01$, likewise for the DSS-96 and Water-96 animals, $F(1,16)=20,79$, $p<.001$ (Bonferroni test, $p<.01$). The DSS-96 animals had significant higher GMP levels than the DSS-36 animals, $F(1,16)=9,14$, $p<.01$ confirmed by a Bonferroni test, $p<.05$.

Macroscopic pathology

Clinical evaluation after 36 hrs on DSS revealed no pathological findings in the colons. Loose stools

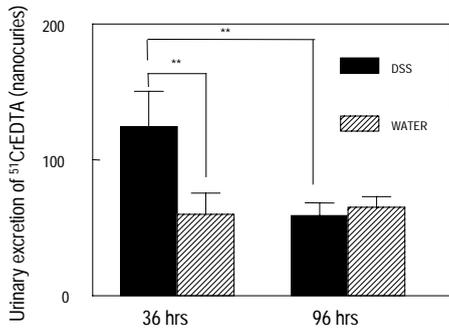


Figure 1. Urinary excretion of ⁵¹CrEDTA (nanocurie) yield during 6 hours urine collection after 36 or 96 hours access to DSS or water. **p<.01

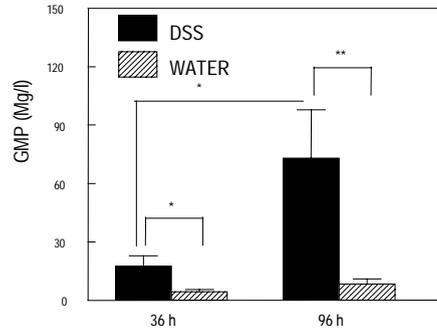


Figure 2. Faecal granulocyte marker protein (GMP) after 36 or 96 hours access to DSS or water. *p<.05, **p<.01.

were observed after 96 hrs on DSS. Colonic abnormalities were visible only in these animals. Four of the five DSS-96 animals had visible erosions together with the highest levels of GMP (Table 1). Erosions were seen in various parts of the colon; 2 of the animals had erosions in the distal part, one in the mid- and one in the proximal part of the colon. Gross bleeding was not noted in any of the animals.

Discussion

Our results show an elevated level of GMP after 36 hours access to DSS, and an even greater elevation after 96 hours. The inflammatory effect of DSS is well documented (*Elson et al., 1995; Gaudio et al., 1999; Araki et al., 2000*) and the elevation in GMP in all likelihood reflects release of granulocytes from the blood into the gut lumen. It should be noted that GMP elevation was greatest in those animals that showed visible morphological changes in the colon. The main inflammatory site following oral administration of DSS is the colon, which presents characteristic pathological findings.

The effect of DSS consumption for 36 hrs was reflected in the elevated excretion of isotopes in urine samples over 6 hrs after oral administration of ⁵¹CrEDTA. A question remains however whether the effect is due to permeability changes in the upper or lower gut.

Surprisingly, our data show that the elevated levels of ⁵¹CrEDTA diminish after 96 hrs of DSS consumption, suggesting a return to normal permeability despite the presence of morphological changes and high levels of GMP. Understanding these relations remains a challenge. We expected the time course of ⁵¹CrEDTA measurements to correlate with GMP, previously reported by Berstad and co-workers (2000). ⁵¹CrEDTA and granulocytes pass through the same intercellular junctions, although it is not clear whether inflammation or increased permeability is the primary process. Our findings indicate that different processes are involved despite the fact that experimentally sensitised mucosa enlarges the tight junctions causing both abnormal permeability and inflammation. The disease activity fluctuates and our assessment was limited to 96 hrs. How the GMP and ⁵¹CrEDTA levels are altered within a broader range remains to be studied. 36 hrs of DSS consumption may affect segments of the upper part of the intestines, while the lower parts still remain unaffected. The elevated ⁵¹CrEDTA level may therefore reflect an early process of increased permeability in the upper part. 96 hrs on DSS reverses the results, and might reflect that ongoing exposure to DSS causes increased damage to the lower part of the intestines, or more specific, the distal part of the colon assessed by elevated faecal GMP levels.

In summary, the present preliminary study shows that DSS has an acute effect upon intestinal permeability where 36 hrs access is sufficient to give an increase in urinary excretion of $^{51}\text{CrEDTA}$. 96 hrs on 4% DSS solution is sufficient to induce inflammation which is indicated by elevated GMP levels and macroscopical findings in rats. While voluntary oral administration reduces possible stress factors, we are uncertain as to where in the gastrointestinal tract permeability occurs. Further studies utilising the principles of this model will perhaps result in a greater understanding of the mechanisms by which inflammatory responses are triggered. Furthermore, measurements of intestinal permeability and inflammation may have an important clinical value in predicting symptom relapse. Based on experience from this study, our next question will incorporate the role of stress in addition to methodological refinement.

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