Cysteinyl leukotrienes play a part in inflammatory processes such as inflammatory bowel diseases. The present study aimed to evaluate the effects of the cys-LT-1 receptor antagonist montelukast on a mild colitis model in rats. Colitis was induced by administering 4% dextran sulphate sodium (DSS, MW 45 000) in drinking water for 9 days. Montelukast (10 mg/kg/day) or vehicle was given by gastric gavage once daily simultaneously with DSS administration. A healthy control group receiving water as drinking fluid and vehicle by gastric gavage was included. Body weight loss, consistency of faeces (loose/diarrhoea) and occult blood in the faeces/ gross bleeding were assessed on days 6 - 9. After sacrifice, the following were assessed: colonic histology, the expression of inducible nitric oxide synthase, macrophage/monocyte marker ED1, cyclooxygenase-1 and cyclooxygenase-2, as well as the production of leukotriene B$_4$ and prostaglandin E$_2$, its metabolite bicyclic-prostaglandin E$_2$ and thromboxane B$_2$ in the colonic tissue incubation in vitro. Rats receiving DSS exhibited bloody diarrhoea from day 6 onwards. Montelukast significantly reduced the occult blood in the faeces/ gross bleeding, maintained normal body weight gain and tended to decrease the ratio of leukotriene B$_4$/ prostaglandin E$_2$ production in the colon in vitro. The results indicate that montelukast has some potential to ameliorate mild experimental colitis induced by DSS.

**Key words:** cysteinyl leukotrienes, cys-LT-1 receptor, dextran sulphate sodium (DSS) induced colitis, leukotriene B$_4$, montelukast, prostaglandin E$_2$

**INTRODUCTION**

The cysteinyl leukotrienes, leukotrienes C$_4$, D$_4$ and E$_4$, secreted mainly by eosinophils, mast cells, monocytes and macrophages (1 - 5), produce a variety of...
actions which emphasize their importance as pathogenic elements in inflammatory states, such as the recruiting of eosinophils and stimulating the extravasation of plasma (6 - 9). They also increase chloride secretion in colonic mucosa (10) and cause smooth muscle contractions in a number of tissues, including the colon (7, 11, 12).

Chronic inflammatory bowel diseases - Crohn’s disease and ulcerative colitis - are associated with the increased mucosal formation of cysteinyl leukotrienes (13 - 16), as well as leukotriene B₄ and prostaglandins, the more traditional mediators of inflammation (17). Enhanced generation of cysteinyl leukotrienes has been demonstrated in several experimental models of colitis: immune complex (18), trinitrobenzene sulphonic acid (TNBS) (19, 20), acetic acid (19, 21), sulphhydryl blocker (22), and lipopolysaccharide (23) -induced models. In colitis induced with immune complexes, the generation of leukotriene C₄ has been reported to correlate with the severity of inflammatory cell infiltration (18). However, with the same models, controversial reports also exist: the production of cysteinyl leukotrienes is not always described as being enhanced in the inflamed colon (21, 24, 25) and even decreased levels have been reported (26).

There are not many reports on the effects of cys-LT-1 receptor blockade in experimental colitis models. The cys-LT-1 receptor antagonists SKF-104,353 and SR 2640 have been shown to prevent early colonic transit disturbances associated with TNBS-induced colitis, in contrast to synthesis inhibitors and receptor antagonists of other eicosanoids, indicating that cysteiny1 leukotrienes mediate these alterations (27). Another cys-LT-1 receptor antagonist, pranlukast (ONO-1078), has been reported to reduce the severity of chronic TNBS-induced colitis (28) and yet another cys-LT-1 receptor antagonist, zafirlukast, has shown prophylactic potential against acetic acid –induced colitis (29). In addition, our study (30) suggests that although cys-LT-1 receptor antagonist montelukast has only limited potential to ameliorate acute, severe colitis in a TNBS model, it exerts some beneficial actions. This was seen as enhanced production of gastroprotective PGE₂ (30), which potentially is capable of antagonizing the inflammatory reaction in a milder colitis model. Thus, the present study aimed to evaluate whether montelukast is able to reduce the severity and modify the production of different eicosanoids in mild colitis induced with dextran sulphate sodium (DSS) in rats.

 MATERIALS AND METHODS

Animals

Male rats (150-190 g) of outbred HsdBrlHan:WIST stock (Harlan, Horst, The Netherlands) were housed two to a cage in a standard animal laboratory at 22 ± 2°C at a relative humidity of 50 ± 10%, with artificial light from 6 a.m. to 8 p.m. The animals had free access to standard rat chow
Colitis induction

The rats received 4% DSS (MW 45 000, TdB Consultancy, Uppsala, Sweden) in their drinking water for nine days. The duration of DSS administration was selected on the basis of previous reports (31, 32) and our preliminary studies. The healthy control rats received water without DSS.

Experimental design

The non-fasted animals were randomly divided into three treatment groups \((n = 6\) in each), one healthy control group and two with colitis - one as a diseased control. The groups received by gastric gavage (volume 0.2 - 0.3 ml) either montelukast (kindly donated by Merck & Co, Rahway, NJ, USA) dissolved in water (10 mg/kg/day) (montelukast) or water (controls). The dose of montelukast was selected on the basis of previous studies (average dose) (33, 34) and of the recommendation of the manufacturer. Montelukast treatment began concurrently with DSS administration and was given daily before noon throughout the experiment.

Assessment of inflammation

The animals were weighed daily, and the consistency of faeces (normal, loose, diarrhoea), gross bleeding and the presence of occult blood (Hemolex, Oriola, Espoo, Finland) in the faeces of each rat were evaluated on days 6 - 9 using the scoring by Cooper et al. (35). An index of loose faeces/ diarrhoea was created by giving the values 0 if the faeces were normal, 2 if the faeces were loose, and 4 if there was diarrhoea. (Normal faeces = well-formed pellets; loose faeces = pasty and semiformed stools which do not stick to the anus; diarrhoea = stools, that stick to the anus). An index of occult blood in the faeces/ gross bleeding was created by giving the values 0 if there was no occult blood and no gross bleeding, 2 if there was occult blood in the faeces, and 4 if there was gross bleeding. The animals were killed on the ninth day after the beginning of DSS administration, by decapitation preceded by an overdose of CO\(_2\). The colon was removed by cutting at the pubic symphysis and at the caecum, and immediately transferred into Krebs buffer (pH 7.5), which was oxygenated with 95% O\(_2\) - 5% CO\(_2\). The colon was incised along its mesenteric border and gently washed. A 5-mm segment was cut from the distal end for the eicosanoid production assays, and a 2-mm segment was taken from the adjacent section for histological assessment. The remaining colon was then weighed. Colon-to-body weight was calculated as an index of tissue oedema. Immediately after weighing, the colon was cut 7 cm proximally from the distal end. The remaining parts of the 7 - cm segment were cut into strips longitudinally for measurement of myeloperoxidase activity (indicator of neutrophil infiltration), and inducible nitric oxide synthase (iNOS), cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) protein expression. One unit of myeloperoxidase activity was defined as that which degraded 1 µmol of hydrogen peroxide in 1 min and was expressed as [unit/ g] of tissue (36). The tissue samples for histology and immunohistochemistry (macrophage/ monocyte marker ED1 and COX-2) were suspended in 4% formaldehyde, embedded in paraffin, cut, and, for histology, stained with hematoxylin and eosin.

Assessment of eicosanoid production

The colon sample (40-80 mg, whole thickness of the wall) for eicosanoid measurements was placed in a pre-weighed tube containing Krebs buffer, and weighed. It was then first pre-incubated in 3 ml Krebs buffer (pre-oxygenated with 95% O\(_2\) - 5% CO\(_2\)) for 15 min at 37°C to minimise the effect
of sample handling, after which the buffer was discarded and the tissue was incubated in 3 ml oxygenated Krebs buffer for another 15 min at 37°C. After incubation the media were collected, centrifuged (5 min, 2000 g, at 4°C) and stored at -80°C. The sample was then cut into 5-mm pieces and incubated in 3 ml of RPMI 1640 medium for a further 24 hours at 37°C. After incubation the media were collected, centrifuged (5 min, 2000 g, at 4°C) and stored at -80°C. Prostaglandin E₂ and bicyclic-prostaglandin E₂ were assayed by radioimmunoassay kits (Institute of Isotopes, Budapest, Hungary) as described previously (30). Bicyclic-prostaglandin E₂ (13,14-dihydro-15-keto-11β,16-epoxy-prostaglandin E₂) is a stable end product of the main prostaglandin E₂ metabolite found in the circulation, 13,14-dihydro-15-keto-prostaglandin E₂. The leukotriene B₄ enzymeimmunoassay kit was purchased from Amersham (Buckinghamshire, UK). The thromboxane B₂ and leukotriene C₄/D₄/E₄ radioimmunoassays were performed as described previously (30), using antibody raised against the bovine serum albumin conjugate of leukotriene C₄/D₄/E₄, with cross-reactivity data as follows: leukotriene C₄ 100%, leukotriene D₄ 100%, leukotriene E₄ 70%, other leukotrienes negligible.

Western blot analysis of iNOS, COX-1 and COX-2

The colon samples were homogenised in boiling buffer (pH 7.4) containing Tris-HCl, Na₃VO₄ and sodium dodecyl sulphate, and centrifuged. Western blots of iNOS and COX-2 were performed as described previously (30), and a Western blot of COX-1 was performed according to the same protocol. The primary antibody used was either rabbit polyclonal anti-iNOS IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat polyclonal anti-COX-1 IgG (Santa Cruz Biotechnology) or goat polyclonal anti-COX-2 IgG (Santa Cruz Biotechnology); the secondary antibody was either horseradish peroxidase-coupled anti-rabbit IgG (Santa Cruz Biotechnology) (iNOS) or anti-goat IgG (Zymed Laboratories, San Francisco, CA, USA) (COX-1, COX-2). Bound antibodies were detected using an enhanced chemiluminescence reaction. The optical density of each band was quantified using specific computer programmes (GeneSnap and GeneTools, Synoptics, Cambridge, UK).

Immunohistochemistry for ED1 and COX-2

Colonic tissue was fixed as described above. The deparaffinised sections were first incubated in the blocking serum, followed by incubation with the primary antibody, either mouse monoclonal anti-ED1 IgG (Serotec, Oxford, UK) or rabbit polyclonal anti-COX-2 IgG (Cayman Chemical, Ann Arbor, MI, USA). After the primary antibody, the sections were incubated in the biotinylated secondary antibody, either anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA) (ED1) or anti-rabbit IgG (Vector Laboratories) (COX-2). The sections were then incubated with peroxidase labelled biotin-avidin-complex using a commercial Elite ABC kit (Vector Laboratories). The colour was developed by incubation in a 3-amino-9-ethyl carbazole solution containing hydrogen peroxide. Finally, sections were counterstained with Mayer’s hemalum and mounted in aqueous mounting medium. Sections incubated with either normal mouse IgG (ED1) or normal rabbit IgG (COX-2) served as negative controls. The specimens were examined by Leica DMR microscope (Leica Microsystems, Wetzlar, Germany), photographed (the whole specimen) and digitised using specific computer programmes. The percentage of staining of the whole area was quantified from all the photographs taken, and the results are presented as the mean of these percentages.

Statistical analysis

Data are expressed as means ± SEM. Statistical analyses were carried out using the Kruskal-Wallis test, and when this indicated a significant effect, the Mann-Whitney U-test was used. P values of < 0.05 were considered statistically significant.
RESULTS

Severity of inflammation

DSS induced bloody diarrhoea from day 6 onwards (Fig. 1A and 1B), but did not significantly reduce the weight gain of the animals compared to the healthy control group, indicating a mild form of inflammation (Fig. 2). Macroscopically, the colons of the colitis control group did not differ from those of the healthy control group and the colon wet weight was similar in all the groups (Table 1). Histological changes were also small except for the increased inflammatory cell infiltration. No myeloperoxidase activity was detected (data not shown), indicating that these cells were not neutrophils.

Montelukast decreased occult blood in the faeces/ gross bleeding at the end of the experiment (P < 0.05) (Fig. 1A), but it did not affect diarrhoea (Fig. 1B). Montelukast inhibited the reduction of weight gain seen in the colitis control group (P < 0.05) (Fig. 2), indicating a beneficial effect.

Eicosanoid production

The in vitro colonic production of leukotriene B$_{4}$ in 15 min increased in the colitis control group (P < 0.05), whereas the cyclooxygenase products, prostaglandin E$_{2}$, bicyclic-prostaglandin E$_{2}$ and thromboxane B$_{2}$, remained at the same level with the healthy control group (Table 1). Cysteinyl leukotriene production was not measurable in the 15-minute incubation but in the 24-h incubation high levels of cysteinyl leukotrienes were produced. This appeared to be higher in the colitis control animals than in the healthy ones (40 ± 9 pg/mg and 31 ± 2 pg/mg, respectively). There were no statistically significant differences in the levels of cyclooxygenase products measured in the 24-h incubation (data not shown). The ratio of leukotriene B$_{4}$/ prostaglandin E$_{2}$ production during the 15-min incubation period increased 2.5-fold in the colitis control group compared to the healthy control group (Mann-Whitney P = 0.076; Kruskal-Wallis P = 0.108). A similar increase (2.5-fold) in the colitis control group was seen in the ratio of leukotriene B$_{4}$/ bicyclic-prostaglandin E$_{2}$ (Mann-Whitney P = 0.05; Kruskal-Wallis P = 0.075).

Montelukast did not significantly influence eicosanoid production, but it reduced the ratio of leukotriene B$_{4}$/ prostaglandin E$_{2}$ production within 15 min by 60% (Mann-Whitney P = 0.076; Kruskal-Wallis P = 0.108) (Table 1).

Colonic iNOS, ED-1, COX-1 and COX-2 protein expression

Expression of iNOS (Fig. 3A) and of the protein recognized by ED-1 (Fig. 3B) increased in the inflamed colon, but there were no differences between the montelukast group and the colitis control group. Interestingly, we did not detect any COX-2 by Western blot in the inflamed or in the healthy colons. COX-2 expression detected by immunohistochemistry in the mucosal layer did not markedly differ between the healthy and the colitis control groups, nor was it
Fig. 1. Occult blood in faeces/ gross bleeding (A) and loose faeces/ diarrhoea (B) 6 - 9 days after the beginning of DSS administration. An index of occult blood/ gross bleeding: 0 = no occult blood in the faeces and no gross bleeding, 2 = occult blood in the faeces, and 4 = gross bleeding. An index of loose faeces/ diarrhoea: 0 = faeces normal, 2 = faeces loose, and 4 = diarrhoea. Data are means ± SEM (n = 6 in each group). C, colitis control; M, montelukast (10 mg/ kg). *P < 0.05 vs. colitis control.
affected by montelukast (Fig. 3C). COX-1 was expressed in every specimen and the levels were comparable between the different groups (data not shown).

Table 1. Colon wet weight (g/kg) and in vitro eicosanoid production in 15-min incubation of the colon (pg/mg) after DSS administration for nine days.

<table>
<thead>
<tr>
<th></th>
<th>Healthy control</th>
<th>Colitis</th>
<th>Montelukast</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Colon wet weight</strong></td>
<td>4.2 ± 0.1</td>
<td>4.2 ± 0.2</td>
<td>4.3 ± 0.1</td>
</tr>
<tr>
<td><strong>LTB₄</strong></td>
<td>0.37 ± 0.04*</td>
<td>0.87 ± 0.12</td>
<td>0.58 ± 0.15</td>
</tr>
<tr>
<td><strong>LTE₄</strong></td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td><strong>PGE₂</strong></td>
<td>4.3 ± 1.0</td>
<td>4.2 ± 1.0</td>
<td>6.0 ± 1.3</td>
</tr>
<tr>
<td><strong>Bicyclic-PGE₂</strong></td>
<td>18 ± 2</td>
<td>14 ± 1</td>
<td>17 ± 2</td>
</tr>
<tr>
<td><strong>TXB₂</strong></td>
<td>29 ± 4</td>
<td>32 ± 5</td>
<td>40 ± 3</td>
</tr>
<tr>
<td><strong>LTB₄/ PGE₂</strong></td>
<td>0.13 ± 0.04</td>
<td>0.32 ± 0.08</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td><strong>LTB₄/ bicyclic-PGE₂</strong></td>
<td>0.023 ± 0.005</td>
<td>0.058 ± 0.010</td>
<td>0.038 ± 0.010</td>
</tr>
</tbody>
</table>

Values are means ± SEM (n = 5 - 6 in each group). LTB₄, leukotriene B₄; LTE₄, leukotriene E₄; PGE₂, prostaglandin E₂; TXB₂, thromboxane B₂; *Cysteinyl leukotrienes are presented as the end-product LTE₄; n.d., not detected. *P < 0.05 vs. colitis control.

Fig. 2. Body weight change (%) during DSS administration. Data are means ± SEM (n = 6 in each group). The montelukast dose was 10 mg/kg. *P < 0.05 vs. colitis control.
In the present study, we investigated the effects of the oral treatment of the cys-LT-1 receptor antagonist montelukast on the severity of the colonic damage
and on the expression and synthesis of various inflammatory markers in mild DSS-induced colitis in rats. DSS induced a light inflammation in the colon, with increased macrophage infiltration and iNOS expression. Montelukast decreased the occult blood in the faeces/ gross bleeding and inhibited body weight reduction, supporting the benefits of montelukast treatment. One possible explanation to anti-inflammatory effect is the reduced ratio of leukotriene B$_4$/prostaglandin E$_2$ production.

Leukotriene B$_4$ is a very potent chemoattractive agent for leukocytes, stimulating adhesion, chemotactic movement, aggregation, enzyme release and the generation of superoxide (37), and there are data to support its direct involvement in the pathophysiology of inflammatory bowel diseases (17). Prostaglandins, on the other hand, suppress various cellular immune functions in vitro (17), accelerate cell production and differentiation after mucosal damage, stimulate mucus production, and might act as modulators of local blood flow (38). In fact, exogenous prostaglandins have been shown to ameliorate intestinal damage in experimental models of colitis (26, 39 - 42). Arachidonate cascade is very complex, and subtle changes in the profile of its products may be important. Therefore, it is possible that the 60% decrease in the ratio of leukotriene B$_4$/prostaglandin E$_2$ production by montelukast may explain the moderate beneficial effects on colitis that were seen in the present study.

In the present study, colonic prostaglandin E$_2$ production in vitro was not enhanced in the DSS-treated animals. The reports on prostaglandin E$_2$ tissue levels and colonic production in DSS-induced colitis model are not entirely consistent. When DSS is administered for seven days, prostaglandin E$_2$ levels in colonic tissue are either reduced (43, 44) or not affected (45), whereas administration for two weeks results in increased levels (46). Furthermore, DSS administration for five days has been reported to increase the in vitro production of prostaglandin E$_2$ in the colon during 24-hour incubation (47).

In this study, cyclooxygenase-1 and cyclooxygenase-2 expressions remained unchanged in DSS-induced mild colitis, in line with the results of prostaglandin E$_2$ production. COX-2 is not apparently up-regulated in the epithelium in this model (44, 48), and it is not clear whether the number of COX-2-expressing mononuclear cells increases either (44, 48). Cyclooxygenase-1 expression, in contrast, is completely eliminated in the crypt epithelium in most areas in DSS-induced colitis, but not affected in mononuclear cells (44, 48), which explains the lack of differences between healthy and diseased animals in the present study. In contrast, we have found only one study reporting an increase in COX-2 expression in an acute (two days) DSS-model (49) (vs. the present study with DSS administration for nine days). Therefore the role of COX-2 in DSS-induced colitis remains largely unsolved.

Previous studies by us (30) and others (28) have suggested that cys-LT-1 receptor antagonists can increase prostanoid synthesis in TNBS-induced colitis. In the present study, montelukast had no effect on colonic prostaglandin E$_2$
production per se, but it modified the ratio of leukotriene B₄/prostaglandin E₂ production. In our previous study, montelukast did not modify prostaglandin E₂ production in vitro in healthy animals in contrast to animals with severe acute colitis (30). These differences may explain the seemingly inconsistent findings.

Although the exact role of iNOS - another enzyme induced in inflammatory reactions - remains to be discovered (50, 51), high levels of nitric oxide, produced by iNOS, are believed to cause intestinal injury (52). This accords with studies, where a selective iNOS inhibitor reduced damage in TNBS-induced (53) as well as in DSS-induced colitis (54) in rats, whereas a non-selective inhibitor was ineffective (53, 54). In the present study, iNOS was induced after DSS treatment, but montelukast had no effect on its expression in the colon.

In conclusion, we suggest that montelukast has moderate beneficial effects on some of the inflammatory variables in mild colitis induced by DSS.

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