Mucosal factors inducing neutrophil movement in ulcerative colitis: the role of interleukin 8 and leukotriene B₄

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Abstract
Background—The movement of neutrophils into the colonic mucosa in ulcerative colitis is induced by chemokines including interleukin 8 (IL8) and leukotriene B₄ (LTB₄).

Aims—To compare the ability of mucosa from ulcerative colitis patients and controls to stimulate neutrophil movement, to define the contribution of LTB₄ to this, and to define the relative biological importance of LTB₄ and IL8.

Patients—Resected mucosa was obtained from seven control patients and 10 patients with ulcerative colitis.

Methods—Mucosal homogenate supernatants were used to stimulate isolated neutrophils and the effect assessed by the neutrophil shape change response. Responses were inhibited with either the LTB₄ receptor antagonist SC41930 or neutralising anti-IL8 antibody. LTB₄ was extracted and assayed by RIA.

Results—Homogenate supernatants from inflamed mucosa were more bioactive (median 1·2 mg/ml−1 induced 50% response) than those from uninfamed mucosa (4·25 mg/ml−1 induced 50% response; difference 2·8 mg/ml−1 (95·6% CI 0·5 to 6·1, p<0·05)). Maximal inhibition by SC41930 of the response was significantly greater in inflamed mucosa (54% median) than in uninfamed mucosa (34%). This inhibition correlated with the level of immunoreactive LTB₄ (r=0·78). Anti-IL8 reduced bioactivity of homogenate supernatants from inflamed mucosa (at 1:10 dilution) by 11·4% (IQR 1·2 to 51·8, p=0·021) whereas SC41930 reduced it by 54·8% (35·6 to 77·5, p=0·008).

Conclusions—Inflamed colonic mucosa releases more neutrophil movement inducing bioactivity than uninfamed mucosa, and has greater LTB₄ dependent activity. It yields both IL8 and LTB₄ dependent activity but greater LTB₄ dependent activity. (Gut 1996; 39: 248–254)

Keywords: neutrophils, interleukin 8, leukotriene B₄, ulcerative colitis, colonic mucosa.

Granulocytes migrate into the mucosa in colitis.¹ This movement is promoted by various chemotactic cytokines. Two of the most potent of these are interleukin 8 (IL8)² and leukotriene (LT)B₄.³ IL8 is produced by several cell types including macrophages, endothelial cells, and neutrophils.⁴ Immunoreactive IL8 is raised in inflamed mucosa from patients with active ulcerative colitis.⁵ LTB₄ has been shown to be the major lipid extractable chemotactic component of homogenised mucosa in ulcerative colitis.⁶ Other mediators such as bacterial peptides,⁷ neuropeptides,⁸ and cyclic endoperoxides⁹,¹⁰ may be important modulators of inflammation in colitis.

We have compared the bioactivity of homogenate supernatants of uninfamed colonic mucosa with that of homogenate supernatants of inflamed colonic mucosa. We have also investigated the relative contribution of IL8 and LTB₄ to the bioactivity of homogenate supernatants from inflamed mucosa. The bioactivity of supernatants was measured with an in vitro neutrophil shape change assay; a measure of the neutrophil motile response.¹¹

Methods
Inflamed colonic mucosa was obtained from 10 patients (three male), age 21 to 72 having operations for colitis (eight with treatment failure, one proctitis and sigmoid colon cancer); uninfamed colonic mucosa was obtained from seven patients (one male) age 43 to 94, having colectomy for cancer (one rectal, two rectosigmoid, one sigmoid, two descending colon) or benign tumour (sesseal adenoma of ascending colon one). Of the patients with colitis six were taking salicylates, nine corticosteroids, and four azathiprine. None of the control patients were taking anti-inflammatory medications. Uninfamed control tissue was collected from parts of the cancer resection specimens as far removed from the pathological lesions as possible. Mucosa was cut from the specimen and snap frozen in liquid nitrogen. Acute and chronic inflammation was scored by a histopathologist blinded to the assay results on a scale of 0–3 derived from quantitative histology.¹² ¹³ Tissue was thawed, weighed, and homogenised in Hanks’s balanced salt solution (without Ca²⁺ or Mg²⁺)+10⁻² mol/l 3-[N-morpholino] propanesulphonic acid (HBSS/MOPS) using an Ultra-Turrax homogeniser (Janke and Kunkle, Whyteleafe Scientific, UK). The homogenate was centrifuged for 15 minutes at 750 g and the supernatant collected. Homogenates concentrations were expressed as milligrams tissue wet weight per millilitre of homogenates.

Two separate series of experiments were performed. Homogenate supernatant bioactivity was compared between homogenate
supernatants of inflamed (n=6) and uninflamed mucosa (n=7) using a neutrophil shape change assay. The bioactivity was assessed for its LTB4 dependence in these experiments with a, leukotriene receptor antagonist, SC41930 (Searle, Skokie Illinois). In the second series of experiments we investigated whether there was both a contribution of LTB4 and IL8 to the bioactivity of homogenate supernatants from inflamed mucosa (n=9). For these experiments we used supernatant at a concentration of 40 mg wet weight of tissue homogenised per millilitre buffer at 1:10, 1:20, and 1:40 dilutions. IL8 was blocked with a blocking anti-IL8 antibody (goat polyclonal anti-IL8 antibody, Sandoz Forshungs Institute, Vienna) and LTB4 was blocked with SC41930.

We used a modification of the method of Haston as a bioassay to assess induced neutrophil shape change, a measure that correlates well with other measures of neutrophil movement. Neutrophils were obtained from healthy volunteers. Blood was taken using a 19 gauge needle (1-1X50 mm, Becton Dickinson, Dublin, Ireland) and syringe (Becton Dickinson) and red cells sedimented with 2:1 blood:dextran (dextran 70, 0:9% NaCl, Baxter Healthcare, Thetford, UK) for 45 minutes. The upper granulocyte rich fraction was layered onto lymphocyte separation medium, density 1:077, 7 ml cells:3 ml of lymphocyte separation medium (Flow Laboratories, Rickmansworth, UK) and spun at 400 g for 30 minutes in a capped sterile 12 ml tube (Sterilin, Stone, UK). The supernatant fluid and lymphocyte/monocyte layer were discarded. The cells in the pellet were resuspended in 1 ml of buffer solution, transferred to a fresh 12 ml tube, and further 9 ml buffer added. The buffer used was Hank’s balanced salt solution without calcium and magnesium (Northumbrian Biologicals, Cramlington, UK) buffered with 10⁻² mol/l morpholino-propanesulphonic acid (HBSS/MOPS). Neutrophils and contaminating red blood cells were spun down and the supernatant removed; the red cells in the pellet were haemolysed with 1 ml of deionised water for 45 seconds; the remaining neutrophils were diluted to 10 ml with buffer and again spun down. The pellet was resuspended in 2 ml of buffer. Neutrophils were counted using a Coulter Counter ZM (Coulter Electronics, Luton, UK). The viability of the neutrophils in a preparation was assessed by trypan blue exclusion and a preparation was not used if viability was less than 90%. An aliquot of cells were fixed with an equal volume of 2-5% glutaraldehyde in buffer to assess activation. If greater than 10% polarised cells were observed before incubation the preparation was not used. The neutrophil preparation may be contaminated by red blood cells, which are clearly distinguished microscopically. Isolated cells were shown to be predominantly neutrophil polymorphonuclear leucocytes by light microscopy of May-Grunwald-Giemsa stained smears of the cell preparation (92% polymorphonuclear leucocytes 4% lymphocytes 4% eosinophils).

Neutrophils were diluted in buffer to 2X10⁶ ml⁻¹ for assay and incubated in a ratio of 0-4 ml of neutrophils plus 50 μl of test homogenate supernatant (or control agonist) and with 50 μl SC41930 10X10⁻⁶ mol/l (or control buffer). For the anti-IL8 antibody experiments agonists and antagonists were preincubated together for 20 minutes, separate aliquots of neutrophils were then incubated with control antagonist, each homogenate alone, with each homogenate and each antagonist (SC41930 or IL8 Ab), and with homogenate plus both antagonists. Cells were incubated for 30 minutes at 37°C and the samples then fixed with an equal volume of 2-5% glutaraldehyde (Sigma) in buffer.

Colitis and control homogenates were assayed together to avoid bias due to differences in the test neutrophil preparations used. Each homogenate was always assayed at three different dilutions 1:10, 1:20, and 1:40.

After the experimental stimulation and fixation of the cells the change in morphology from round to polarised was assessed. Fixed cells were mounted in a Neubauer haemocytometer. Cells were categorised as being round or not round 'polarised' at X125 magnification (Leitz Ortholux microscope, Leica UK, Milton Keynes, UK), under transmission illumination. A mean of two cell counts were taken (>100 cells each). The percentage change not round to the total count was expressed as a percentage - % neutrophil shape change. Experiments were counted blind on coded samples to avoid unconscious bias.

The reproducibility of counting cell shape change was assessed by comparing repeated counts within observer and between two observers each blind to the counts of the other observer. Repeated counts of seven separate neutrophil experimental tubes, stimulated by different concentrations of LTB4, to develop a range of 60 to 67-5% shape change, were made by each observer.

Dose response experiments were performed for this assay using synthetic LTB4 (Cayman, Reading, UK) and IL8 (Sandoz, Forschungs Institute, Vienna). We investigated the specificity of inhibition of LTB4 by SC41930 in comparison with n-formyl-methionine-leucine-phenylalanine (fMLP) and IL8 in the assay and similarly the specificity of inhibition by goat-IgG anti-IL8-antibody of synthetic IL8 induced responses in comparison with LTB4. Non-specific goat IgG antibody (Sigma, Poole, UK) was used as the control. For the inhibition experiments described anti-IL8 antibody was used at 0-5X10⁻⁶ g t⁻¹ to block IL8 at 10⁻⁴ M SC41930 was used to inhibit LTB4 induced responses (published data give an IC50 of 0-3X10⁻⁸M. For the experiments comparing homogenate bioactivity between colitic and uninflamed patients, the LTB4 dependence was determined as the maximal reduction in neutrophil shape change observed with SC41930 for any one homogenate supernatant.
**LTB₄ assay**

LTB₄ was assayed by a validated radioimmunoassay.¹⁷ Homogenates supernatants were extracted using the method of Powell.¹⁸ Results are corrected for recovery measured by spiking each sample with [³H]-LTB₄. The radioimmunoassay has a sensitivity of 5 pg/0·1 ml at 90% B/BO and has low cross reactivity for other lipoxygenase products (20-OH-LTB₄ has 3-9% and 12-R-HETE 0-4% crossreactivity).

**Ethics**

Ethical approval was obtained from the Hospital and University Ethics Committee for the use of resected specimens and for neutrophil isolation from healthy volunteers.

**Statistical analysis**

The concentration of homogenate required to give 50% activation was derived by interpolation of data at different homogenate dilutions (bioactivity was defined as the reciprocal of this concentration). Differences between the bioactivities of colitic and normal mucosa homogenates were compared using the Mann-Whitney U test. Correlations were assessed by the Spearman rank correlation coefficient (r). Data from experiments on the inhibition of IL8 and LTB₄ were analysed for each dilution by Friedman two way analysis of analysis of variance and differences between control and inhibitors compared by Wilcoxon signed rank sum test. For comparisons of the percentage of induced activity inhibited by SC41930, allowing for the effects in unstimulated cells, the following calculation was used for each individual set of data.

\[
\%\text{ inhibitable activity} = 100\% \times \frac{(H - H_{0}) - (B - B_{0})}{(H - B)}
\]

where \(H\) = homogenate stimulated shape change; \(H_{0}\) = homogenate stimulated shape change in the presence of the inhibitor; \(B\) = unstimulated shape change and \(B_{0}\) = unstimulated shape change in the presence of the inhibitor SC41930. B = unstimulated shape change and \(B_{0}\) = unstimulated shape change in the presence of the inhibitor SC41930.

Data are expressed as medians and interquartile ranges.

**Results**

**Reproducibility of scoring shape change**

There was no significant difference between blinded observers in assessing neutrophil shape change. For duplicate counts of the percentage neutrophil shape change, the ratio for counts made by one observer compared with another was 0·85 (mean, 95% confidence intervals (CI) 0·671 to 1·07) showing that different observers made similar assessments. Repeated counting by one observer was also reproducible; the within observer ratio for repeated counts was 1·22 (0·948 to 1·57).

**Effects of standards and inhibitors**

Figure 1 shows the dose response to IL8 and LTB₄. Control experiments showed that SC41930 (10⁻⁶ M) inhibits the neutrophil shape change induced by LTB₄ 0·6×10⁻⁹ M by and the absence of an effect on the other neutrophil chemoattractants fMLP 10×10⁻⁹ M and IL8 (0·1×10⁻³ g/l⁻¹) (Fig 2A). Anti-IL8 specifically inhibited IL8; in two experiments, each performed in duplicate, IL8 3×10⁻⁶ g/l⁻¹ caused 77% neutrophil shape change and 40% neutrophil shape change, this was inhibited by 97% and 96% respectively by goat anti-IL8 antibody 0·5×10⁻³ g/l⁻¹ (Fig 2B).

At higher concentrations of IL8 inhibition by anti-IL8 was not complete; IL8 at 10×10⁻⁶ g/l⁻¹ in two experiments induced 91% and 77% neutrophil shape change. This induced activity was inhibited by 45% and 64% respectively by anti-IL8 antibody (0·5×10⁻³ g/l⁻¹). Anti-IL8 did not inhibit 1 nM LTB₄ (Fig 2B). Non-specific goat IgG antibody (0·5×10⁻³ g/l⁻¹) did not inhibit the IL8 induced response (Fig 2B).

**Unstimulated control experiments**

In the neutrophil preparations used for these experiments there was detectable LTB₄ dependent activity consistent with the known ability of neutrophils to synthesise LTB₄; unstimulated controls had an activity of 20·3% (median, IQR 12·3 to 27·4) polarised forms. This activity was reduced by SC41930 alone to 15·5% (2·9 to 21·5), a difference from in inhibited controls of 4·9% (median, p=0·06).

There was less detectable intrinsic neutrophil IL8 dependent activity; unstimulated neutrophils plus anti-IL8 antibody had an activity of 17·5% polarised forms (12·7 to 26·5) (median difference from controls 1·2%, p=0·05).

**Inflamed mucosa compared with uninfamed mucosa**

Homogenate supernatants from uninfamed mucosa were less active than homogenate from controls.
supernatants from inflamed mucosa at inducing in vitro neutrophil shape change (Fig 3A). Homogenate supernatants from uninflamed mucosa produced a 50% neutrophil shape change response at median 4-25 mg/ml−1 (range 2-1 to >8) mg/ml−1. Inflamed mucosal homogenates induced a 50% neutrophil shape change response at 1-2 mg/ml−1 (0-1 to 2-6, difference 2-8 mg (96-5% CI 0-5 to 6-1, p=0-007).

Because of the age differences between the patients with inflamed mucosa and uninflamed mucosa we examined the effects of age on the ability of homogenate supernatants to induce neutrophil activation. There was a positive correlation between patient age and the potency of homogenate supernatants from control patients (Spearman r=0.98, p=0.001) but there was no relation with the samples from patients with colitis.

Homogenates of inflamed colon from patients with active colitis also had a greater percentage of LTB4 dependent activity than homogenate supernatants from uninflamed colon (Fig 3B). SC41930 reduced the shape change induced by supernatants of homogenates of uninflamed mucosa by median 34% (22 to 38), whereas it inhibited neutrophil shape change by 54% median (37 to 72) when neutrophils were stimulated with supernatants from inflamed colon (difference inflamed:uninflamed 25% (96-5% CI 10 to 37%).

**Figure 2:** (A) Effect of SC41930 on LTB4 induced neutrophil shape change. SC41930 10−6 mol/lt LTB4 10−8 mol/l; fMLP 10−6 mol/l; IL8 10−8 g/l (1.25×10−8 mol/l). Percentage inhibition of induced response shown on vertical axis. Error bars (SEM). (B) Effect of anti-IL8 antibody on IL8 (3 ng/ml) and LTB4 (10−9 M) induced neutrophil shape change (goat anti-IL8 antibody and non-specific control antibody ×10−3 g/l). Percentage inhibition of induced response shown on vertical axis (mean of two experiments each performed in duplicate).
**Effects of inhibitors of IL8 and LTB4**

Homogeneous supernatants induced neutrophil shape change that was dependent on the concentration of homogenate. The 1:10 dilution of supernatant induced 66.7% polarised forms (median, IQR 50.6-83.2), whereas the 1:20 dilution induced 50.6% (33.1 to 66.9) and the 1:40 induced 29% (17 to 40). This activity was reduced significantly at each dilution both by anti-IL8 (p=0.016 at 1:10) and SC41930 (p<0.005). When neutrophils were stimulated with the 1:10 dilution of homogenate supernatant, anti-IL8 inhibited the induced neutrophil shape change response by 9.1% (3.7 to 36.3) whereas SC41930 inhibited the induced shape change response by 42.2% (31.9 to 73.3) and anti-IL8 antibody along with SC41930 inhibited the induced neutrophil shape change by 53.8% (45.1 to 83.4). With 1:20 and 1:40 dilutions similar results were obtained (Fig 4). When corrected for the effects of inhibitable activity in the control samples, the proportion of the activity induced by homogenate supernatants (1:10) that was sensitive to SC41930 was 54.8% of induced activity (35.6 to 77.5) whereas the proportion that was sensitive to anti-IL8 antibody was 11.4% of induced activity (1.2 to 51.8).

**Discussion**

Neutrophil shape change measurement is a validated method for assessing the motile response of neutrophils and has the advantage over Boyden chamber assays that it does not confuse motility with adhesive interactions and movements along surface bound chemotactic gradients (haptotaxis).\(^1\) We have previously used the neutrophil shape change response to assess the role of LTB\(_4\) in rectal dialysates from healthy volunteers and colitis patients.\(^1\)

Our data show that in active colitis there is an increase in the bioactivity of supernatants of mucosal homogenates to induce motile responses in neutrophils. This parallels the finding in vivo of an increase in the number of polarised neutrophils in the peripheral blood\(^2\) and would be expected from work showing raised LTB\(_4\) synthesis from ex vivo incubations of mucosal biopsy specimens.\(^2\) The results also correspond with our previous findings of increased rectal dialysate LTB\(_4\) dependent bioactivity in ulcerative colitis. Mucosal homogenate supernatants have previously been shown to have a high proportion of neutrophil chemotactic bioactivity which co-elutes with synthetic LTB\(_4\) on high pressure liquid chromatography.\(^6\)

Our data on mucosal homogenates from active colitis show that these contain both IL8 and LTB\(_4\) dependent bioactivity able to induce neutrophil motile responses. At the dilutions of supernatant used in these experiments causing submaximal stimulation, the inactivation by SC41930 or anti-IL8 antibody of LTB\(_4\) or IL8 stimulated responses would have been virtually complete. However, it is not possible to interpret all the homogenate induced activity that is inhibited by SC41930 as being due to LTB\(_4\) in the homogenates because there is some SC41930 sensitive activity under unstimulated conditions. Allowing for this reduction in unstimulated activity, SC41930 sensitive (LTB\(_4\) dependent) activity accounted in these experiments for 54% of the homogenate supernatant induced bioactivity. This may be an underestimate of the proportion of LTB\(_4\) dependent activity as SC41930 is a competitive antagonist (higher concentrations were not used as a higher concentration of SC41930 (10\(^{-5}\) M) will inhibit fMLP.\(^14\) Anti-IL8 antibody sensitive activity accounted for a lower proportion (median 11%) of the induced activity.

Although there is an age bias between the control and colitis groups in these experiments, this does not account for the differences we have observed. In fact, the bias would have tended to reduce the observed differences because we found that increasing age was associated with increased homogenate supernatant bioactivity in the control subjects. The reason for the apparent relation of homogenate bioactivity with age is unknown.

Our data will not show the influence of ephemeral compounds such as thromboxane A\(_2\). Thromboxane A\(_2\) is probably present in vivo in colitis as the inactive product thromboxane B\(_2\) is increased in ulcerative colitis.\(^2\)\(^2\) It has an important role in the recruitment of...
inflammatory cells in animal models but because thromboxane A₂ has very short half life is unlikely to be present in our test supernatants and in processed stored samples used by others.6 Also it is possible that agonists in the homogenates exert their influence via LTβ₄ dependent autocrine and paracrine effects as has been shown for thromboxane A₂ in vivo.23 These indirect effects mediated via neutrophil LTβ₄ release could be investigated by pretreating neutrophils with a 5-lipoxygenase inhibitors.

By contrast E series prostaglandins, though also present in raised concentrations in colitis, have anti-inflammatory effects.24 PGE₂ in concentrations up to 10⁻⁶ mol/L does not effect the response of neutrophils to 10⁻³ mol/L LTβ₄ or fMLP in this shape change assay (data not shown). It therefore seems unlikely that it would have attenuated the responses in comparison of active disease supernatants and controls.

Many of the colitic patients from whom colonic mucosa was obtained were taking anti-inflammatory drugs including corticosteroids, azathioprine, and 5-aminosalicylic acid preparations. These drugs may conceivably have reduced the differences observed between the two groups but are unlikely to be the reason for the observed differences as these drugs are described as attenuating rather than activating inflammation.

Our data show that there is IL8-like bioactivity in inflamed mucosa in ulcerative colitis so confirming that the IL8 immuno-reactivity detected in previous studies of colonic homogenates is bioactive. Though homogenate supernatant IL8 was found to account for less neutrophil shape change activity than LTβ₄ in these experiments, it may have an important role in the acute inflammatory responses in ulcerative colitis. It is also probable that at a cellular level these two neutrophil chemotactic agents have complementary roles. LTβ₄ promotes the adhesion of neutrophils to the endothelium by increasing neutrophil CD11/18 expression, whereas IL8 in high concentrations may unstick the neutrophil by causing conformational change in the ligands for the CD18 adhesion molecule and thereby allowing diapedesis of the adhered cell onwards to an inflammatory site.25 IL8 may also have a longer duration of action than LTβ₄ in tissue.4 As IL8 is produced by lipopolysaccharide stimulated epithelium and endothelium,2 as well as by macrophages and neutrophils it is conceivable that it could be up regulated earlier in the inflammatory process than LTβ₄ which has been shown to predominantly derive from infiltrating neutrophils in animal models.27 However our data suggest that in these supernatants with homogenised mucosa that LTβ₄ is the most prominent neutrophil movement inducing factor.

The comparison of the data presented here from mucosal homogenates with data from rectal diaysates samples is of interest. We have previously found equivalent neutrophil activating bioactivity in rectal diaysates obtained from healthy volunteers and patients with active ulcerative colitis19 but that when diaysates were collected from patients who had had bowel preparation prior to colonoscopy, those from patients with ulcerative colitis had greater neutrophil activating potency.28 These data fit in with the data presented here indicating that the mucosa is a major source of chemotactic factors in active colitis, but that the luminal content of the healthy bowel also has potent neutrophil activating and chemotactic activity. In ulcerative colitis the transmigration of neutrophils from mucosa to lumen may be driven both by the release of mucosal factors into the lumen and by the presence of normal luminal factors in conjunction with abnormal epithelial function.

Our data support a role for leukotriene synthesis inhibitors and receptor antagonists as therapeutic agents in ulcerative colitis, though the presence of detectable amounts of anti-IL8 sensitive activity illustrates the significant parallel proinflammatory pathways present in colitis. This redundancy may limit the usefulness of any one specific anti-inflammatory treatment.

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