Increased faecal mucin sulphatase activity in ulcerative colitis: a potential target for treatment

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Abstract
Colonic mucin is heavily sulphated and it has been shown that enzymatic desulphation by faecal bacterial sulphatases greatly increases its susceptibility to degradation by faecal glycosidases. A possible role for faecal mucin sulphatase in the pathogenesis of inflammatory bowel disease has therefore been explored. Faecal mucin sulphatase activity assayed using 35S mucin as substrate was increased in ulcerative colitis (median 80-2 units/g pellet weight (range 6-9-1063; 95% confidence intervals (CI): 45-2 to 293-8, n = 22) compared with 11-3 units/g (range 3-0-53-5; 95% CI: 8-7 to 29-8, n = 17) in healthy controls (p < 0.01), where one unit released 1000 dpm free sulphate/hour from 35S mucin (1680 dpm/μg). Patients with active ulcerative colitis had higher sulphatase activity (median 146; 95% CI: 98 to 253 units/g, n = 10) than those with inactive ulcerative colitis (median 42-2; CI: 22-5 to 81-6 units/g, n = 12) (p < 0.05). Longitudinal studies in patients with ulcerative colitis show fluctuations of faecal mucin sulphatase activity corresponding to clinical disease activity in six of seven patients. Faecal mucin sulphatase activity was not significantly increased in Crohn's disease (median 36-6, range 5-7-106-6; 95% CI: 22-9 to 65-3 units/g, n = 14). The bismuth salts, bismuth subcitrate and bismuth subsalicylate were found to inhibit faecal mucin sulphatase activity at concentrations achievable therapeutically. The increased faecal mucin sulphatase activity in ulcerative colitis could be the result of greater intraluminal substrate (mucin) availability leading to bacterial enzyme induction, but would probably result in more rapid degradation of secreted mucin and represents a potential target for treatment.

Methods

PATIENTS AND CONTROL SUBJECTS
Faecal samples were collected from 17 healthy volunteers, 22 patients with ulcerative colitis, and 14 patients with Crohn's disease, six with ileocolonic disease and eight with ileal disease. Only three of the 10 ulcerative colitis patients were not receiving sulphasalazine. None of the patients or controls had a history of infective diarrhoea in the previous 12 months and none had received antibiotics within the previous month.

Diagnoses of Crohn's disease or ulcerative colitis were based on conventional pathological and radiological criteria. Clinical information was obtained from the patients by history and examination and information recorded onto a standard protocol. The mean stool frequency in the week before obtaining the sample was recorded. In patients with Crohn's disease the disease activity was assessed by the simple Crohn's disease activity index devised by Harvey and Bradshaw. Disease severity of patients with ulcerative colitis was graded clinically by the scoring method described by Powell-Tuck et al. All patients with ulcerative colitis had rigid sigmoidoscopy performed for clinical reasons using a disposable Lloyd-
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Davies sigmoidoscope carried out by the same observer. Grading of sigmoidoscopy was according to Gomes et al.11 The median ages of the normal subjects was 38 years (range 19–52), ulcerative colitis 37·5 years (range 22–78), and Crohn’s disease 32·5 years (range 26–65). There was no significant age difference between the three groups (p=0·15; Kruskal-Wallis one way analysis of variance).

Ethical approval was granted by the Royal Liverpool University Hospital ethical committee.

FAECAL COLLECTION

A morning sample of faeces was collected in sterile containers from the subjects and frozen within two hours of voiding. The samples were kept at −70°C until batch analysis.

SULPHATASE ASSAY METHOD

Extraction

Faecal samples of about 1–2 g wet weight were homogenised in 50 ml 0·05 M TRIS/maleate pH 6·5 in a loose fitting glass homogeniser. The homogenate was centrifuged at 25 000×g for two hours in a refrigerated centrifuge. The supernatant was passed through filters culminating in a 0·2 micron filter to obtain a bacteria free filtrate. Protein analysis was carried out on all samples using the bichinonic acid assay.12 The centrifuge tubes containing the precipitate were weighed after desiccation in an 80°C oven and reweighed after washing and drying and the difference taken as the dry weight of the faecal pellet.

Preparation of 35S human colonic mucin as substrate

The method of biological radiolabelling of human colonic mucin has been previously described and validated.7 Colonic biopsy specimens were obtained with informed consent from patients undergoing routine colonoscopy who were found to have macroscopically and histologically normal rectal mucosa. The specimens were then cultured in the presence of NaH235SO4 to achieve biological radiolabelling of colonic sulphomucin based on the method of Trier et al.13 After 24 hours the mucin was extracted by ultrasonication, desalted, and pooled before purification. The same pooled [35S] mucin sample was used as substrate for all the sulphatase assays described here. Mucin was purified by high performance gel filtration. This technique has been shown to produce mucin of high purity without contamination by mannose containing glycoprotein or proteoglycans.14,15 Sulphatase assay was carried out using 35S mucin as substrate. Two hundred and fifty μl of the stock 35S mucin (50 000 dpm/ml) were incubated with 250 μl of the faecal filtrate in 0·05 M TRIS/maleate pH 6·5 for 24 hours at 37°C. The reagents were then applied onto a small gel filtration column (PD-10 G25M, Pharmacia, Milton Keynes, UK) with exclusion size of 5000 daltons. The intact mucus glycoprotein eluted first in the void volume with clear separation from the liberated sulphate and sulphated monosaccharides or oligosaccharides. Recovery of [35S] from the desalting column averaged 98% (range 96–99%). One ml of 0·1 M barium chloride was added to the pooled low molecular weight fractions (total volume 5 ml) to separate by precipitation the free sulphate from any sulphated monosaccharides. The excess barium was precipitated by 1 ml of 0·1 M potassium sulphate and the precipitate centrifuged and washed before resuspension in scintillant for β counting on an LKB 1219 Rackbeta (LKB, Turku, Finland). All assays were controlled against heat inactivated samples.

There is no technique that is sufficiently sensitive to permit accurate estimation of the number of sulphated sugar residues per molecule of mucin in the small samples extracted from biopsy specimens. Enzyme activity cannot therefore be expressed conventionally in terms of micromoles of substrate catalysed. One stock sample of 35S mucin was used for all the experiments in the study and enzyme activity was defined arbitrarily as: 1 unit is that enzyme activity which releases 1000 dpm of free sulphate per hour at 37°C. The stock substrate had an activity of 1680 dpm/μg glycoprotein. The mucin sulphatase assay had an interassay coefficient of variation of 8·8%.

Faecal mucin sulphatase activity was expressed as units/g faecal pellet weight, units/mg protein content, and as ratio of sulphatase/α-glucosidase activity.

α GLUCOSIDASE ASSAY

α Glucosidase is an ubiquitous bacterial secreted enzyme, which is not relevant to mucus degradation and that may serve as a useful guide to the total faecal bacterial load. α Glucosidase activity was measured using 4-methylumbelliferyl-α-D-glucoside (Sigma, Poole, UK) by the method of Potier.16 Samples of 0·1 ml were incubated in 0·1 ml of 10 mmol/l solution of 4-methylumbelliferyl-α-D-glucoside and incubated for one hour at pH 6·5, 37°C and emission at 450 nm after excitation at 363 nm was fluorospectrophotometrically measured.

INHIBITOR ASSAYS

Bismuth subcitrate (kindly supplied by Brocades Pharma, Liederdorp, Netherlands) at a concentration of 1, 2, 3, 4, and 5 g/l in 0·05 M TRIS/maleate pH 6·5, a suspension of bismuth subsalicylate (kindly supplied by Proctor and Gamble, Egham, UK) of 4 g/l in 0·05 M TRIS/maleate pH 6·5, dextran sulphate of molecular weights 5000 daltons, 8000 daltons, and 500 000 daltons (Sigma, Poole, UK) at a concentration of 0·2, 0·4, 0·6, 0·8, 1·0, and 5·0% weight/weight in 0·05 M TRIS/maleate pH 6·5 were each incubated at 37°C for 24 hours in the presence of mucin sulphatase purified from a single ulcerative colitis faecal sample and containing 52 μg protein/ml and
50,000 dpm/ml $^{35}$S mucin. Release of sulphate from the substrate was then assayed as above. In the case of the insoluble bismuth subsalicylate, constant agitation of the reactants was carried out throughout the incubation period.

LEUCOCYTE STUDIES
Experiments to find out if leucocytes contain mucin desulphating enzymes were performed. Human leucocytes were prepared from 20 ml of venous blood taken from a healthy volunteer collected into lithium-heparin tubes. Erythrocytes were sedimented with 1% dextran (Mr=70,000, Sigma, Poole, UK) in 0.9% sodium chloride solution for 30 minutes at room temperature. The supernatant was then centrifuged on a lymphocyte separation medium (Flow Laboratories, High Wycombe, UK) at 400 g for 30 minutes. Neutrophils were obtained from the cell pellet and incubated with 0.87% ammonium chloride to lyse residual erythrocytes. The mononuclear cells were then harvested from the interface. Neutrophil and lymphocyte preparations were then ultrasonicated in 0.05 M TRIS/maleate buffer yielding 23 μg protein/ml and 4 μg/ml respectively.

A crude faecal extract (79 μg), serum (0.1 ml), the polymorphonuclear leucocyte, and mononuclear cell preparations (2.3 μg and 0.4 μg protein respectively) were incubated in the presence of 25,000 dpm $^{35}$S mucin for 24 hours and the release of $^{35}$S sulphate assayed as above.

STATISTICAL ANALYSES
Data comparisons between two groups were performed using Wilcoxon's rank sum test. Multiple group comparisons were performed using the Kruskal-Wallis one way analysis of variance (ANOVA) by ranks. Correlation of sulphatase activity to clinical indices was performed by Kendall's rank correlation for non-parametric data. Two tailed tests of significance were used throughout with p values of <0.05 regarded as significant. Ninety five per cent confidence limits are quoted where relevant.

Enzyme inhibitor kinetics were calculated using a method of least squares regression and comparison with models of inhibitor kinetics using the software package Regression (Blackwell Scientific, Oxford, UK) as previously described.17

Results
Faecal sulphatase in inflammatory bowel disease and controls
The median faecal mucin sulphatase activity when expressed in terms of units/g pellet weight was significantly higher in ulcerative colitis (80-2 (range 6-9–1063; 95% CI: 45-2 to 293-8) units/g, n=22) compared with healthy controls (11-3 (range 3-0–53-5; 95% CI: 8-7 to 29-8) units/g, n=17 (p<0.01)) but...
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Figure 4: Longitudinal study showing serial faecal mucin sulphatase activity in seven patients in varying degrees of disease activity showing that faecal mucin sulphatase varies with disease activity in all except patient C. This study was performed on a different batch of 35S labelled mucin from the population studies in Figs 1–3.

not significantly changed in Crohn’s disease (36.6 (range 5.7–106.6; 95% CI: 22.9–65.3) units/g, n=14). Similarly, when expressed in terms of the protein content of the faecal filtrate, the faecal mucin sulphatase activity was significantly higher in ulcerative colitis; 14.2 units/mg (95% CI: 4.2 to 28.8) compared with healthy controls; 3.6 units/mg (95% CI: 2.4 to 4.3) (p<0.01) but not significantly raised in Crohn’s disease 4.1 units/mg (95% CI: 3.4 to 9.5 units/mg) (Fig 1). The faecal ratio of sulphatase:α glucosidase activity was also significantly raised in ulcerative colitis (median ratio; 23, 95% CI: 15 to 34, p<0.01) but not in Crohn’s disease (12, 3 to 18) compared with controls (6, 1 to 11).
Relation between disease activity and faecal mucin sulphatase activity

Patients with active ulcerative colitis (n=12) had a significantly higher faecal mucin sulphatase activity (median 146 units/g pellet weight, 95% CI: 98 to 253) compared with those with inactive disease (n=10) (median 42-2 units/g pellet weight; 95% CI: 22-5 to 81-6) (p<0.05, Wilcoxon’s rank sum test). Faecal sulphatase activity in inactive ulcerative colitis was significantly greater than in healthy controls (median 11.3; 95% CI: 8-7 to 29.8, n=17, p<0.05). Sulphatase activity also correlated well with the more detailed St Mark’s Hospital clinical scoring described by Powell-Tuck et al.\textsuperscript{10} (r=0.41; p<0.01, Kendall’s rank correlation) (Fig 2). There was a significant positive correlation between sigmoidoscopic score and faecal mucin sulphatase activity (Kendall’s t=0.45, p<0.01) (Fig 3). Of the ulcerative colitis group studied, only three patients were not receiving sulphasalazine and this was too few for subgroup analysis.

There was no correlation between disease activity and faecal mucin sulphatase activity in Crohn’s disease. Faecal sulphatase activity of those with colonic Crohn’s disease (median 11.0; range 5.7–96 units/g pellet weight, n=6) did not differ significantly from those without colonic Crohn’s disease (median 68; range 6.9–106 units/g, n=8), however, the numbers in each group were small.

Longitudinal study

Seven patients with ulcerative colitis were followed up with serial faecal samples analysed for mucin sulphatase activity. Figure 4 shows the faecal sulphatase activity over the time course and disease activity. In six patients the sulphatase activity closely mirrors clinical disease activity.

Effect of leucocyte preparations and serum on $^{35}$S mucin

The removal of $^{35}$S sulphate groups from $^{35}$S mucin by faecal extract, serum, polymorphonuclear leucocyte, and mononuclear cell preparations compared with buffer alone was assessed. The faecal extract removed 13% of the sulphate groups but there was negligible mucin sulphatase activity detected in the serum and leucocyte preparations.

Inhibitor studies

Bismuth subcitrate was assayed for its effects on the sulphatase using nine substrate concentrations (5, 6-25, 8-33, 12-5, 15, 25, 50, 100×10$^3$ dpm/ml) to determine its inhibitor kinetics. Bismuth subcitrate inhibits mucin sulphatase and the kinetic profile of this inhibitor best fits a non-competitive model of enzyme inhibition. The inhibitor constant K$_{\text{EI}}$ derived from the Dixon plot\textsuperscript{18} was 1.5 mmol/l. The concentration/effect relation was assessed by incubation of sulphatase with five different concentrations of bismuth subcitrate (0–8 g/l; 0–14.1 mmol/l) (Fig 5A). Sulphatase activity was inhibited 50% by bismuth subcitrate (that is, E$_{50}$) 1.09 mmol/l and maximal inhibition in excess of 95% was caused by bismuth subcitrate 13 mmol/l (Fig 5B).

Bismuth subsaliclyate in a suspension of 4 g/l inhibited 95% of mucin sulphatase activity when continuously agitated. Because of the insolubility of the drug, however, it was not possible to determine its K$_{\text{EI}}$ with any certainty or to ascertain its inhibitor characteristics. The concentration of this suspension is similar to that used therapeutically in bismuth subsaliclyate enemas.\textsuperscript{19}

Both low molecular weight varieties of sulphated dextrans caused 90% inhibition of mucin sulphatase activity at a concentration of 5% wt/wt. Their inhibition kinetic characteristics best fit a non-competitive model. High molecular weight sulphated dextran inhibits 94% of mucin sulphatase activity at a concentration of 5% wt/wt. Its kinetic characteristics best fit a competitive model.

Discussion

This study shows that there is increased faecal mucin sulphatase activity in patients with ulcerative colitis. Patients with Crohn’s disease also had a slightly raised faecal mucin sulphatase activity but this did not achieve statistical significance. Subgroup analysis showed no difference between Crohn’s colitis and small bowel Crohn’s disease. This finding is of interest as it makes it less likely that an increase
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in faecal mucin sulphatase merely reflects some non-specific inflammatory activity in the colon. In ulcerative colitis the faecal sulphatase activities were highest in those with active disease. To minimise error resulting from faecal dilution by diarrhoea and increased inflammatory debris in faeces of patients with inflammatory bowel disease we have expressed the results in three ways, as units/g faecal pellet weight, units/mg protein content, and as ratio of sulphatase/\( \alpha \) glucosidase activity. Similar results were obtained using each method. \( \alpha \) glucosidase is expressed by most colonic flora and therefore reflects faecal bacterial load.\(^{20}\)

The increased sulphatase/\( \alpha \) glucosidase ratio therefore implies a specific increase in sulphatase production or sulphatase producing bacteria rather than a non-specific increase in bacterial activity.

Human serum and leucocytes have been shown not to contain mucin sulphatases. Aryl sulphatases A and B are found in human leucocytes,\(^{21}\) but these do not seem to be active on sulphated human colonic mucin.

There may be two possible explanations for the increased faecal sulphatase found in patients with active ulcerative colitis. The first is that an increase in faecal mucin sulphatase activity is secondary to the disease process. If the mucin sulphatase is of bacterial origin, then perturbations of the faecal flora could account for an isolated proliferation of sulphatase producing bacteria. Such perturbations may be as a result of a changed luminal environment such as increased faecal lactate.\(^{22}\) Most workers, however, have reported no significant differences in the faecal flora of patients with ulcerative colitis compared with controls.\(^{23,24}\)

The second possible explanation is that in some patients this increase in faecal sulphatase activity itself contributes to the colitic process by increasing mucin degradation and thus upsetting the balance between mucus production and bacterial degradation. Enzymatic desulphuration might well be the rate limiting step in colonic mucin degradation. This could be compounded by mucus changes such as the reduced sulphation that has been shown in cultured mucosal explants from patients with ulcerative colitis\(^{25}\) and by the increased susceptibility of ulcerative colitis mucin to degradation by faecal extracts that has been reported by others.\(^{26}\) It would still be necessary to postulate an external environmental factor that triggers relapse of colitis, perhaps a gastroenteritic infection.\(^{27}\) This explanation is still highly speculative and would require further careful, prospective studies.

The faecal mucin sulphatase has previously been shown to be probably mainly of bacterial origin, particularly *Bacteroides* ssp.\(^{28}\) A similar mucin sulphatase has also been isolated from a *Prevotella* strain (RS2) isolated from pig colonic mucosa.\(^{29}\) A change in the colonic flora by antibiotics might be expected to change the course of ulcerative colitis if this enzyme has a role in the pathogenesis. Intravenous metronidazole\(^{30}\) and oral vancomycin\(^{31}\) have so far not been shown to be effective treatment in ulcerative colitis. Oral tobramycin has been shown in one study, however, to produce promising results when given in combination with corticosteroids.\(^{32}\) None of these antibiotic regimens are capable of sterilising the colon\(^{30}\) but permit different bacterial species to proliferate preferentially. The possible change in faecal mucin sulphatase activity by antibiotics would clearly require further study.

Previous assessment of faecal sulphatase activity in inflammatory bowel disease has been attempted using high iron diamine histochemical staining as a semi-quantitative estimate of mucin sulphatase.\(^{33}\) This showed that high iron diamine staining was often lost after treatment of rectal mucosal sections with bacteria free faecal extracts but failed to show any significant increase in this effect in ulcerative colitis. It is probable, however, that high iron diamine staining, which is dependent on charge, may be affected by other changes in mucin such as sialylation.\(^{34}\) This is supported by the discrepancy between the pattern of staining seen in the normal rectum, where high iron diamine positivity is confined to the lower crypts in contrast with autoradiography after mucosal culture in the presence of \(^{35}S\)O\(_4\), which shows incorporation of sulphate into mucin, although not necessarily into mucin, throughout the crypts.\(^{35}\)

Increased faecal sulphatase activity has also been shown in ulcerative colitis using lactitol 6-sulphate as substrate\(^{26}\) but it is probable that the enzymes that act on this substrate are different from those that act on the sulphated residues in mucin.\(^{29}\)

Longitudinal studies showed that mucin sulphatase activity mirrored relapse and remission of the disease in six of seven patients in this study. Whether or not this increase in sulphatase activity is the cause or the result of the relapse, it is likely to contribute to the perpetuation of the disease by increasing mucin depletion and leaving the mucosa vulnerable to damage by toxic colonic contents.

The bacterial sulphatase, because of its critical role in determining the rate of degradation of secreted mucus, is a primary target for the development of new therapeutic agents for colitis. The sulphated dextrans are not appropriate for intraluminal treatment, because like other sulphated polysaccharides (for example, carrageenan), they tend to cause colitis.\(^{36}\) The explanation for the difference between the competitive inhibition seen with high molecular weight dextran and the non-competitive inhibition with lower molecular weight dextrans is unclear; but it is possible that the higher molecular weight dextrans mimic sulphated mucin and so are an alternative substrate for the sulphatase. Further experiments with appropriately labelled dextran sulphate would be required to assess this. Bismuth salts like other heavy metals, act as non-specific enzyme inhibitors, and are shown here to be effective sulphatase inhibitors and have been shown to have promising effects when used in patients with corticosteroid resistant colitis.\(^{19}\)

Faecal mucin sulphatase is probably critical in determining the rate of degradation of secreted mucus and the increased concentrations in ulcerative colitis, whether a primary
phenomenon or secondary to disease, are probably relevant to the reduction in mucus gel thickness that has recently been reported.\textsuperscript{37} Conversely, the comparative lack of increased faecal sulphate in Crohn's disease is in keeping with the comparatively thick mucus layer\textsuperscript{37} and the comparative lack of histological mucus depletion\textsuperscript{18} that help distinguish Crohn's disease from ulcerative colitis.

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