Evaluating the antioxidant potential of new treatments for inflammatory bowel disease using a rat model of colitis

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Abstract

Background—Reactive oxygen species may mediate tissue injury in inflammatory bowel disease. Aminosalicylates have antioxidant activity and the antioxidants, superoxide dismutase and allopurinol, are of reported benefit in inflammatory bowel disease.

Aim—To develop a convenient technique for testing the antioxidant potential of standard and novel therapeutic agents for use in inflammatory bowel disease.

Methods—Amplified chemiluminescence was used to measure reactive oxygen species production by colonic biopsy specimens from rats with acetic acid induced colitis and to assess the *in vitro* effect of conventional antioxidants, standard therapies and proposed novel therapies for inflammatory bowel disease.

Results—The model was validated by demonstrating that the profile of effects on chemiluminescence of acetic acid induced colitis biopsy specimens given by conventional antioxidants (sodium azide, catalase, copper-zinc superoxide dismutase, dimethyl sulphoxide, *N*-acetyl-cysteine and ascorbate) and standard therapies (5-aminosalicylate and hydrocortisone) resembled that previously reported using biopsy specimens from ulcerative colitis. Human recombinant manganese superoxide dismutase did not alter chemiluminescence. Two novel compounds, LY231617 (10 mM) and amflutizole (20 mM), reduced chemiluminescence by 98% ($n=5$, $p=0.009$) and 88% ($n=5$, $p=0.03$), respectively.

Conclusions—The similarity of the chemiluminescence responses of colonic biopsy specimens from acetic acid induced colitis and ulcerative colitis to a range of conventional antioxidants and standard treatments suggests that this model is a useful method for testing the antioxidant potential of new therapies for inflammatory bowel disease. The antioxidant actions of dimethyl sulphoxide, ascorbate, and the novel compounds, amflutizole and LY231617 in this model suggest that these agents merit further assessment in the treatment of inflammatory bowel disease.

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It is now well recognised that reactive oxygen species (ROS) such as superoxide ($O_2^{\cdot-}$), the hydroxyl radical ($OH^{\cdot}$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and oxidant derivatives, such as *N*-chloramines (RNHCl), are produced in excess by the inflamed mucosa in inflammatory bowel disease (IBD) and may be pathogenic.^{1–4} The predominant sources of ROS in the inflamed mucosa are probably activated mucosal phagocytic leucocytes⁵ and episodes of ischaemia reperfusion⁶ (Fig 1). Increased arachidonic acid metabolism⁷ may also lead to excess mucosal ROS production. The resulting oxidant stress may overwhelm the endogenous defences that regulate ROS production during normal metabolism, particularly if there are comparatively low tissue levels of endogenous antioxidants, as in the colonic mucosa.⁸ Consequent tissue injury by ROS⁹ results from their direct reaction with carbohydrates, lipids, proteins, and DNA, stimulation of phospholipase A_2 ,¹⁰ 5-lipoxygenase,¹¹ and neutrophil chemotaxis,¹² and activation of transcription factors involved in cytokine release and cell growth, such as NF κ B,¹³ *c-fos*, *c-myc* and *c-jun*.¹⁴

It has been suggested that the efficacy of current standard treatments is related to their antioxidant actions.⁴ 5-ASA is a potent antioxidant and reduces mucosal ROS production by inflamed human colorectal biopsy tissue *in vitro*,¹ as well as mucosal lipid peroxidation in ulcerative colitis (UC) *in vivo*.¹⁵ Hydrocortisone and other glucocorticoids do not directly scavenge ROS but their anti-inflammatory effects include inhibition of neutrophil function.¹⁶

Recent, albeit uncontrolled, trials have suggested that specific antioxidant treatment may be therapeutically effective in IBD. In corticosteroid resistant Crohn's disease (CD) a combination of superoxide dismutase and desferrioxamine was reportedly effective,¹⁷ while allopurinol has been used successfully in acute and chronic pouchitis.¹⁸ Antioxidants have been used successfully in experimental models of intestinal inflammation.^{19–20} Compounds with antioxidant activity should therefore be investigated as potential treatments for IBD. There are many compounds with known antioxidant activity and many new potential therapies whose ability to reduce ROS production by inflamed colon is unknown. It would therefore be useful to have a method for screening potential treatments for this action.

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Figure 1: Schematic representation of the major sources of ROS production by the inflamed mucosa in inflammatory bowel disease. Enzymes are shown in *italics* and three examples of exogenous scavengers in plain text. Superoxide produced by activated neutrophils or ischaemia reperfusion dismutates to hydrogen peroxide, catalysed by superoxide dismutase. Superoxide reduces Fe^{3+} to Fe^{2+} thus facilitating the Haber-Weiss reaction by which hydroxyl ions are produced. Hydrogen peroxide is catalysed by myeloperoxidase to hypochlorous acid.

Recent findings from this laboratory have shown that ROS production by rectal biopsy specimens from patients with UC and CD can be measured in vitro using a chemiluminescence technique, and that addition of conventional antioxidants reduces the measurable ROS.¹ Biopsy material from patients with active UC is, however, too limited for screening multiple compounds.

Acetic acid induced colitis in rats resembles UC in histology, eicosanoid production,²¹ and response to sulphasalazine.²² Inflamed colonic mucosa in acetic acid induced colitis is also known to produce excess ROS and antioxidants will reduce measurable ROS²³; in these studies, however, mucosal scrapings rather than biopsy specimens were used and no comparison was made with human material, thus limiting the comparability with IBD.

This study used full thickness colonic biopsy specimens from acetic acid induced colitis and validated the screening technique by comparing results with previous studies using biopsy specimens from patients with active UC, in which the conventional antioxidants (sodium azide, taurine, dimethyl sulphoxide, *N*-acetylcysteine, ascorbate and the enzymes catalase and CuZn superoxide dismutase) and two standard therapies for IBD (5-amino-salicylate (5-ASA) and hydrocortisone), were evaluated.^{1 24 25} In addition, we studied the effects of potential new antioxidant approaches with human recombinant manganese superoxide dismutase (Rh-Mn superoxide dismutase) and two novel antioxidants, LY231617 and amflutizole, whose structures are shown in Figure 2.

Methods

REAGENTS

All chemical reagents were of analytical grade and were obtained from Sigma Chemical,

Figure 2: The chemical structures of amflutizole and LY231617.

Poole, Dorset, unless otherwise stated. The CuZn superoxide dismutase was from human erythrocytes (specific activity: 3610 U/mg protein). Catalase was from bovine liver attached to 4% agarose beads (specific activity: 150 000–200 000 U/g agarose). Rh-Mn superoxide dismutase (specific activity: 3500 U/mg protein) was provided by Bender, GesmbH, Vienna, Austria. Amflutizole (3-(3-trifluoromethyl)-4-aminoisothiazole-5-carboxylic acid) and LY231617 (2,6-bis(1,1-dimethylethyl)-4-[(1-ethyl)amino]methylphenol hydrochloride) (Fig 2) were obtained from Lilly Research Laboratories, Indianapolis, USA.

Table I shows the compounds used and their postulated mechanisms of antioxidant activity.

Luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) was maintained as stock solution (50 mg in 1 ml of DMSO) for up to one month. Luminol and lucigenin (*N,N'*-dimethyl-9,9'-biacridinium dinitrate) were prepared prior to the experiment in Dulbecco's phosphate buffered saline (D-PBS) with added calcium (1.13 mM) and glucose (5 mM) and oxygenated for 10 minutes with 95% O_2 , 5% CO_2 to maintain tissue viability, following which the pH was adjusted to 7.4 with NaOH or HCl (1M).

INDUCTION OF EXPERIMENTAL COLITIS IN RATS
Acetic acid induced colitis was induced in male Wistar rats (150–200 g, Charles River, UK) using a modification of the method described by MacPherson and Pfeiffer.²⁶ The animals were fasted for 16 hours with access to water ad libitum. Each rat was sedated by brief respiration of 20–25% CO_2 followed by anaesthesia with 35 mg/kg intraperitoneal pentobarbital. An infant feeding tube (Pennine Healthcare FT-1608/40, outside diameter 2 mm) was inserted into the colon to 8 cm and 2 ml of acetic acid (3% v/v in 0.9% saline) or saline alone (control animals) infused into the colon. The acetic acid/saline was retained in the colon for 30 seconds, after which fluid was withdrawn. The rats were killed at 24 hours by CO_2 asphyxiation.

ASSESSMENT OF COLITIS

Macroscopic scoring

At postmortem laparotomy, 6 cm of colon extending proximally from 2 cm above the anal margin was removed, split longitudinally, pinned out on card, and the macroscopic

TABLE I Study compounds

Drug/compound (study concentration)	Antioxidant action (reference)	Soluble in aqueous solution	Chemiluminescence amplifier	Control solution
<i>Conventional antioxidants</i>				
Sodium azide (1 mM)	‡ Myeloperoxidase ⁵⁵ /OH• ³⁴	+	Luminol	D-PBS
Catalase (3000 U/ml)	‡ H ₂ O ₂	+	Luminol	HI* enzyme
Taurine (20 mM)	‡ HOCl ⁵⁶ , OH• ⁵⁷	+	Luminol	D-PBS
CuZn superoxide dismutase (30/300 U/ml)	‡ O ₂ ^{•-}	+	Lucigenin	HI* enzyme
Dimethylsulphoxide (1/5/10%)	‡ OH• ⁵⁸	+	Luminol	D-PBS
N-Acetylcysteine (20 mM)	‡ Glutathione peroxidase/‡ HOCl ⁴⁶	+	Luminol	D-PBS
Ascorbate (20 mM)	‡ HOCl ⁴⁷ /thiyl/peroxyl/O ₂ ^{•-} ⁴⁶	+	Luminol	D-PBS
<i>Standard treatments in IBD</i>				
5-ASA (1/10/20 mM)	‡ OH• ⁵⁹ /O ₂ ^{•-} ⁶⁰ /HOCl ⁶¹ /Fe chelator ⁶²	+	Luminol	D-PBS
Hydrocortisone (1 mM)	‡ Neutrophil function ¹⁶	+	Luminol	D-PBS
<i>Proposed treatments in IBD</i>				
Rh-Mn superoxide dismutase (300 U/ml)	‡ O ₂ ^{•-}	+	Lucigenin	HI* enzyme
Amflutizole (1/10/20 mM)	Inhibits xanthine oxidase ⁵²	–	Luminol	D-PBS
LY231617 (1/10 mM)	Not known	–	Luminol	D-PBS

*=Heat inactivated, ‡=scavenges or inhibits, †=stimulates. All compounds and controls were dissolved in Dulbecco's phosphate buffered saline (D-PBS). For most compounds control was D-PBS alone.

appearances of the colonic mucosa scored on a scale adapted from Morris *et al*²⁷ ranging from 0–4: (0) no macroscopic change, (1) mucosal erythema alone, (2) mild mucosal oedema, slight bleeding or small erosions, (3) moderate oedema, bleeding ulcers or erosions, (4) severe ulceration/erosions, oedema, and tissue necrosis. Intermediate values reflected intermediate appearances.

Microscopic scoring

In some experiments randomly distributed full thickness biopsy specimens were fixed in 10% formol saline prior to wax embedding, sectioning, and staining with haematoxylin and eosin. Biopsy specimens (n=27) from 14 animals were scored by a histopathologist blinded to the macroscopic score and chemiluminescence responses of the sections, using the scoring system shown in Table II.

CHEMILUMINESCENCE ASSAY

Luminol and lucigenin react with oxidants, such as ROS, to form 3-aminophthalate and N-methylacridone, respectively. Electrons in the luminol and lucigenin are raised to higher energy levels during the reaction with oxidants. On reverting to the ground state, energy is

released in the form of photons, which are detected by the photomultiplier tubes and photon detectors that comprise the scintillation counter.²⁸ Lucigenin responds more specifically to superoxide than luminol²⁹ and was therefore used to detect changes in chemiluminescence induced by superoxide dismutase. Luminol and lucigenin were used at the same concentration (300 μM) as used in previous studies using human biopsy specimens.¹

Full thickness biopsy specimens were taken at random by cutting cross sectional strips (median (IQR) wet weight; 44 (31–78) mg) of the dissected, inflamed colon. Biopsy specimens from colons of macroscopic score ≥2 were used for drug studies. Such samples were initially placed in pre-oxygenated (95% O₂, 5% CO₂ for 10 minutes) D-PBS, with added calcium (1.13 mM) and glucose (5 mM) at ambient temperature and then transferred to 300 μM luminol or lucigenin immediately prior to assessment of the chemiluminescence response, which was measured in a Packard Tri Carb 1600 CA liquid scintillation counter analyser operated in 'the out-of-coincidence' mode for two minutes.

ASSESSMENT OF EFFECTS OF TEST COMPOUNDS ON CHEMILUMINESCENCE RESPONSE

All compounds and appropriate controls were prepared prior to the experiment and adjusted to pH 7.4 by addition of 1M NaOH or HCl. Controls for these experiments were vehicle (D-PBS) for all compounds except CuZn superoxide dismutase, Rh-Mn superoxide dismutase, and catalase, in which heat inactivated enzyme was used (Table I). Heat inactivated enzymes were prepared by heating in D-PBS at 100°C for two hours.³⁰

Water soluble compounds

The effect of test compounds on the chemiluminescence response of rat acetic acid induced colitis biopsy specimens was compared with the response of a paired biopsy to the control for that compound. The chemiluminescence response was measured in 1.8 ml

TABLE II Histological assessment of full thickness biopsy specimens from acetic acid colitis in rats

<i>Neutrophil infiltrate</i>			
None 0	Slight increase 1	Marked increase 2	
	Epithelium		(0–2)
	Lamina propria		(0–2)
	Muscularis mucosa		(0–2)
	Submucosa		(0–2)
	Muscularis propria		(0–2)
	Serosa		(0–2)
<i>Fibrin deposition</i>			
Absent 0	Present 1		
	Mucosa		(0–1)
	Submucosa		(0–1)
<i>Submucosal neutrophil margination</i>			
Absent 0	Present 1		(0–1)
<i>Submucosal oedema</i>			
Nil 0	Patchy 1	Confluent 2	(0–2)
<i>Epithelial necrosis</i>			
Nil 0	Localised 1	Extensive 2	(0–2)
<i>Epithelial ulceration</i>			
Absent 0	Present 1		(0–1)
Maximum score			20

of 300 μ M luminol or lucigenin and then immediately repeated after addition of 200 μ l of the test compound, at 10 times the final concentration, or appropriate control. After counting, samples were blotted, weighed, and in some experiments, placed in 10% formol saline for subsequent histological examination.

Water insoluble compounds

LY231617 and amflutizole were insoluble in aqueous solution at the required concentrations. Suspensions were therefore prepared in 10% acacia dissolved in D-PBS, oxygenated for 10 minutes in 95% O₂, 5% CO₂ and adjusted to pH 7.4 with 1M NaOH. Acacia is a demulcent gum used to suspend or emulsify water insoluble pharmacological agents. Oxygenation of these suspensions was required as the biopsy specimens were transferred to the acacia suspensions between the chemiluminescence counts rather than remaining in the vial as with the water soluble agents. After the initial chemiluminescence count, as described above, five specimens were placed together in 10% acacia with or without the addition of the test compound in suspension. The specimens were grouped to avoid disparity in dosage exposure due to heterogeneity in the suspension. Incubation was carried out for 30 minutes in a shaking water bath at 37°C. At the end of this time the samples were removed, washed twice in oxygenated D-PBS to remove excess acacia, and luminol amplified chemiluminescence recounted. As a comparator, 5-ASA was also assessed in acacia.

CALCULATIONS AND STATISTICS

Chemiluminescence response of biopsy specimens

Luminol and lucigenin amplified chemiluminescence is expressed as the number of photons/min/mg wet weight of tissue after subtraction of the background count. The background count was taken as the average photons/min for two vials of each 12 analysed containing 300 μ M luminol or lucigenin in oxygenated D-PBS alone. Comparisons between groups of untreated control animals, saline treated animals, and acetic acid induced colitis were made using the Kruskal-Wallis and Dunn's multiple comparison tests.

To assess the variability of the chemiluminescence response of colonic biopsy specimens from acetic acid induced colitis, the coefficient of variation was calculated from the chemiluminescence response in luminol produced by two to six biopsy specimens from each of 22 rats.³¹ Comparisons between animals are expressed as the median and interquartile range (IQR) of the coefficient of variation. A similar calculation for the response to lucigenin was made in two to six specimens from each of 10 animals.

The macroscopic appearances, the histological score, and the chemiluminescence response were compared using Spearman's rank correlation coefficient.

Effect of water soluble compounds

For each compound at each concentration a pair of biopsy specimens from each of ≥ 5 rats was used to assess the percentage change in chemiluminescence. The chemiluminescence counts before and after addition of test compound are given as t_1 and t_2 , respectively, and for the specimen treated with control, c_1 and c_2 , respectively. To take account of small changes in control values during the assay, the change in chemiluminescence produced by the test compound on one biopsy specimen was adjusted by the change in chemiluminescence produced by the control solution with its pair. Accordingly, the percentage change in chemiluminescence response induced by the test compound ($\Delta\%t$) is calculated as follows: $\Delta\%t = ((t_2 \times c_1 / c_2) - t_1) \times 100$.

The results are expressed as median % (IQR). The difference in chemiluminescence response between biopsy specimens treated with test compounds and controls is calculated by comparing the percentage change in chemiluminescence induced by the test agent and control using the Wilcoxon signed rank test for paired variables. A p value of less than 0.05 (two tailed) was taken as significant.

To investigate the variability of the change in chemiluminescence response produced by vehicle alone, the coefficient of variation of the response to D-PBS (control for most of the compounds tested) was calculated for two to six biopsies from each of 10 animals and the median (IQR) expressed for the coefficient of variation of the luminol amplified chemiluminescence response to D-PBS in all 10.³¹

Effect of water insoluble compounds

Results are expressed as the mean percentage change in chemiluminescence for each group of biopsy specimens exposed to the test compound after correction of the initial count for the percentage change induced by acacia alone (control). No range is given because grouping the biopsy specimens together resulted in loss of animal identity. The chemiluminescence counts before and after exposure to the test compound were compared using the Mann-Whitney U test. A p value of less than 0.05 (two tailed) was taken as significant.

To investigate the variability of the chemiluminescence response induced by incubation of biopsy specimens in 10% acacia, the median coefficient of variation was calculated for eight groups of five biopsies from five rats incubated in 10% acacia alone that were used as controls in experiments assessing the effect of water insoluble compounds.

Results

VALIDATION OF THE ACETIC ACID INDUCED COLITIS MODEL

Chemiluminescence response in acetic acid induced colitis compared with controls

We compared the chemiluminescence response of 71 colonic biopsy specimens from 23 rats

with acetic acid induced colitis to eight non-inflamed colonic biopsy specimens from four rats given intracolonic 0.9% saline and 22 biopsy specimens from 10 untreated control rats (Fig 3). The median (IQR) for each of these groups was 17 449 (6498–38 880) photons/min/mg, 74 (0–154), and 0 (0–66) respectively. For lucigenin amplified chemiluminescence, 26 colonic biopsy specimens from 10 rats with acetic acid induced colitis were compared with eight specimens from four rats given 0.9% saline and to 10 specimens from seven untreated controls (Fig 3). The median (IQR) for these groups was 819 (518–1471) photons/min/mg, 347 (157–759), and 143 (48–227), respectively. Biopsy specimens from acetic acid induced colitis produced significantly more luminol amplified chemiluminescence than specimens from saline treated and untreated controls and more lucigenin amplified chemiluminescence than untreated controls ($p < 0.001$) (Fig 3). There was no difference in the chemiluminescence response of colonic tissue from the two control groups.

Chemiluminescence related to the severity of inflammation

To assess the relation between ROS production as detected by chemiluminescence and inflammation in the acetic acid induced colitis model we compared the luminol amplified chemiluminescence response of full thickness biopsy specimens with the macroscopic colonic appearances and the histological grade of inflammation in rats with acetic acid induced colitis. Figures 4 and 5 show significant positive correlations between the macroscopic and histological scores, respectively, of

Figure 3: Log transformed luminol and lucigenin amplified chemiluminescence counts in full thickness colonic biopsy specimens from rats pre-treated with 3% intracolonic acetic acid (AAC) or 0.9% saline (Saline), assessed at 24 hours and in untreated control animals (Control). For luminol 71 colonic biopsy specimens from 23 rats with acetic acid induced colitis were compared with eight colonic biopsy specimens from four rats given intracolonic 0.9% saline and 22 biopsy specimens from 10 untreated controls. For lucigenin amplified chemiluminescence, 26 specimens from 10 animals with acetic acid induced colitis were compared with eight specimens from four animals given 0.9% saline and 10 specimens from seven untreated controls. * $p < 0.001$, Dunn's multiple comparison test.

Figure 4: Correlation of luminol amplified chemiluminescence with the macroscopic score in 71 biopsy specimens from 23 rats pre-treated with 3% acetic acid induced colitis at 24 hours. Data shown as macroscopic score against the log of the chemiluminescence, showing the regression line, $y = 0.3x + 3.4$, $r = +0.5$, $p = 0.0001$, Spearman's rank correlation coefficient.

inflamed rat colons and the chemiluminescence response of tissue from the same specimens. The macroscopic and histological score were also positively correlated ($r = +0.7$, $p = 0.0001$; data not shown).

Variability of the initial chemiluminescence response

The median (IQR) of the coefficient of variation of the initial chemiluminescence response of two to six specimens from 22 rats, with acetic acid induced colitis, to luminol was 66 (39–83)% and of two to six specimens from 10 rats to lucigenin was 29 (17–49)%. These variations are small in comparison to the percentage increase in luminol and lucigenin amplified chemiluminescence response of acetic acid induced colitis biopsy specimens compared with control (mean, +20 000% and +500%, respectively).

Variability of the change in chemiluminescence after addition of Dulbecco's PBS

The coefficient of variation of the change in luminol amplified chemiluminescence resulting from incubation in D-PBS (control for most of the experiments) calculated from the

Figure 5: Correlation of luminol amplified chemiluminescence with the histological score (see Table II) in 27 biopsy specimens from 14 rats pre-treated with 3% acetic acid induced colitis at 24 hours showing the regression line, $y = 0.1x + 3.5$, $r = +0.7$, $p = 0.0004$, Spearman's rank correlation coefficient.

10 animals in which there were two or more control experiments was 18 (11–41)%.

Variability of the change in chemiluminescence after incubation of acetic acid induced colitis biopsy specimens in 10% acacia

The coefficient of variation for the change in chemiluminescence of eight groups of five biopsy specimens each, incubated in 10% acacia alone (controls), was 48%, which reflects the variability of this system compared with incubation in D-PBS.

COMPARISON OF RESPONSE OF BIOPSY SPECIMENS FROM ACETIC ACID INDUCED COLITIS AND UC TO CONVENTIONAL ANTIOXIDANTS

Significant reductions in luminol amplified chemiluminescence in the acetic acid induced colitis model were observed with sodium azide (–83 (–85 to –73)% $p=0.03$), catalase (–43 (–47 to –41)% $p=0.03$), 10% (1.28 M) DMSO (–67 (–78 to –50)% $p=0.03$), and ascorbate (–54 (–69 to –35)% $p=0.01$) and in lucigenin amplified chemiluminescence with CuZn superoxide dismutase (–26 (–32 to –15)% $p=0.03$) (Fig 6). *N*-acetylcysteine produced no significant change in luminol amplified chemiluminescence. The profile of responses in acetic acid induced colitis demonstrate a striking resemblance to that previously seen in UC.^{1 24 25} With one compound, taurine, a small, but significant, reduction was produced in UC biopsy specimens¹ but not in acetic acid induced colitis.

COMPARISON OF RESPONSE OF BIOPSY SPECIMENS FROM ACETIC ACID INDUCED COLITIS AND UC TO STANDARD TREATMENTS

As for UC specimens, 5-ASA produced a significant reduction in luminol amplified

chemiluminescence response in acetic acid induced colitis biopsy specimens (maximum –88 (–89 to –70)% at 20 mM 5-ASA, $p=0.03$) with a limited dose response producing an estimated IC_{50} of 4 mM (Fig 7). Hydrocortisone did not alter luminol amplified chemiluminescence in either acetic acid induced colitis or UC biopsy specimens²⁴ (Fig 7).

RESPONSE OF BIOPSY SPECIMENS FROM ACETIC ACID INDUCED COLITIS TO POTENTIAL NEW TREATMENTS FOR IBD

Rh-Mn superoxide dismutase had no demonstrable antioxidant activity in this system, altering the lucigenin amplified and luminol amplified chemiluminescence response, respectively by –9 (–17 to +3)% and +17 (+10 to +37)%, respectively. However, two novel water insoluble antioxidant compounds, LY231617 (10 mM) and amflutizole (20 mM), produced noticeable reductions in luminol amplified chemiluminescence after incubation of the specimens in suspensions of the compounds in 10% acacia (means, –98%, $p=0.009$ and –88%, $p=0.028$, respectively) (Fig 8). 5-ASA (20 mM), which had previously been shown to reduce luminol amplified chemiluminescence in aqueous solution, reduced the chemiluminescence by a similar amount (mean, 83% $p=0.002$) when tested in acacia.

Discussion

These findings show that full thickness colonic biopsy specimens from rats with acetic acid induced colitis produce increased levels of ROS, as detected by amplified chemiluminescence, compared with control tissue. In addition, we have found that the level of ROS production detected by luminol amplified chemiluminescence correlates with the grade

Figure 6: The percentage change in chemiluminescence response of inflamed biopsy specimens from acetic acid induced colitis to conventional antioxidants compared with results using mucosal biopsy specimens from active UC (†data redrawn from references^{1 24 25}). Chemiluminescence was measured before and after exposure to test compound and compared with a second biopsy specimen exposed to vehicle ($n \geq 5$; *denotes $p < 0.05$ drug compared with vehicle, Wilcoxon signed rank test). Luminol was used as the amplifier for chemiluminescence for all compounds except CuZn superoxide dismutase, for which lucigenin was used. NAC=*N*-acetylcysteine.

Figure 7: The percentage change in the luminol amplified chemiluminescence response of inflamed biopsy specimens from acetic acid induced colitis to standard treatments in IBD compared with results using mucosal biopsies from active UC (†data redrawn from references^{1, 24, 25}). Chemiluminescence was measured before and after exposure to test compound and compared with a second biopsy specimen exposed to vehicle ($n \geq 5$; *denotes $p < 0.05$ drug compared with vehicle, Wilcoxon signed rank test).

of mucosal inflammation, as assessed by the macroscopic and histological score. These findings confirm previous studies using mucosal scrapings from rat acetic acid induced colitis.²²

We have now shown that colonic biopsy specimens from the acetic acid induced colitis model respond to conventional antioxidants and standard treatments for IBD, 5-ASA, and hydrocortisone, in a similar fashion to mucosal specimens from patients with active UC.^{1, 24, 25} The estimated IC_{50} of 5-ASA in this model compares favourably with the intraluminal concentrations recorded after oral administration of aminosalicylates (5–10 mM) in UC.³² Hydrocortisone had no antioxidant

Figure 8: Mean percentage change in luminol amplified chemiluminescence response of inflamed biopsy specimens from acetic acid induced colitis to proposed new treatments, LY231617, amflutizole, and to 5-ASA. Chemiluminescence was measured before and after incubation of colonic biopsy specimens in a suspension of the test compound in 10% acacia for 30 minutes and compared with specimens exposed to acacia alone ($n = 5$; *denotes $p < 0.05$ drug compared with vehicle, Mann-Whitney U test).

activity in the present short-term in vitro experiments in this model or with UC biopsy specimens.²⁴ However, other studies, using prolonged incubation with dexamethasone in vitro, have shown inhibition of superoxide generation by stimulated phagocytes³³; the therapeutic effect of hydrocortisone in vivo may thus be partly due to an indirect antioxidant action by inhibition of neutrophil function.¹⁶

Of the conventional antioxidants examined, the greatest inhibition occurred with azide, a potent inhibitor of myeloperoxidase,²⁸ which also inhibits other haem proteins such as catalase and quenches singlet oxygen and the hydroxyl ion.³⁴ Although the antioxidant action of azide could result from a cytotoxic effect, azide has been shown to reduce the luminol amplified chemiluminescence of stimulated neutrophils to a greater effect when added before the stimulus (100%) than after (20%),³⁵ an effect that would not occur if the reduction in chemiluminescence was purely due to cytotoxicity.

Superoxide is produced by the activated neutrophil, partly as the result of NADPH activity,³⁶ and as a result of episodes of ischaemia reperfusion, which may be important in the pathogenesis of IBD³⁷ (Fig 1). Superoxide generation has been located to the epithelium, vascular endothelium, and infiltrating mononuclear cells in inflamed UC mucosa³⁸ and levels of superoxide dismutase are reduced in inflamed intestinal resection tissue from patients with IBD.³⁹ Administration of exogenous superoxide dismutase is of benefit in vivo in the acetic acid induced colitis model¹⁹ and there is preliminary, uncontrolled, clinical evidence that liposomal encapsulated CuZn superoxide dismutase may be of benefit in CD¹⁷ and UC.⁴⁰ The present studies using acetic acid induced colitis biopsy specimens support previous findings showing that CuZn superoxide dismutase reduces lucigenin amplified chemiluminescence by mucosal biopsy specimens in vitro from UC.²⁴

Rh-Mn superoxide dismutase has theoretical advantages over CuZn superoxide dismutase as a potential therapeutic agent for IBD because of its longer half life⁴¹ and its human origin should lessen the risk of in vivo antigenicity. In two models of chronic inflammation, adjuvant induced arthritis and bleomycin induced lung damage, Rh-Mn superoxide dismutase was shown to be more effective than CuZn superoxide dismutase.⁴² Mn superoxide dismutase has been shown to reduce leucocyte adhesion in a model of intestinal ischaemia⁴³ and, in high dose, to improve trinitrobenzene sulphonic acid/ethanol induced colitis.²⁰ The failure of Rh-Mn superoxide dismutase to reduce ROS and production by acetic acid induced colitis biopsy specimens in vitro may be due to poor tissue penetration of this large tetradimer: this may also limit its potential use in vivo.

Hydrogen peroxide, produced by superoxide dismutation and peroxidases, is injurious to the rectal mucosa in humans when applied topically⁴⁴ and intraperitoneal catalase improves inflammation in rat acetic acid induced

colitis,¹⁹ suggesting that hydrogen peroxide and its metabolites may contribute to colonic inflammation. An antioxidant effect for catalase can be demonstrated in both acetic acid induced colitis and UC biopsy specimens,¹ further supporting the suggestion that ROS produced by neutrophils are important in IBD.

N-acetylcysteine replenishes intracellular stores of glutathione, is a weak scavenger of superoxide and hydroperoxide but reacts avidly with hypochlorous acid and the hydroxyl radical.⁴⁵ However, N-acetylcysteine was ineffective in reducing ROS production by acetic acid induced colitis or UC biopsy specimens under the present experimental conditions.²⁴

The physiological level of ROS is regulated by both enzymatic systems and direct oxygen scavengers, such as ascorbate, which reacts rapidly with superoxide, the hydroxyl radical,⁴⁶ and hypochlorous acid.⁴⁷ Ascorbate levels are decreased in the plasma in IBD.^{48, 49} In this investigation ascorbate produced similar reductions in chemiluminescence in acetic acid induced colitis and human UC biopsy specimens.²⁴ However, ascorbate is readily oxidised to the potentially damaging ascorbate radical in the presence of iron,⁵⁰ present in the bowel lumen in millimolar quantities.³ This reaction could limit the potential usefulness of ascorbate as a treatment in UC.

The predominant antioxidant action of DMSO in vitro is scavenging of the hydroxyl ion.⁵ As the hydroxyl ion is extremely reactive, the local concentration of any scavenger would probably have to be prohibitively high to compete with biological molecules in the vicinity of its production. This may explain evidence from in vivo studies in which oral 5% (0.64 M) DMSO failed to improve acetic acid induced colitis.²² Nevertheless, the antioxidant effects of DMSO obtained here, and in UC biopsy specimens,¹ suggest that evaluation of its potential efficacy when given in enema formulation in high concentration to patients with distal UC may be worthwhile.

The hypochlorite scavenger taurine had little effect on ROS production by UC biopsy specimens¹ and no effect in this model, possibly because the reaction product of hypochlorous acid and taurine, taurine-N-chloramine, is itself an oxidant.²⁹

The novel agent, LY231617, protects against cerebral ischaemia in an animal model in vivo and ameliorates hydrogen peroxide induced neuronal damage in vitro.⁵¹ The potent antioxidant activity shown here suggests that it should be further evaluated as treatment in UC, perhaps initially in an enema or suppository formulation to achieve the necessary intraluminal concentration (10 mM).

We studied another antioxidant, amflutizole, which is a non-competitive inhibitor of xanthine oxidase and has also shown efficacy in a model of neuronal ischaemia.⁵² Ischaemia reperfusion with increased superoxide generation through xanthine oxidase activation³⁶ may have a pathogenic role in IBD. Multifocal vascular microinfarction is a feature of CD³⁷ and microvascular thrombi have been detected

in both UC and CD.⁵³ The competitive xanthine oxidase inhibitor, allopurinol, has been of reported benefit in pouchitis.¹⁸ In the present studies amflutizole was at least as effective as 5-ASA in reducing the chemiluminescence response of biopsy specimens from acetic acid induced colitis. Xanthine oxidase inhibition with amflutizole should, therefore, be further explored as a therapeutic option in IBD; again initial studies using topical treatment in distal UC would be desirable.

In conclusion, this in vitro system using biopsy specimens taken from rats with acetic acid colitis provides a convenient method for the screening of the antioxidant potential of new treatments for IBD. Agents that reduce ROS production by inflamed human colorectal biopsy specimens, such as CuZn superoxide dismutase, catalase, and DMSO also ameliorate inflammation in acetic acid induced colitis. Previous workers have suggested that the ROS production in both UC¹ and acetic acid induced colitis^{19, 22} is largely mediated by neutrophils, and the results here with azide, catalase, and CuZn superoxide dismutase support this proposal. The additional evidence of an antioxidant effect with the xanthine oxidase inhibitor, amflutizole, and previous evidence that allopurinol reduces ROS production by inflamed colonic biopsy specimens from acetic acid induced colitis²² suggests that, at least in this animal model, ischaemia reperfusion may also contribute to mucosal oxidant stress.

The limited antioxidant effect of antioxidant enzymes in both acetic acid induced colitis and UC biopsy specimens¹ may be because of poor tissue penetration or inactivation by hypochlorous acid⁵⁴ produced by activated neutrophils; these factors may restrict their therapeutic potential.

Of the agents assessed in this study, dimethyl sulphoxide, ascorbate, amflutizole, and LY231617 seem most suitable for further evaluation in IBD.

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