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THE REACTION OF 5-AMINO-SALICYLIC ACID WITH HYPOCHLORITE

IMPLICATIONS FOR ITS MODE OF ACTION IN INFLAMMATORY BOWEL DISEASE

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Abstract—Although 5-amino-salicylic acid (5-ASA) provides effective treatment for inflammatory bowel disease, its mode of action is unestablished. 5-ASA inhibits luminol-dependent chemiluminescence triggered by activated neutrophils, hydrogen peroxide plus peroxidase or sodium hypochlorite. The concentrations required for 50% inhibition of the cells was approximately 3.6 μM. In the non-cellular system, the concentration of 5-ASA required for total inhibition being approximately equivalent to the concentration of sodium hypochlorite. The reaction of 5-ASA with hypochlorite or activated neutrophils resulted in the production of a non-fluorescent product of 5-ASA. The production of this metabolite by cells was dependent upon the activity of the peroxidase and occurred with a time course which was coincident with oxygen consumption. It was concluded that by reacting with hypochlorite, 5-ASA would provide protection against the potentially damaging effects of activated neutrophils in the inflamed bowel.

5-Amino salicylate delivered to the site of inflammation in ulcerative colitis either by the breakdown of sulphasalazine by colonic bacteria [1] or by release from a pH-sensitive coated capsule [2] provides an effective treatment for the disease, inducing remission in the acute phase and also reducing relapse rate in quiescent disease [3–6]. Its mechanism of action, however, remains unestablished.

Ulcerative colitis is characterised by an inflammatory infiltrate in the mucosa, containing both neutrophils and macrophages. We have recently shown that in inflammatory bowel disease, these cells retain the ability to produce reactive oxygen metabolites and are "primed", cells at the inflammatory sites, producing up to 3 times the response of neighbouring cells in non-inflamed tissue [7]. The oxygen products generated include superoxide (O_2^-), hydrogen peroxide (H_2O_2) and in the presence of peroxidase, (hypochlorite) OCl^- [8]. This latter metabolite may play an important role in mediating tissue damage. Not only is it a powerful oxidant but also it has been shown to inhibit α-1-antitrypsin [9, 10] and activate the latent neutrophil collagenase [11]. Reduction of the extracellular concentration of this reactive metabolite will therefore provide protection against phagocyte-mediated tissue damage.

In this paper, we demonstrate that 5-ASA inhibits luminol-dependent chemiluminescence by reacting with hypochlorite ions. This inhibitory effect is demonstrated with activated neutrophils, and enzymatically generated hypochlorite or sodium hypochlorite. We further demonstrate that this reaction produces a non-fluorescent metabolite of 5-ASA, which may provide an *in-vivo* marker for 5-ASA hypochlorite scavenging.

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EXPERIMENTAL

Preparation of human neutrophils and assays of activity. Neutrophils were isolated from blood taken from healthy volunteers. After dextran sedimentation and centrifugation through Ficoll-Hypaque, the contaminating erythrocytes were removed by hypotonic lysis. The resultant cell population (>95% neutrophils) was suspended in HEPES-buffered Krebs medium containing 120 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM, Mg_2SO_4, 1.3 mM CaCl, 0.1% BSA, 25 mM HEPES, pH adjusted to 7.4 with NaOH. Luminol-dependent chemiluminescence was measured on a purpose-built luminometer using 11 μM luminol as indicator as previously described [12]. Oxygen consumption by neutrophils was measured using a Clark-type electrode chamber as previously described [13]. Superoxide dismutase-sensitive reduction of cytochrome *c* was monitored using a dual beam spectrophotometer in a thermostatically controlled sample housing as previously described [14]. Myeloperoxidase was measured using guaicol as substrate and β-glucuronidase using an umbelliferon substrate as previously described [15].

Fluorimetric measurements. Fluorescence measurements were performed using a Spex Fluorolog III (Glen Creston, U.K.). Fluorescence spectra were corrected for the lamp intensity by comparison with a reference detector positioned to record lamp intensity entering the sample housing. Quartz cuvet-

tes were used throughout and samples containing cell suspension were stirred and maintained at 37° by circulating water. In cell experiments emisson at the 5-ASA peak of interest (500 nm) and at 680 nm, which was insensitive to 5-ASA (i.e. cell scatter and autofluorescence) was employed to eliminate the possibility that fluorescence changes observed also contained effects resulting from baseline shifts due to changes in autofluorescence, scatter etc. This was achieved using a T-format with two monochromators and detectors (Glen Creston, U.K.).

Materials. 5-ASA (Sigma) was dissolved in dimethyl sulphoxide for use in experiments, and dissolved from solid freshly on each day. Ficoll-Hypaque was purchased from Pharmacia; luminol, guaicol, cytochalasin B and formyl-methionyl-leucyl-phenylalanine (f met-leu-phe) from Sigma; umbelliferon substrate from Koch-light and all other reagents were "Analar" grade from BDH (Poole, U.K.).

RESULTS

5-ASA inhibits neutrophil luminol-dependent chemiluminescence

5-ASA (10–100 μM) had no significant effect on the secretion of myeloperoxidase or β-glucuronidase from human neutrophils maximally stimulated with the peptide, f-met-leu-phe (1 μM) plus cytochalasin B (5 μg/ml). The production of superoxide ions by f met-leu-phe stimulated neutrophils, measured by the reduction of cytochrome *c*, was minimally inhibited, 100 μM 5-ASA producing a 22% inhibition in the rate of production of superoxide. Similarly, cyanide-insensitive oxygen consumption triggered by this stimulus was only slightly inhibited, 100 μM 5-ASA producing 20% reduction. In contrast, however, luminol-dependent chemiluminescence produced by neutrophils stimulated with similar concentrations of f met-leu-phe + cytochalasin B was totally abolished by 100 μM 5-ASA. The $K_{i_{50}}$ for 5-ASA inhibition of the chemiluminescent response of stimulated neutrophils was 3.6 ± 1.8 μM (mean ± SD from 6 separate samples of neutrophils; Fig. 1a). This striking discrepancy suggested that this inhibitory effect of 5-ASA was the result of interference with the triggering of luminol-dependent chemiluminescence, rather than an effect on the neutrophils. This conclusion was strengthened by the observation that the drug inhibited luminol-dependent chemiluminescence equally well when added at any time after cell stimulation. This conclusion was further tested by determining the effect of 5-ASA on luminol-dependent chemiluminescence when triggered in a cell-independent system.

5-ASA inhibits luminol-dependent chemiluminescence triggered by hypochlorite

The mechanism by which neutrophils trigger luminol-dependent chemiluminescence is dependent not only on the activity of the neutrophil oxidase [16, 17] but also on the activity of the peroxidase [15, 18, 19]. Luminol-dependent chemiluminescence is also triggered by hypoxanthine/xanthine oxidase plus microperoxidase (Fig. 1b) or more simply hydrogen peroxidase plus peroxidase (Fig. 1c). In these cell-free systems, 5-ASA strongly inhibited chemiluminescence, 50 μM also totally abolishing the response. The $K_{i_{50}}$ for 5-ASA in these systems was 0.23 ± 0.02 μM and 0.43 ± 0.01 μM respectively (Figs 1b and c). The major product of the cell-free system in the presence of chloride, is OCl^-, which alone can trigger luminol-dependent chemiluminescence. The addition of sodium hypochlorite to luminol produced a spike of luminescence, which was also inhibited by 5-ASA, the concentration required for total inhibition being approximately equivalent to the concentration of OCl^- present (Table 1).

Table 1. Effect of 5-ASA on hypochlorite-triggered luminol-dependent chemiluminescence

Hypochlorite 5-ASA Conc. (μM)	Concentration (μM) 25	50	100	200
	(% maximum luminescence response)			
25	0*	17.9 ± 1.8	48.3 ± 17.2	60.9 ± 6.1
50	0	0	27.5 ± 2.9	45.4 ± 9.5
100	0	0	0	8.3 ± 1.45
200	0	0	0	0

* Luminescence not significantly different from background rate.

The data shown are the percentages of the luminol-dependent chemiluminescence triggered by hypochlorite in the absence of 5-ASA. For each column recorded luminescence counts were 3.6, 6.0, 7.8 and 9.2 × 10 c.p.s. for 25, 50, 100 and 200 μM hypochlorite respectively. The data shown are the mean ± SD of three replicate experiments. It can be seen that the luminescence was totally inhibited when the concentration of 5-ASA was equal to or exceeded the concentration of hypochlorite.

5-ASA fluorescence is lost by interaction with hypochlorite or activated neutrophils

The fluorescence of 5-ASA excited by 340 nm was lost by the reaction with equimolar concentrations of hypochlorite and also by the reaction of hypoxanthine/xanthine oxidase in the presence of peroxidase and chloride (Fig. 2a). The time course of conversion of 5-ASA to a non-fluorescent product, following addition of peroxidase to the reaction mixture containing hypoxanthine/xanthine oxidase, was monitored by measurement of emitted fluorescence at the peak of 500 mm (Fig. 2b).

The non-fluorescent product of 5-ASA was also produced by the interaction with neutrophils maximally stimulated with f-met-leu-phe (1 μM) and cytochalasin B (5 μg/ml) (Fig. 3a). The reaction was prevented by the peroxidase inhibitor, azide (1 mM) and proceeded with a time course approximately equivalent to the rate of oxidase activity as measured by oxygen consumption (Fig. 3b).

DISCUSSION

The results presented here have shown that 5-ASA reacts with a metabolite generated by activated neutrophils, preventing it from triggering luminol-dependent chemiluminescence. This interaction of

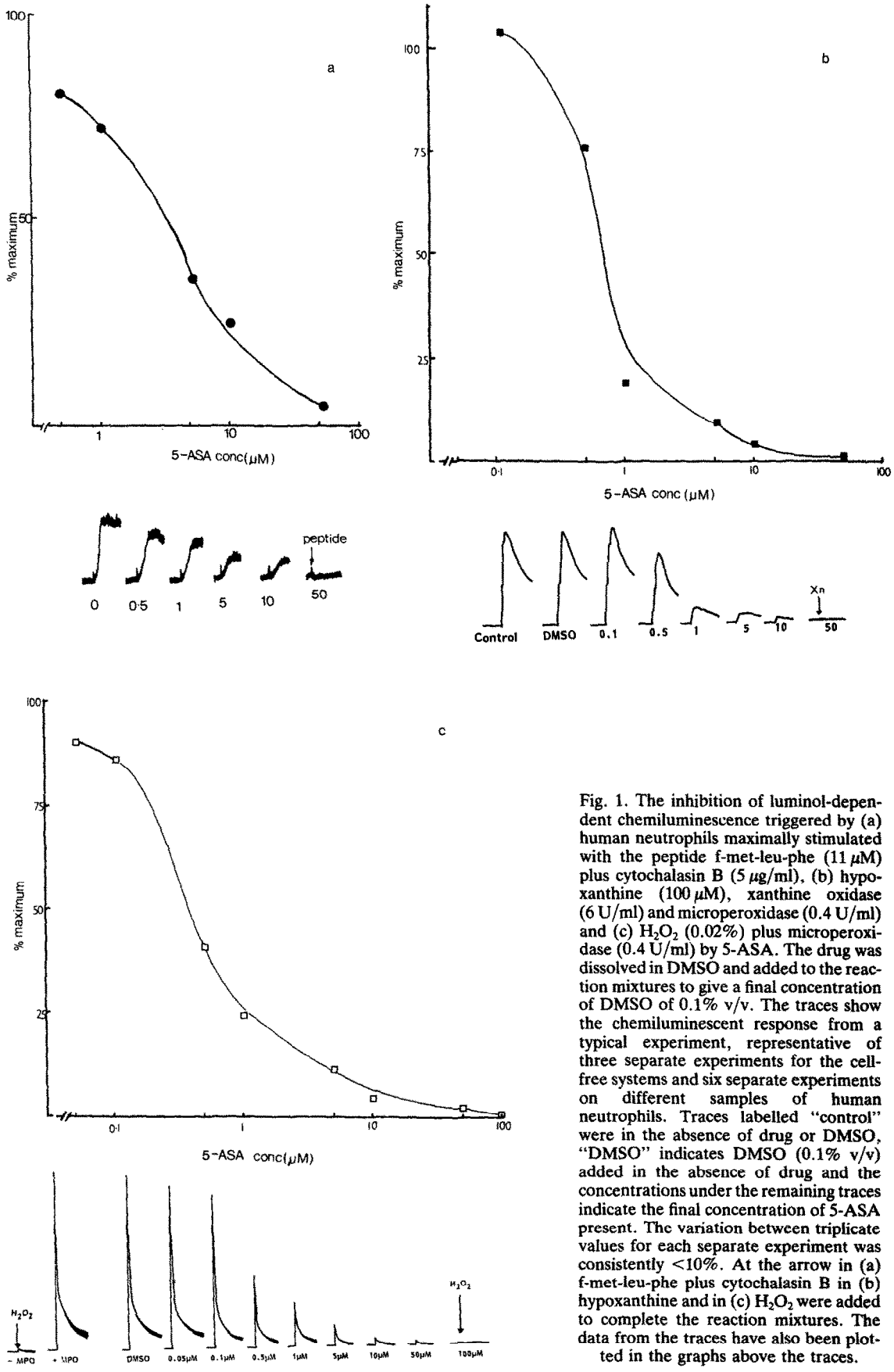

Fig. 1. The inhibition of luminol-dependent chemiluminescence triggered by (a) human neutrophils maximally stimulated with the peptide f-met-leu-phe (11 μM) plus cytochalasin B (5 μg/ml), (b) hypoxanthine (100 μM), xanthine oxidase (6 U/ml) and microperoxidase (0.4 U/ml) and (c) H_2O_2 (0.02%) plus microperoxidase (0.4 U/ml) by 5-ASA. The drug was dissolved in DMSO and added to the reaction mixtures to give a final concentration of DMSO of 0.1% v/v. The traces show the chemiluminescent response from a typical experiment, representative of three separate experiments for the cell-free systems and six separate experiments on different samples of human neutrophils. Traces labelled "control" were in the absence of drug or DMSO, "DMSO" indicates DMSO (0.1% v/v) added in the absence of drug and the concentrations under the remaining traces indicate the final concentration of 5-ASA present. The variation between triplicate values for each separate experiment was consistently <10%. At the arrow in (a) f-met-leu-phe plus cytochalasin B in (b) hypoxanthine and in (c) H_2O_2 were added to complete the reaction mixtures. The data from the traces have also been plotted in the graphs above the traces.

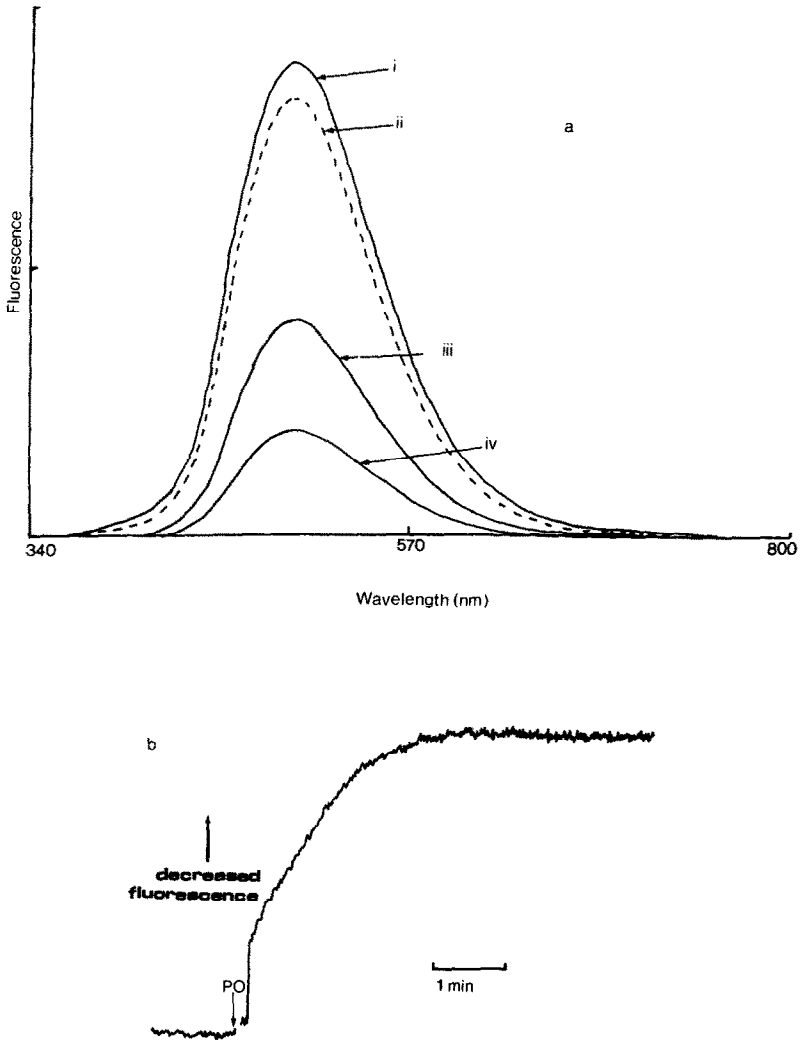

Fig. 2. (a) The fluorescence emission spectra of 5-ASA excited at 340 nm, (i) in 150 mM NaCl, (ii) plus hypoxanthine (100 μM) and xanthine oxidase (6 U/ml) (iii) also plus horseradish peroxidase (1 U/ml) incubated for 1 min (iv), as (iii) after a further min. All spectra were corrected for the lamp spectrum. (b) The time course for the disappearance of fluorescence monitored at 500 nm emission. At the arrow indicated, horseradish peroxidase was added to the reaction mixture containing 150 mM NaCl, hypoxanthine (100 μM) and xanthine oxidase (1 U/ml). Fluorescence intensity at 500 nm was monitored continuously and for clarity is shown as the rate of production of the non-fluorescent product.

the drug results in the production of a non-fluorescent product of 5-ASA, the appearance of which is co-incident with oxygen consumption from activated neutrophils and is dependent upon peroxidase activity. These effects were shown to result from the ability of the drug to react with hypochlorite.

During the course of this work, Dull *et al.* [20] showed that activated neutrophils and monocytes oxidised 5-ASA to produce several products distinguishable by HPLC. The major products were identified as gentisate (0.2%) and salicylate (0.14%). However, the possibility that the non-fluorescent product reported here was either of these was excluded as both gentisate and salicylate are fluorescent when excited at 340 nm, peak emission being at 453 nm and 412 nm respectively. The reaction of stimulated neutrophils with 5-ASA resulted in the disappearance of the emission peak at 500 nm, without the appearance of a detectable fluorescence peak in the range 350–800 nm (Fig. 3). Furthermore, gentisate and salicylate, like 5-ASA, react with ferric chloride, whereas the metabolite produced after neutrophil activation did not.

The concentration of 5-ASA required for inhibition was found to depend upon the concentration of hypochlorite, approximately equivalent concentrations of 5-ASA being required for total inhibition (Table 1). In the *in-vitro* cell system, approximately 100 μM 5-ASA was required to inhibit totally luminol-dependent chemiluminescence by neutrophils maximally stimulated with chemotactic peptide in the presence of cytochalasin B (Fig. 1), where oxidase activation results in extracellular production of luminol-reactive oxygen metabolites [15],

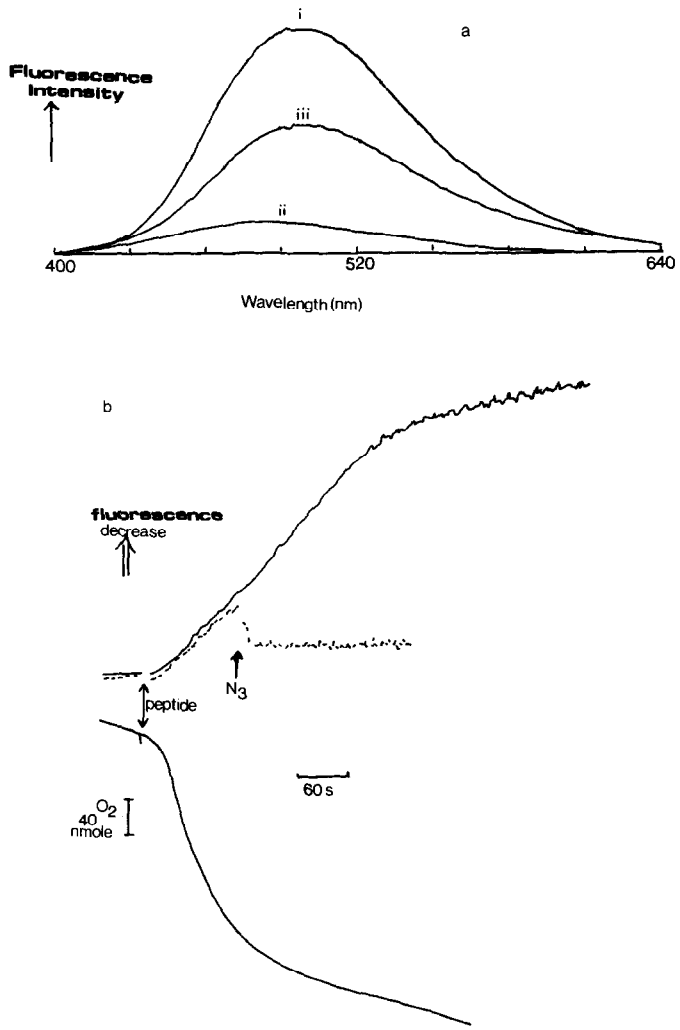

Fig. 3. (a) The emission spectra of 5-ASA excited at 340 nm (i) in the presence of unstimulated neutrophils, (ii) after maximal cell stimulation with f-met-leu-phe (1 μM) plus cytochalasin B (5 μg/ml) and (iii) as (i) but sodium azide (1 mM) added at 1 min after cell stimulation. All spectra were corrected for the lamp spectrum. (b) The upper traces show the time course of the fluorescence decrease of 5-ASA emitting at 500 nm. At the first arrow, the chemotactic peptide and cytochalasin B were added. The broken trace shows a repeat experiment in which sodium azide (1 mM) was added at the second arrow. The lower trace shows the decrease in oxygen concentration following cellular stimulation of the neutrophil oxidase. The effect of azide on oxygen consumption was not significantly different from that shown here.

suggesting that the local concentration of hypochlorite around the cell to be approximately 100 μM. A similar concentration was required to inhibit luminol-dependent chemiluminescence triggered by latex particles, where the reaction with luminol is intra-phagosomal [15]. This suggests an intra-phagosomal concentration of hypochlorite of approximately 100 μM, a value similar to that estimated for intra-phagosomal H_2O_2 based on bactericidal activity [21]. Interestingly, in cells treated with phorbol myristate acetate, higher concentrations of 5-ASA were required to inhibit chemiluminescence, suggesting that either this agent produced a higher local concentration of hypochlorite or that the site of production was inaccessible to the drug. In the clinical situation, the concentration of 5-ASA has been reported to be 10 mM in the faecal stream [22] and 15 μM in the plasma [23]. At the site of action of 5-ASA in the inflamed mucosa of the bowel, it is therefore proposed that the *in-vitro* phenomena demonstrated here would also occur *in-vivo*.

Hypochlorite has been proposed to mediate tissue damage at inflammatory sites. It is a powerful oxidant, reacting with many biological molecules. It also has been shown to inhibit α-1-antitrypsin [9, 10] and to directly activate the latent activity of the neutrophil collagenase [11]. It is therefore proposed that by reacting with hypochlorite, 5-ASA will provide protection against the potentially tissue damaging products of activated neutrophils in the inflamed bowel. The continued presence of the drug may also reduce relapse by preventing accumulation of OCl^-

generated by cells in quiescent mucosa. It is now important to establish the identity of the 5-ASA metabolite and to search for its production in patients receiving the drug. The presence of this metabolite will provide evidence for hypochlorite production in inflammatory disease and will also provide evidence for "hypochlorite-scavenging" as the mode of action of 5-ASA.

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