Toxic oxygen metabolite production by circulating phagocytic cells in inflammatory bowel disease

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Abstract

To investigate the possibility that the oxidative capacity of phagocytic cells may be defective in inflammatory bowel disease, toxic oxygen metabolite production by circulating neutrophils and monocytes has been measured by luminol dependent chemiluminescence. Neutrophils from patients with Crohn's disease and ulcerative colitis produced significantly lower chemiluminescent responses after chemotactic stimulation with formyl-methionylleucylphenylalanine (fMLP) than neutrophils from control patients, $p=0.018$ and 0.043 respectively. Chemiluminescent responses of neutrophils from patients with inflammatory bowel disease, however, were similar to control responses when cells were stimulated with latex beads or phorbol myristate acetate. Monocytes from patients with Crohn's disease produced significantly greater levels of chemiluminescence than control monocytes when stimulated with either fMLP ($p<0.002$), phorbol myristate acetate ($p<0.0005$) or latex beads ($p<0.002$). Monocytes from patients with ulcerative colitis also produced significantly greater levels of chemiluminescence than controls when stimulated with latex beads ($p<0.5$) or phorbol myristate acetate ($p<0.0005$), although there was no difference in the level of chemiluminescence in response to fMLP. These results exclude a generalised defect in phagocytic cell oxidase activity in inflammatory bowel disease and suggest that circulating monocytes are 'activated'.

The aetiology of Crohn's disease and ulcerative colitis remains uncertain. Phagocytic cells are prominent in the inflamed bowel wall in both conditions.¹ Since the work of Metchnikoff,² neutrophils and macrophages have been implicated in the pathogenesis of tissue damage at inflammatory sites. These cells are able to generate highly reactive metabolites of oxygen such as hydrogen peroxide (H_2O_2) and hypochlorite (OCl^-) as well as the superoxide (O_2^-) and hydroxyl radicals ($OH^\cdot$).^{3,4} Although these products of oxygen are involved in bacterial killing, their production outside the cell may also cause tissue damage.⁵

One feature of Crohn's disease is the typical non-caseating granuloma.⁶ Parallels have been drawn between Crohn's disease and chronic granulomatous disease, where granulomas similar to those seen in Crohn's disease have been described in the rectum and the terminal ileum of symptomatic patients.⁷ The oxidative capacity of circulating phagocytic cells from patients with

chronic granulomatous disease is defective, these cells being unable to produce toxic oxygen metabolites when stimulated.⁸ This has led to speculation that phagocyte function in inflammatory bowel disease is in some way defective.⁹

Neutrophils and macrophages migrating into the tissues originate from the circulating pool of neutrophils and monocytes. Therefore, any generalised defect in phagocytic function should be present in the circulating cells.

In this paper, the oxidative capacity of circulating neutrophils and monocytes has been assessed by a sensitive chemiluminescent technique to investigate the possibility that the oxidative capacity of these cells may be defective in inflammatory bowel disease.

Methods

PATIENTS

The diagnosis of Crohn's disease and ulcerative colitis was based on accepted clinical, radiological, endoscopic, and histological criteria. The severity of Crohn's disease was assessed by the criteria of DeDombal *et al*¹⁰ and the severity of ulcerative colitis by the criteria of Truelove and Witts.¹¹ Both these systems identify patients with mild, moderate, and severe disease. Patients with moderate and severe disease were grouped together as having active disease and patients with mild disease as having inactive disease. Control subjects included healthy volunteers, and patients with other non-inflammatory gastrointestinal pathologies – for example, haemorrhoids, fissure *in ano*, colonic polyps, and gastrointestinal malignancies.

Neutrophil chemiluminescence and monocyte chemiluminescence was studied in different cohorts of patients with Crohn's disease and ulcerative colitis, as well as different control groups.

NEUTROPHIL STUDIES

In this study there were 25 control subjects (mean age 47 years, range 25–79) and 33 patients with Crohn's disease (mean age 42 years, range 16–78), of which 10 were asymptomatic. Four of these were receiving no treatment, four were on prednisolone alone, one was on 5-amino-salicylic acid (5ASA) and prednisolone and a further patient was on 5ASA alone. Six patients had Crohn's disease confined to the small bowel and in the remaining four, only the colon was involved. Of the patients with active disease, six were not taking any medication, 11 were taking prednisolone alone, four were taking 5ASA in combination with prednisolone, and one patient

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5ASA alone. Seventeen patients had only small bowel disease, four only colonic disease and the remaining two had both small and large bowel disease. Of the 19 patients with ulcerative colitis (mean age 45 years, range 21–72), five were asymptomatic, one of which was off treatment and two were on prednisolone and two were on salazopyrin. Of the patients with active disease, two were on prednisolone alone, and the remaining patients were taking prednisolone in combination with 5ASA.

MONOCYTE STUDIES

In this study there were 16 control subjects (mean age 50 years, range 22–81) and 20 patients with Crohn's disease (mean age 46 years, range 27–76), of which five were asymptomatic, two of whom were off treatment, two on 5ASA and one on prednisolone. Four of these patients had Crohn's disease confined to the small bowel and in the fifth, both the small and large bowel were involved. The remaining patients had active disease, three of whom were off treatment, eight were on prednisolone and two on 5ASA as sole therapy and a further two were on a combination of the two drugs. Four patients had Crohn's disease confined to the small bowel, in nine only the large bowel was involved and the remaining two patients had both large and small bowel involvement. There were 17 patients with ulcerative colitis (mean age 46 years, range 22–72). Six were asymptomatic, two of these were off treatment, one on prednisolone and one on sulphasalazine and two on a combination of the two drugs. Of the patients who had active disease, two were off treatment, and the remainder were on prednisolone and 5ASA or sulphasalazine.

ISOLATION OF PHAGOCYTIC CELLS

Neutrophils

Neutrophils were isolated from 20 ml freshly drawn heparinised (10 IU/ml) whole blood, after dextran sedimentation of erythrocytes at 1 g for 45 min at room temperature.¹² The leucocyte rich plasma was layered over an equal volume of Ficoll-paque (Pharmacia, Upsala, Sweden) and centrifuged at 200 g for 30 minutes. Erythrocytes, contaminating the neutrophil pellet, were destroyed by osmotic lysis with water. Neutrophils were washed twice in HEPES (N-2-Hydroxyethylpiperazine-N-2-ethanesulphonic acid) buffered Krebs solution (HBK) containing 120 mM NaCl, 4.8 mM KCl, 1.2 mM Mg₂SO₄, 1.2 mM KH₂PO₄, 1.3 mM CaCl₂, 0.1% bovine serum albumin and 25 mM HEPES, pH adjusted to 7.4 with NaOH. Cells were resuspended in fresh HBK (1–6 × 10⁶/ml) and stored on melting ice until required.

Purity was assessed in fixed preparations of the isolated cells (>95%). Viability was assessed by the exclusion of trypan blue 0.1% and (>95%).

Monocytes

Monocytes were isolated from 30–40 ml freshly

drawn heparinised blood (10 IU/ml), diluted 1:1 with HBK. Blood was gently layered over an equal volume of Ficoll/paque and centrifuged at 200 g for 30 minutes. Mononuclear cells were retrieved from the interface of the plasma and Ficoll, and washed twice in HBK before being stored on melting ice. Cytological preparations were made of each sample of mononuclear cells and stained with non-specific esterase to identify monocytes in each sample.¹³ The proportion of monocytes in each sample was calculated (7–50%), and from this, the number of monocytes/ml (0.5–3 × 10⁶). Neutrophil contamination was <5% and viability of mononuclear cells >95%.

MEASUREMENT OF TOXIC OXYGEN METABOLITE PRODUCTION

Toxic oxygen metabolite production by both neutrophils and monocytes was measured by luminol dependent chemiluminescence¹⁴ in purpose built apparatus.¹⁵ This consisted of a light-tight, thermostatically controlled specimen chamber, exposed to a photomultiplier tube (Thorn EMI 30 mm, bialkaline front surface no. 9924B. High voltage supply 940V, discriminator 0.1V). Quantitative readings of luminescence were made off a digital read-out and recorded continuously with a pen chart recorder.

One millilitre of cell suspension was placed in a plastic tube and luminol (Sigma Chemical Co, Poole, England), dissolved in DMSO, was added (final concentration luminol 11 μM 0.2% v/v DMSO). The tube was incubated for five minutes at 37°C before being placed in the specimen chamber. The level of resting chemiluminescence was recorded before addition of the stimulus.

Three different stimuli were used: (1) 4β phorbol-myristate-acetate (PMA) dissolved in DMSO (final concentration 1 μg/ml) (2) The chemotactic peptide formyl-methionyl-leucyl-phenylalanine (fMLP) (Sigma, final concentration 1 μM). (3) Latex beads (Polysciences inc. Northampton) 0.74 μm diameter, 10⁹ beads/ml.

For each cell sample and stimulus, peak stimulated chemiluminescence was recorded. Triplicate measurements were made on separate aliquots of the cells studied. The basal level was subtracted from the peak level and the mean chemiluminescent response per 10⁶ cells calculated.

As the chemiluminescent responses of each group of patients were not normally distributed, median values were calculated and statistical comparisons between the three groups were made using the Mann Whitney U test.

The relationship between cell number and chemiluminescent response was assessed using the Pearson correlation coefficient, by measuring the chemiluminescent response of increasing concentrations of neutrophils (1 × 10⁶–1.2 × 10⁷/ml, four subjects) and monocytes (0.5 × 10⁶–6 × 10⁶/ml, four subjects).

Results

Neutrophils and monocytes from each patient studied produced detectable chemiluminescence

Figure 1: Cell concentration and chemiluminescent response. The relationship between the number of (a) neutrophils and (b) monocytes stimulated with phorbol myristate acetate (PMA) and the magnitude of the chemiluminescent response. Each point represents the mean (SE) triplicate measurements.

after each stimulus. There was no difference in the pattern of chemiluminescent response between controls and inflammatory bowel

disease cells. There was direct, linear correlation between the magnitude of the peak chemiluminescent response and the number of neutrophils per millilitre in the four samples of neutrophils, $r=0.995$, 0.995 , 0.996 and 0.907 (Fig 1a). Similarly, there was also direct, linear correlation between the number of monocytes and the magnitude of their chemiluminescent response in the four samples of mononuclear cells, $r=0.98$, 0.998 , 0.978 and 0.985 (Fig 1b).

CHEMILUMINESCENT RESPONSE TO CHEMOTACTIC STIMULATION

The magnitude of the median chemiluminescent response of both neutrophils and monocytes from each subject to chemotactic stimulation with fMLP is shown in Figure 2.

The median response of neutrophils from control subjects was 551 counts per second per 10^6 neutrophils (cps). The response of neutrophils from patients with Crohn's disease to fMLP was significantly lower, median 312 ($p=0.018$), as was the response of neutrophils from patients with ulcerative colitis, median 265 ($p=0.043$).

In contrast, the chemiluminescent response of monocytes from patients with Crohn's disease was significantly greater than those of controls (control median response 72 cps, Crohn's disease 313 cps, $p<0.002$). There was no significant

Figure 2: Chemiluminescent response to chemotactic stimulation. The chemiluminescent response of neutrophils and monocytes from controls (■) and patients with active Crohn's disease and ulcerative colitis (●), and inactive inflammatory bowel disease (○), to stimulation with the chemotactic peptide formyl-methionyl-leucyl-phenylalanine (fMLP, $1 \mu\text{M}$). Each point represents the mean of triplicate measurements. Horizontal bar represents the median value for each group of patients.

Figure 3: Chemiluminescent response to phagocytic stimulation with latex beads $0.7 \mu\text{m}$ diameter, $>10^2/\text{cell}$. See legend Figure 2 for details and text for significance values.

difference between the response of monocytes from patients with ulcerative colitis (median 159 cps) and controls.

CHEMILUMINESCENT RESPONSE TO PHAGOCYTIC STIMULATION

The median chemiluminescent response of both neutrophils and monocytes from each subject to phagocytic stimulation with latex beads is shown in Figure 3. There was no significant difference between the chemiluminescent response of neutrophils from controls and patients with inflammatory bowel disease (median responses; control, 366 cps; Crohn's disease 452 cps and ulcerative colitis 681 cps).

The chemiluminescent response of monocytes, however, was significantly greater for patients with inflammatory bowel disease compared to controls. Control median response 280 cps, Crohn's disease 1803 ($p < 0.002$), ulcerative colitis 1460 ($p < 0.0005$).

CHEMILUMINESCENT RESPONSE TO PHORBOL MYRISTATE ACETATE

The responses of neutrophils and monocytes to phorbol myristate acetate are shown in Figure 3. There was no significant differences between the responses of neutrophils from controls and patients with inflammatory bowel disease

(median responses; controls, 7014 cps; Crohn's disease 6194 cps and ulcerative colitis 8234 cps).

The response of monocytes from patients with inflammatory bowel disease was significantly greater than control responses. Control median 280 cps, Crohn's disease 1883 cps ($p < 0.0005$), ulcerative colitis 1060 cps ($p < 0.05$).

EFFECT OF DISEASE ACTIVITY ON CHEMILUMINESCENT RESPONSE

There was no difference in the response of neutrophils from patients with inactive disease compared to the response of neutrophils from patients with active disease for any of the three stimuli used (Figs 2–4). Similarly, drug therapy did not appear to influence the magnitude of the chemiluminescent response of neutrophils or monocytes.

The chemiluminescent response of monocytes from patients with active Crohn's disease however was significantly greater than the response of patients with inactive disease after stimulation with fMLP (inactive median response 207 cps, active 526 cps, $p < 0.02$) and after stimulation with phorbol myristate acetate (inactive 535 cps, active 2419 cps, $p < 0.05$). The difference in response after stimulation with latex beads was not statistically significant (inactive 591 cps, active 1942 cps). For each stimulus there was no significant difference in the magnitude of the

Figure 4: Chemiluminescent response to stimulation with phorbol myristate acetate (PMA, 1 µg/ml). See legend Figure 2 for details and text for significance values.

response of monocytes from patients with inactive Crohn's disease and control subjects.

A similar relationship was seen in ulcerative colitis, with significantly greater activity by monocytes from patients with active disease compared to patients with inactive ulcerative colitis. After stimulation with phorbol myristate acetate, the response of monocytes from patients with inactive disease was 174 cps and from patients with active disease 1655 cps ($p<0.0002$); after fMLP, 58 cps and 305 cps respectively ($p<0.01$) and after latex beads 655 cps and 1881 cps respectively ($p=0.02$). The response of monocytes from patients with in active ulcerative colitis was not significantly different from the response of control monocytes.

EFFECT OF TREATMENT WITH STEROIDS
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TABLE I Effect of treatment with steroids on the chemiluminescent response of phagocytic cells from patients with active inflammatory bowel disease

Crohn's disease	Neutrophils		Monocytes	
Steroid treatment	Yes	No	Yes	No
Patients(n)	15	7	10	5
Phorbol myristate acetate	5857	5577	1687	3330
fMLP	325	258	467	592
Beads	375	258	1837	2717
Ulcerative colitis	Neutrophils		Monocytes	
Steroid treatment	Yes	No	Yes	No
Patients(n)	11	3	8	2
Phorbol myristate acetate	5442	8613	2658	1027
fMLP	263	489	327	802
Beads	1143	394	1649	2691

Median chemiluminescent response expressed as counts per second per 10^6 cells.

steroids, on the chemiluminescent response of neutrophils and monocytes, was investigated in patients with active Crohn's disease and active ulcerative colitis. The results are shown in the Table. There was no significant difference in the magnitude of the chemiluminescent response of either neutrophils or monocytes in Crohn's disease or ulcerative colitis for any of the three stimuli. The number of patients not being treated with steroids was, however, small.

Discussion

In this paper, we have shown that circulating neutrophils and monocytes from patients with inflammatory bowel disease generate toxic metabolites of oxygen, measured by luminol dependent chemiluminescence, in response to three different stimuli. Toxic oxygen metabolite production by phagocytic cells after stimulation with fMLP is dependent on a transient rise in intracellular calcium.¹⁶ Latex beads, and other phagocytic stimuli, activate phagocytes by a mechanism independent of changes in intracellular calcium, but which involve the enzyme C-kinase.^{16 17} Phorbol myristate acetate is a potent stimulus of toxic oxygen metabolite production by phagocytes and activates C-kinase directly. Thus it appears that activation of toxic oxygen metabolite production by phagocytic cells occurs via at least two different intracellular pathways.

There was no difference in the response of neutrophils from patients with inflammatory bowel disease and controls after stimulation with

latex beads or phorbol myristate acetate. The response to fMLP was slightly depressed in both Crohn's disease and ulcerative colitis. Although this was statistically significant, there was considerable overlap between the three groups. Thus, it would appear that a generalised defect of toxic oxygen metabolite production by neutrophils is not present in either Crohn's disease or ulcerative colitis. Depressed chemotaxis of neutrophils *in vitro* in inflammatory bowel disease has been previously reported^{18, 22} and depressed toxic oxygen metabolite production therefore may be secondary to a defective neutrophil response to chemotactic stimulation.

Increased chemiluminescence in response to zymosan has been reported²³ although the numbers studied were small and included patients with both Crohn's disease and ulcerative colitis as one group. Other studies, however, have reported no difference in the levels of luminol dependent chemiluminescence between controls and patients with Crohn's disease after stimulation with opsonised zymosan²⁴ and phorbol myristate acetate.²⁵

Diminished production of hydrogen peroxide by neutrophils from patients with untreated Crohn's disease has been reported,²⁶ with normal production of hydrogen peroxide by neutrophils from patients with ulcerative colitis and Crohn's disease on medical treatment.^{26, 27} Superoxide production has also been reported to be significantly depressed in ulcerative colitis^{27, 28} and Crohn's disease²⁸ in some studies, whereas in others the depression of superoxide production by neutrophils in Crohn's disease was only slight.^{26, 27}

Interpreting these findings is difficult. As hydrogen peroxide is produced by reduction of superoxide,¹ a marked diminution in superoxide production would be expected to be associated with similar depression of hydrogen peroxide production.

In vivo, neutrophil activation results in the production of a series of interrelated toxic metabolites of oxygen, coupled with the release of granular enzymes²⁹ and formation of highly reactive metabolites such as hypochlorite.⁵ The generation of luminol dependent chemiluminescence by phagocytic cells requires both the production of oxygen metabolites and enzyme release and probably reflects hypochlorite ion generation.^{30, 31} This method of measuring toxic oxygen metabolite production *in vitro*, therefore, more accurately reflects events *in vivo*.

Toxic oxygen metabolite production by circulating monocytes has received less attention in inflammatory bowel disease than neutrophils. This is surprising in view of the relationship between monocytes and tissue macrophages. In this study, we have shown that toxic oxygen metabolite production by circulating monocytes is significantly greater in both Crohn's disease and ulcerative colitis compared with normal controls. Furthermore, this enhanced production of oxygen metabolites was confined to monocytes from patients with active inflammatory bowel disease. Increased chemiluminescence was seen in response to each of the three different stimuli used, suggesting a generalised increased oxidative potential of these cells. Two

other studies have shown increased luminol dependent chemiluminescence by circulating monocytes in Crohn's disease^{34, 35} but in neither study was a relationship to disease activity shown.

In mice, activation of the mononuclear phagocyte system by *C parvum*, vaccinia or murine cytomegalo virus was associated with enhanced chemiluminescence by stimulated peritoneal macrophages.³² Similarly, increased superoxide and hydrogen peroxide production by macrophages activated *in vivo* and *in vitro* has been reported.^{33, 35}

The results presented here suggest that circulating monocytes in patients with inflammatory bowel disease are 'activated'. This is supported by studies relating to other monocyte functions in inflammatory bowel disease including; – increased phagocytosis of *C albicans* and *S aureas*,^{36, 38} increased lysosomal hydrolase content,^{39, 41} increased enzyme release,^{39, 40} increased random motility and chemotaxis⁴² and increased IgG and C3b receptor activity.⁴³ Similar 'activation' of circulating monocytes has been shown in other chronic inflammatory conditions such as rheumatoid arthritis.⁴⁴ Therefore a generalised defect in toxic oxygen metabolite production by phagocytic cells is unlikely to be involved in the aetiology of inflammatory bowel disease. The response of the two types of circulating phagocytes studied, however, differed in inflammatory bowel disease. Neutrophils appeared to be functioning normally, whereas monocytes produced an enhanced response when stimulated. It is of interest that pulmonary mononuclear phagocytic cells also have enhanced oxidative metabolism.⁴⁴ Further study is required to define the precise role of phagocytic cells in the aetiology of inflammatory bowel disease, especially of phagocytes at the site of inflammation in the bowel wall.

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