Diminished neutrophil function in Crohn’s disease and ulcerative colitis identified by decreased oxidative metabolism and low superoxide dismutase content

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SUMMARY Features of the neutrophil oxidative metabolism and enzyme activity in peripheral blood neutrophils were studied in 43 patients with Crohn’s disease, 13 with ulcerative colitis and 33 healthy controls. The production of superoxide anion (O$_2^-$) by phorbol-myristate-acetate stimulated neutrophils from patients with Crohn’s disease and ulcerative colitis was significantly diminished compared with controls mean (SE)=47.1 (3.6) and 38.0 (3.8) nM 67.4 (7.5) nmol/10$^7$ cells/min, p≤0.02, respectively, while the production of hydrogen peroxide was normal. The neutrophil content of superoxide dismutase (SOD), a cytoprotective enzyme, was also markedly diminished in Crohn’s disease mean (SE)=7.11 (0.23) ng SOD/μg DNA, p<0.05, and ulcerative colitis mean (SE)=5.74 (0.42) compared with controls 7.84 (0.27), p<0.001. In contrast, the concentration of neutrophil elastase, a neutral protease, was found to be normal when compared with neutrophils from controls. The neutrophil O$_2^-$ production and the SOD concentrations were significantly and negatively correlated with the disease activity in Crohn’s disease and ulcerative colitis. The results indicate diminished neutrophil function in peripheral blood of patients with Crohn’s disease and ulcerative colitis as illustrated by a diminished oxidative system, which correlates with the disease activity.

In Crohn’s disease and ulcerative colitis various neutrophil functions have been shown to be impaired. In vivo and in vitro assays on the mobility of neutrophils revealed normal or reduced skin window influx, random mobility, and chemotaxis. The basis of a reduced moving pattern has, in part, been shown to be caused by serum mediators and not by decreased cellular activity. On the other hand, recent studies with Indium labelled leucocytes to localise the disease process in these disorders seem to be very efficient and give no indication of severe reduction of the in vivo mobility of these cells.

Phagocytosis by neutrophils has been analysed in only few studies and in most of them was found to be normal. In contrast, the production of oxygen metabolites, which are used for the intracellular killing of microorganisms, was to some extent impaired as measured by nitro blue tetrazolium reduction and by more quantitative assays for superoxide anion and hydrogen peroxide production. When neutrophils accumulate, however, they may produce local toxic levels of oxygen metabolites and can cause an inflammatory process. This is counteracted intracellularly and extracellularly by superoxide dismutase, the reason why this enzyme is called cytoprotective and anti-inflammatory.

It was recently reported that human leucocyte elastase, a neutral protease, was demonstrable in the circulation of patients with inflammatory bowel disease in significantly higher quantities than in controls. Elastase cleaves many basal membrane components as well as fibrin and other proteins and it is thought to be involved in the pathogenesis of destructive lung disease, like emphysema, on the basis of a protease-antiprotease imbalance.

The present study was undertaken to determine...
the neutrophil function based on oxygen metabolite production, intracellular superoxide dismutase and elastase concentrations of neutrophils in Crohn's disease and ulcerative colitis patients. Furthermore, these parameters were correlated with the disease activity.

Methods

PATIENTS
Forty three patients with Crohn's disease (CD) and 13 patients with ulcerative colitis (UC) participated in this study. The diagnosis was based on standard clinical, radiological, endoscopic and histological features. The CD patients consisted of 27 women and 16 men with a mean age of 33 years (range 19–55). Eleven were untreated and 32 received sulphasalazine, prednisolone, or both. The UC patients consisted of seven women and six men, mean age 35 years (range 17–54), three patients were untreated and 10 were treated with sulphasalazine, prednisolone, or both. The disease activity of both groups was scored as described by Best et al.\textsuperscript{16} for the CD patients, and by Truelove and Witts\textsuperscript{17} for the UC patients. Twenty nine of the CD patients had a CDAI below 150, whereas 14 had a CDAI above 150. For the UC patients the figures were nine and four respectively. The control group consisted of 33 healthy controls, 14 women and 19 men with a mean age of 32 years (range 21–51).

CELLS
Neutrophils were isolated from heparinised blood by Ficoll/Hypaque density gradient centrifugation and two fold lysis of the erythrocytes from the pellet in a buffered isotonic NH\textsubscript{4}Cl solution at 0°C. The remaining neutrophils were washed extensively with Hanks' balanced salt solution (HBSS, Gibco) with 0.2% bovine serum albumin. Purity of the neutrophils was more than 96% with a viability of greater than 99%. Cells were used either directly for oxygen metabolite production (1×10\textsuperscript{6} cells/incubation) or homogenised by sonication and stored at −70°C for enzyme determinations and DNA measurements.\textsuperscript{18} Storage did not affect the enzyme determinations as shown by repeated analyses.

ELASTASE DETERMINATION
The elastase activity was determined by the lysis of the sensitive and specific chromogenic substrate L-pyroglutamyl-L-prolyl-L-valine-p-nitro-anilide (Kabi) as published by Kramps et al.\textsuperscript{19} In brief, a mixture of 100 μl cell extract, 100 μl HBSS and subsequently 200 μl 0·1 M Tris – 0.96 M NaCl buffer (pH 8·3) were preincubated at 37°C and subsequently 200 μl 0·2 mmol pGlProVal-p-NA was added. The enzyme activity was determined by Δ OD\textsubscript{405}/min/quantity of cells, in duplicate, and expressed as units elastase according to the formula U=311×Δ OD/min. Reproducibility of the assay was assessed by the intra-assay coefficient of variance mean (SE)=2·2% (0·5%) and the interassay coefficient of variance mean (SE)=5·4% (0·9%) over a two years period.

SUPEROXIDE DISMUTASE DETERMINATION
The amount of superoxide dismutase (SOD) was determined by an ELISA method developed in our laboratory. Superoxide dismutase was purified from erythrocytes as described by McCord and Fridovich.\textsuperscript{20} Antibodies to SOD were raised in goats and purified by affinity chromatography with SOD coupled to CNBr-Sepharose 6B. Antibodies were checked on specificity by preincubation of SOD with increasing amounts of antibody and subsequent electrophoresis. At high antibody-SOD ratio's the SOD activity bands completely disappeared. The double antibody sandwich ELISA was carried out as follows. Antibody was coated (10 μg/ml carbonate buffer pH 9·6) overnight at 4°C. After thorough washing 2% pre-immune goat serum was added and incubated for two hours. After again thorough washing standards of purified SOD were added (2·5–1000 ng/ml) as well as the neutrophil homogenates (diluted 1:30, 1:40, and 1:50), in triplicate, and incubated for two hours at room temperature. Antibody to SOD coupled with peroxidase (dilution 1:12500) were added after washing the ELISA plate. A colour reaction was evoked by adding an orthophenylenediamine-H\textsubscript{2}O\textsubscript{2} solution which was stopped after 30 minutes with 2·5 M H\textsubscript{2}SO\textsubscript{4} and the reaction product could be measured spectrophotometrically at 492 nm. The amount of SOD in the cells was calculated from the standard curve. Reproducibility of the assay was high as illustrated by the intra- (4·1±0·3%) and inter-assay (6·1±1·8%) assay coefficient of variance from repeated analyses of the same samples over a two years period. To validate the SOD-ELISA, results obtained with several biological materials – for example, isolated cells,

| Table 1 | Superoxide anion (O\textsubscript{2}−) and hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) production by neutrophils of patients with Crohn's disease, ulcerative colitis, and controls |
|-----------------|-----------------|-----------------|
|                 | Crohn's disease | Ulcerative colitis | Controls |
| O\textsubscript{2}− | 47·1 (3·6) [27]\textsuperscript{f} | 38·0 (3·8) [7]\textsuperscript{f} | 67·4 (7·5) [22] |
| H\textsubscript{2}O\textsubscript{2} | 6·54 (0·33) [25] | 6·78 (0·33) [6] | 7·08 (0·28) [16] |

Mean (SE); *nmol/1×10\textsuperscript{6} cells/min; †significance p=0·02 CD v controls; ‡significance p<0·01 UC v controls. Figures in square brackets indicate numbers studied.
McCord and Fridovich.20 Diminished neutrophil function in intestinal and liver tissues, were compared with the laborious enzymatic-SOD assay as described by McCord et al.10 In the enzymatic assay SOD activity is determined by the inhibition of the reduction of cytochrome C by superoxide anion raised in a xanthine-xanthine oxidase system. The linear correlation coefficient between the SOD-ELISA and the enzymatic-SOD assay was r=0·78.

Superoxide anion (O2) was determined by measuring the reduction of cytochrome C as published previously.10 After incubation of the cells for 30 minutes with or without phorbol-myristate-acetate (50 ng–5 μg) in the presence of cytochrome C the reaction was stopped by rapid chilling and centrifugation. From the reduction of the cytochrome C measured spectrophotometrically at 550 nm the production of O2 could be calculated with the extinction coefficient E=2·1×104 M/cm. The specificity of the superoxide mediated cytochrome C reduction was determined by the inclusion of 100 μg SOD (Sigma)/ml in some of the assays. Inhibition of the reduction was greater than 95%. Hydrogen peroxide was measured by the oxidation of homovanillic acid in the presence of horseradish peroxidase.11 Using the same incubations as for O2 the reaction product could be measured fluorospectrophotometrically (λex=315nm, λem=425nm). The amount of H2O2 produced by the cells could be calculated from the standard-curve of H2O2 added.

Statistical Analysis
The results are expressed as the mean (SE). Significance of the differences in the means was calculated by Student’s t test (similar standard deviations) or the separate variance analysis (when standard deviations were statistically different). The linear correlation coefficients and significances were determined by Pearson’s correlation procedure.

Results
Neutrophils of Crohn’s disease and ulcerative colitis patients produced significantly less superoxide anion, after maximal stimulation with phorbol-myristate-acetate, than those of controls (Table 1). Although neutrophils of ulcerative colitis patients showed a lower superoxide anion production than neutrophils from Crohn’s disease patients (38 nmol v 47 nmol respectively) this difference did not reach statistical significance. The impairment in the superoxide anion production, however, was accompanied by a normal production of hydrogen peroxide by the neutrophils both in Crohn’s disease and ulcerative colitis when compared with controls (Table 1).

The levels of superoxide dismutase in the neutrophils of patients with Crohn’s disease and ulcerative colitis differed significantly (respectively mean (SE) 7·11 (0·23) and 5·74 (0·42) ng SOD/μg DNA, p<0·01; Fig. 1). The superoxide dismutase levels of the neutrophils both from patients with Crohn’s disease and ulcerative colitis were significantly lower than those of controls mean (SE) 7·84 (0·27) ng SOD/μg DNA; CD p<0·05, UC p<0·001.

The partial impairment of the oxidative system, as indicated by the superoxide anion production and the superoxide dismutase concentration was not accom-

Table 2  Neutrophil elastase activity of patients with Crohn’s disease, ulcerative colitis and controls

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>U Elastase/μg DNA</th>
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<tbody>
<tr>
<td>Crohn’s disease</td>
<td>43</td>
<td>30·9 (0·8)</td>
</tr>
<tr>
<td>Ulcerative colitis</td>
<td>12</td>
<td>27·7 (0·9)</td>
</tr>
<tr>
<td>Controls</td>
<td>33</td>
<td>29·5 (1·4)</td>
</tr>
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Mean (SE); n=number of experiments.
panied by changes in the elastase activity in the neutrophils. The neutrophils of patients with Crohn's disease and ulcerative colitis contained as much elastase activity as neutrophils from controls (Table 2).

Consistently, a negative correlation of the neutrophil superoxide anion production, hydrogen peroxide production and SOD level on the one hand with the disease activity on the other hand was found in both Crohn's disease and ulcerative colitis, 

\[ r = -0.251 \text{ to } -0.768 \]

(Table 3, Fig. 2). The neutrophil elastase activity was not significantly correlated with the disease activity in either Crohn's disease or in ulcerative colitis (\( r = 0.026 \) and \( r = -0.339 \) respectively, \( p > 0.1 \)).

**Discussion**

In previous studies we showed that neutrophils of patients with Crohn's disease and ulcerative colitis have a partial impairment of the oxidative metabolism, which could not be attributed to immaturity of the cells or to medical treatment.\(^\text{10, 11}\) The production of superoxide anion by neutrophils was found to be generally decreased in ulcerative colitis and only after maximal stimulation in Crohn's disease. Hydrogen peroxide production was normal in both diseases, although a significant lower production was found in a subgroup of untreated Crohn's disease patients.\(^\text{10}\) In the present study we determined oxygen metabolite production after optimal stimulation with phorbol-myristate-acetate and observed that peripheral blood neutrophils of the patients have a reduced superoxide anion production and also that they have decreased concentrations of superoxide dismutase. In contrast, the hydrogen peroxide production in both patient groups was normal, with again a lower level in the untreated Crohn's disease patients (data not shown). This decrease was not significant, however, in contrast with our previous study, probably as a result of the differences in the disease activity of the untreated patients in both studies or indicating heterogeneity for this defect.

**Table 3** Correlations between neutrophil superoxide anion (\( \text{O}_2^- \)) production, hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) production and superoxide dismutase level on the one hand and the disease activity on the other hand in patients with Crohn's disease and ulcerative colitis

<table>
<thead>
<tr>
<th></th>
<th>nmol ( \text{O}_2^- )</th>
<th>nmol ( \text{H}_2\text{O}_2 )</th>
<th>( \text{ng SOD/} \mu\text{g DNA} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crohn's disease</td>
<td>( r = -0.251 ) (27)*</td>
<td>( r = -0.367 ) (25)*</td>
<td>( r = -0.495 ) (43)*</td>
</tr>
<tr>
<td>Ulcerative colitis</td>
<td>( r = -0.734 ) (7)*</td>
<td>( r = -0.401 ) (6)*</td>
<td>( r = -0.768 ) (12)*</td>
</tr>
</tbody>
</table>

* significance \( p < 0.05 \); t significance \( p < 0.01 \); tnot significant \( p > 0.1 \). Figures in parentheses indicate numbers studied.

The elastase activity of the neutrophils was normal. Therefore, it appears that neutrophils of patients with Crohn's disease and ulcerative colitis have an impaired function related to the oxidative system of the cells which was also found to be negatively correlated with the disease activity. The latter suggests that the neutrophil impairment tends to aggravate with the severity of the disease.

Oxygen radicals are known to play an important role in the intracellular killing of microorganisms by phagocytes.\(^\text{21}\) As the toxic radicals are also produced extracellularly, however, they are directly involved in inflammation and could cause tissue damage and ischaemia.\(^\text{22}\) It has been shown, for instance, that in intestinal ischaemia in cats, in degenerative joint disease, and in destructive lung disease in man, toxic oxygen radicals are important mediators of the inflammatory process.\(^\text{12, 23-24}\) Recent studies on the neutrophil function in Crohn's disease and ulcerative colitis revealed several impairments in relation to oxygen metabolism and chemotactic activity of these cells.\(^\text{25-30, 11}\) Studies with \(^\text{115}\)Indium labelled leucocytes, however, showed rapid migration to the intestine and passing through the intestinal mucosa at the site of inflammation in both Crohn's disease and ulcerative colitis, permitting localisation of the disease process, extent, and disease activity.\(^\text{31}\) It could be that secondary to the high recruitment of neutrophils to the intestinal inflammatory process the cells from the blood might be immature and less activated. In previous studies\(^\text{10, 11}\) we found no indication for this speculation because stimulation of the cells in the presence of cytochalasin E, which normalise oxidative responses of immature neutrophils, revealed a similar diminished oxidative metabolism. It was also found that medical treatment did not affect the \( \text{O}_2^- \)
production and restored the $\text{H}_2\text{O}_2$ production.

Despite the diminished production of oxygen metabolites — that is, superoxide anions, we found the accumulation of these cells at the site of the intestinal inflammation may result in a high local production of these toxic oxygen radicals. This, in itself, would not be harmful, but when the local protective superoxide dismutase level is reduced this could cause tissue damage and ischaemia like reactions. That this process actually can take place is shown by the protective effect of superoxide dismutase administration on experimental intestinal ischaemic tissue damage of the cat and on the beneficial effect of local SOD treatment on degenerative joint disease in man. In Crohn’s disease and ulcerative colitis similar oxygen related processes might play a role as in a preliminary study it was shown by Emerit et al that local administration of superoxide dismutase, encapsulated in liposomes, resulted in an impressive improvement of the intestinal disease process. Moreover, it has been shown by Miyachi et al that sulphasalazine and its metabolites have in vitro anti-oxidant activity to oxygen metabolites produced by neutrophils. It is therefore possible that the local administration of superoxide dismutase, a specific scavenger, will overcome the deficit in superoxide dismutase in the neutrophils at the site of inflammation.

The decrease in neutrophil superoxide dismutase concentrations seems not to be specific for inflammatory bowel disease as it has also been shown in neutrophils of patients with rheumatoid arthritis, in contrast with the increased superoxide dismutase activity in neutrophils of children with bacterial infections.

Adeyemi et al recently reported the interesting finding of increased concentrations of leucocyte elastase in the circulation from patients with inflammatory bowel disease. In the present study we showed that neutrophil elastase concentrations from patients with Crohn’s disease and ulcerative colitis were similar to those of the controls. Thus, the increased serum concentrations of leucocyte elastase found by Adeyemi et al may be caused either by a higher secretion by the neutrophils or by a higher turnover of the cells themselves. Harmful effects of elastase are probably immediately counteracted in serum by antiproteases, however, especially the acute phase protein $\alpha_1$-antitrypsin, which are usually raised in Crohn’s disease and ulcerative colitis during active inflammation.

From the present study it is concluded that patients with Crohn’s disease and ulcerative colitis have a diminished neutrophil function as shown by an impaired superoxide anion production and reduced superoxide dismutase concentrations. Although studies at the intestinal level will have to be undertaken to assess the full significance of these findings, the present observations may have potential implications for the treatment of the inflammatory reaction in Crohn’s disease and ulcerative colitis, as they may be the biological base for the use of SOD in the acute phase of the disease.

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