Partial defect of neutrophil oxidative metabolism in Crohn’s disease

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SUMMARY
Polymorphonuclear leucocytes of patients with untreated Crohn’s disease showed a lower level of oxidative metabolism than polymorphonuclear leucocytes of treated Crohn’s disease patients and controls. Whereas the production of superoxide anion (O$_2^-$) in Crohn’s disease patients was almost normal, polymorphonuclear leucocytes of untreated Crohn’s disease patients showed a significantly deficient production of hydrogen peroxide (H$_2$O$_2$). In the medically treated Crohn’s disease patients, a significant negative correlation was found between H$_2$O$_2$ production by polymorphonuclear leucocytes and disease activity. These findings suggest an intrinsic cellular defect in the neutrophils of Crohn’s disease patients which, together with the decreased locomotor function of these cells in vivo, might contribute to the pathogenesis of the chronic inflammation and granuloma formation in this disease.

Leucocytes are believed to play an important role in the pathogenesis of Crohn’s disease.$^1$ Defective chemotaxis in vivo and an impairment of the phagocytic process are thought to be involved in the formation of the granuloma.$^2$ Using the skin window technique, several groups have found reduced migration of polymorphonuclear leucocytes in Crohn’s disease.$^3$-5 This defect might explain the relative paucity of polymorphonuclear leucocyte infiltration into mucosa affected by Crohn’s disease.

One of the most prominent histopathological features of Crohn’s disease is the presence of granulomas in the gastrointestinal tract, but similar findings have been made in chronic granulomatous disease.$^6$ The prime defect in chronic granulomatous disease concerns the oxidative metabolism – that is, a neutrophil dysfunction which is characterised by a defect in the production of microbicidal oxygen metabolites – for example, superoxide anion (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$); during phagocytosis. It has been postulated that this defective primary defence mechanism is responsible for the granulomatous reactions seen in chronic granulomatous disease.$^6$

These findings raised the question whether the oxidative burst of polymorphonuclear leucocytes is also defective in Crohn’s disease patients. Previous studies had shown that during phagocytosis the nitro blue tetrazolium reduction could be either normal, enhanced, or reduced, and that the luminescence was impaired,$^4$ $^7$-$^9$ but these results were not conclusive, probably because of the ‘non-discriminative’ techniques used. The aim of the present study was to evaluate the oxidative capacity of polymorphonuclear leucocytes from Crohn’s disease patients, with the use of more specific techniques to measure O$_2^-$ and H$_2$O$_2$ production separately.

Methods

Patients
The present series comprised 43 patients with Crohn’s disease. The diagnosis was based on characteristic clinical, endoscopic, histological, and radiological features. The mean age of the 43 Crohn’s disease patients (24 women, 19 men) was 33 years (range 19–67 years). In 14 cases the disease was limited to the ileum, in eight to the colon, and in 21 had ileocolonic involvement. Nineteen patients were taking salazopyrine, five were on corticosteroids, and 10 were on a combination of salazopyrine and corticosteroids. Nine patients had not taken any medication for at least six months because of clinical remission in the past, seven visited the outpatient clinic for routine control and were still in remission, and two were hospitalised owing to clinical relapse.

At the time of venepuncture, disease activity was
evaluated in all of the Crohn’s disease patients according to the Crohn’s Disease Activity Index (CDAI). The control group comprised 29 healthy subjects (14 women, 15 men) with a mean age of 32 years (range 22–51 years).

**OXYGEN METABOLITES PRODUCTION**

Polymorphonuclear leucocytes were isolated from heparinised blood by Ficoll-Hypaque separation and a two-fold lysis of the erythrocytes in buffered ammonium chloride. The cells were washed three times with Hanks’ balanced salt solution containing 0.2% bovine albumin, and counted in a haemocytometer. The polymorphonuclear leucocytes suspension was >96% pure, the remainder being a few contaminating lymphocytes (<4%) and monocytes (<1%). Superoxide anion (O$_2^-$) production was determined as the reduction of ferricytochrome C (cyt C; Sigma). In brief, 1×10$^6$ polymorphonuclear leucocytes were incubated at 37°C in 1 ml Hanks’ balanced salt solution containing 100 μM cyt C and a respiratory stimulus. We determined the O$_2^-$ production with and without stimulation. The stimuli used were: 5 μg phorbol-myristate-acetate, 50 μg concanavalin A (con A) alone or in combination with 10 μg cytochalasin E, and 50 μg lipid polysaccharide derived from *Escherichia coli* 0127:B8 (Difco). The incubations were not performed in duplicates because in most cases too few polymorphonuclear leucocytes were available. The incubation time was 30 minutes except for phorbol-myristate-acetate, for which it was five minutes. All reactions were stopped by rapid chilling of the reaction mixture and cooled centrifugation. Production of O$_2^-$ or reduction of cytochrome C was measured spectrophotometrically and calculated with the extinction coefficient ε$_{550}$=2.1×10$^5$/M/cm. Cell free reaction mixture served as blank. The reproducibility of this assay was 9.2% as determined by quadruplicate incubations with the same stimulus and expressed as intra-assay coefficient of variance:

$$\text{St dev} \times 100 \text{ mean}$$

Hydrogen peroxide (H$_2$O$_2$) production was determined as the oxidation of homovanillic acid, which becomes fluorescent. In brief, 1.5×10$^6$ polymorphonuclear leucocytes were incubated in 3 ml Hanks’ balanced salt solution containing 0.8 mM homovanillic acid, 60 μg horseradish peroxidase, and 2 mM sodium azide (Na$_3$N), to inhibit endogenous cellular peroxidase and catalase activity. Incubation conditions and stimuli were the same as for O$_2^-$, except for phorbol-myristate-acetate, for which the incubation time was also 30 minutes. The fluorescent product was determined fluorospectrophotometrically ($\lambda_{ex}$=315 nm; $\lambda_{em}$=425 nm). Standards of H$_2$O$_2$ were used to determine the amount of H$_2$O$_2$ produced by the cells. The intra-assay coefficient of variance was 5.8%.

**STATISTICAL ANALYSIS**

For statistical comparison, the average values of the Crohn’s disease and control groups were evaluated by Student’s t test. The results are expressed as the mean ± SEM. The linear correlation between cellular activity and disease activity was evaluated by the Pearson correlation procedure.

**Results**

Under all test conditions except phorbol-myristate-acetate stimulation, the mean release of O$_2^-$ by polymorphonuclear leucocytes from the Crohn’s disease groups was the same as for controls (Fig. 1). The maximum stimulation by phorbol-myristate-acetate led to decreased production of O$_2^-$ by the patients’ polymorphonuclear leucocytes (mean 430±91 nmol for untreated and 427±33 nmol for treated Crohn’s disease patients, compared with 607±67 nmol for the controls). This difference reached significance (p<0.05) for the treated group. Concanavalin A incubation revealed no difference in O$_2^-$ production between the control and disease groups. Although cyt E potentiated the con A response distinctly (five to six-fold), as has been shown already by several other studies, the response in the Crohn’s disease groups turned out to be the same as in the control group. Lipopolysaccharide, a potent stimulus for the oxidative metabolism of monocytes (unpublished observations), proved not to be a pre-eminent oxidative stimulus for polymorphonuclear leucocytes as it caused only a moderate (11 to 33%) and non-significant increase in the O$_2^-$ production in all groups with no differences between the groups.

The production of the other oxygen metabolite – that is, H$_2$O$_2$ – was generally depressed in the untreated Crohn’s disease patients, except after stimulation with con A–cyt E (Fig. 2). This impaired H$_2$O$_2$ production was found to be related to both the treated Crohn’s disease group and the control group. For instance, after stimulation with phorbol-myristate-acetate the mean H$_2$O$_2$ production was 52±6 nmol in the untreated Crohn’s disease group, 62±3 nmol in the treated Crohn’s disease group, and 64±2 nmol in the control group. Lipopolysaccharide incubation resulted in a two-fold increase of the H$_2$O$_2$ production in the untreated Crohn’s
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Fig. 1 Superoxide anion (O$_2^-$) production by polymorphonuclear leucocytes of Crohn's disease patients and controls after incubation with PMA (phorbol-myristate-acetate), LPS (lipopolysaccharide from E coli 0127:B8), and con A (concanavalin A) with or without cyt E (cytochalasin E). O$_2^-$ production expressed in nmol/30 min/3x10$^6$ polymorphonuclear leucocytes (mean ± SEM). * CD treated vs controls, p<0.05.

disease group but the mean of the group was still lower than that of the control group and the treated Crohn's disease group (p<0.02). The impairment was seen not only after stimulation of the cells but even in unstimulated cells, which represent the basic state of activity of the cells. In contrast, the polymorphonuclear leucocytes of treated Crohn's disease patients showed normal or even enhanced H$_2$O$_2$ production (see Fig. 2).

The linear relationship between disease activity (CDAI) and the production of oxygen metabolites was also analysed for each of the tests mentioned above. A significant negative correlation was found between the CDAI and H$_2$O$_2$ produced by polymorphonuclear leucocytes after stimulation with phorbol-myristate-acetate (r=-0.575; p<0.01) in the treated Crohn's disease group (Fig. 3). This negative correlation persisted when the two Crohn's disease groups (treated and untreated) were combined (r=-0.373; p<0.05), although production of H$_2$O$_2$ in the latter group was clearly defective. Under the same conditions we also found a negative correlation between the CDAI and O$_2^-$ production (r=-0.237 and r=-0.149, respectively) but in neither case was this correlation significant (p>0.1).

Discussion

Several studies on leucocytes in Crohn's disease have shown depressed movement of polymorphonuclear leucocytes into skin windows, and as no other cellular defect was found, some of the authors ascribed this limited motility to an impaired local inflammatory response. Our untreated Crohn's disease patients, however, showed defective production of H$_2$O$_2$ by polymorphonuclear leucocytes. Although the polymorphonuclear leucocytes of the treated Crohn's disease patients produced normal...
amounts of H₂O₂, there was a significant negative correlation between disease activity and H₂O₂ production. These findings suggest that in Crohn's disease there is at least a partial intrinsic cellular defect that impairs the production of oxygen metabolites by leucocytes, which might be a primary phenomenon in this disease. As this defect can be corrected by medical treatment, however, it could also be a secondary effect on the primary intestinal process. Follow-up studies on Crohn's disease patients with and without medical treatment will help to elucidate the relationship between this polymorphonuclear leucocyte defect and Crohn's disease. The restoration of oxidative metabolism by medication could be either because of a direct effect on the polymorphonuclear leucocytes or an influence on the production of polymorphonuclear leucocytes in the bone marrow. The specificity of our findings requires evaluation in further studies including patients with recurrent infection and other chronic inflammatory conditions such as ulcerative colitis and primary biliary cirrhosis.

It is interesting that in some cases of chronic granulomatous disease, a disease in which the phagocytic cells are incapable of mounting an oxidative burst,12 15 19 20 rectal biopsy specimens showed granulomas which on the basis of morphological criteria could not be differentiated from granulomas seen in Crohn's disease.5 The defect we found seems to be less severe than that in chronic granulomatous disease, as the polymorphonuclear leucocytes did not show abolition of oxidative metabolism but rather an almost normal production of the oxygen radical O₂⁻ and a deficiency in the H₂O₂ production.

In some of the studies on leucocytes in Crohn's disease, both nitro blue tetrazolium reduction and luminescence were found to be impaired,4 8 9 but the techniques used could not discriminate between the different oxygen metabolites produced, and a negative correlation with disease activity was not found. The use of more specific assays to measure the production of both O₂⁻ and H₂O₂ by leucocytes in Crohn's disease enabled us to detect a partial defect in the oxidative burst. Thus, Crohn's disease resembles chronic granulomatous disease as to the impaired leucocyte function, which together with the locomotor dysfunction of the polymorphonuclear leucocytes might give rise to granuloma formation in the intestine as a compensatory mechanism for the clearance of foreign material.

The defect found in the oxidative metabolism in Crohn's disease also appears to differ from that found in certain patients suffering from recurrent bacterial infections.21 22 In these patients, unstimulated polymorphonuclear leucocytes produced low levels of O₂⁻ but normal or raised levels of H₂O₂,21 and under stimulation the production of O₂⁻ was normal or impaired, depending on the stimulus used. These findings indicate that there must be more than one mechanism for the initiation of oxidative metabolism, and also that different pathways are followed for the production of the different metabolites of the respiratory burst. Leucocyte H₂O₂ might be formed by spontaneous or catalysed dismutation of superoxide anion or by
direct divalent reduction of oxygen. The present study has provided further evidence that polymorphonuclear leukocytes have another pathway for the production of H$_2$O$_2$ besides the dismutation of O$_2$. These findings indicate the interesting occurrence of depressed H$_2$O$_2$ production in the presence of normal O$_2$ production in Crohn's disease. Further studies on the oxidative metabolism of polymorphonuclear leukocytes in Crohn's disease may help to elucidate the basic defect and yield new insights into the pathogenesis of Crohn's disease.

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References