Partial defect of neutrophil oxidative metabolism in Crohn's disease

H W VERSPAGET, M A C MIEREMET-OOMS, I T WETERMAN, AND A S PEÑA

From the Department of Gastroenterology, University Hospital, Leiden, The Netherlands

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_2O_2). In the medically treated Crohn's disease patients, a significant negative correlation was found between H_2O_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_2O_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_2O_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 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

Address for correspondence: Dr H W Verspaget, Dept of Gastroenterology, Academisch Ziekenhuis Leiden, Rijnsburgerweg 10, 2333AA, Leiden, The Netherlands.

Received for publication 2 November 1983

evaluated in all of the Crohn's disease patients according to the Crohn's Disease Activity Index (CDAI).¹⁰

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.¹¹ 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).¹² In brief, 1×10^6 polymorphonuclear leucocytes were incubated at 37°C in 1 ml Hanks' balanced salt solution containing 100 μ M cyt C and a respiratory stimulus. We determined the O_2^- production with and without stimulation. The stimuli used were: 5 μ g phorbol-myristate-acetate,¹³ 50 μ g concanavalin A¹⁴ (con A) alone or in combination with 10 μ g cytochalasin E¹⁵ (cyt E; Sigma), and 500 μ g lipopolysaccharide 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 $E_{550} = 2.1 \times 10^4$ 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:

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

Hydrogen peroxide (H_2O_2) production was determined as the oxidation of homovanillic acid, which becomes fluorescent.^{16,17} In brief, 1.5×10^6 polymorphonuclear leucocytes were incubated in 3 ml Hanks' balanced salt solution containing 0.8 mM homovanillic acid, 60 μ g horseradish peroxidase, and 2 mM sodium azide (NaN_3), 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_2O_2 were used to determine the amount of H_2O_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 $\pm$ 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,^{14,15} 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_2O_2 – was generally depressed in the untreated Crohn's disease patients, except after stimulation with con A–cyt E (Fig. 2). This impaired H_2O_2 production was found relative to both the treated Crohn's disease group and the control group. For instance, after stimulation with phorbol-myristate-acetate the mean H_2O_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_2O_2 production in the untreated Crohn's

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/ 3×10^6 polymorphonuclear leucocytes (mean $\pm$ 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_2O_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_2O_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 com-

bined ($r = -0.373$; $p < 0.05$), although production of H_2O_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.^{4 5 18} Our untreated Crohn's disease patients, however, showed defective production of H_2O_2 by polymorphonuclear leucocytes. Although the polymorphonuclear leucocytes of the treated Crohn's disease patients produced normal

Fig. 2 Hydrogen peroxide (H_2O_2) production by polymorphonuclear leucocytes of Crohn's disease patients and controls after incubation with and without respiratory stimuli. H_2O_2 production expressed in nmol/30 min/ 3×10^6 polymorphonuclear leucocytes (mean $\pm$ SEM). * CD untreated vs controls, $p < 0.05$; • CD untreated vs CD treated, $p < 0.02$; * CD treated vs controls, $p < 0.05$.

Fig. 3 Linear correlation between H_2O_2 production by polymorphonuclear leucocytes, after stimulation with phorbol-myristate-acetate, and disease activity (CDAI) in treated Crohn's disease patients. H_2O_2 production expressed in $nmol/30\text{ min}/3 \times 10^6$ polymorphonuclear leucocytes.

amounts of H_2O_2 , there was a significant negative correlation between disease activity and H_2O_2 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.⁶ 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 O_2^- and a deficiency in the H_2O_2 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 O_2^- and H_2O_2 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 O_2^- but normal or raised levels of H_2O_2 ;²¹ and under stimulation the production of O_2^- 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 H_2O_2 might be formed by spontaneous or catalysed dismutation of superoxide anion or by

direct divalent reduction of oxygen.^{23 24} The present study has provided further evidence that polymorphonuclear leucocytes have another pathway for the production of H₂O₂ besides the dismutation of O₂⁻.

These findings indicate the interesting occurrence of depressed H₂O₂ production in the presence of normal O₂⁻ production in Crohn's disease. Further studies on the oxidative metabolism of polymorphonuclear leucocytes in Crohn's disease may help to elucidate the basic defect and yield new insights into the pathogenesis of Crohn's disease.

This study was presented in part at the World Congress of Gastroenterology, Stockholm, Sweden, in June 1982. The authors wish to thank Dr W Beeken for a critical reading of the manuscript and Gabrielle W M Verhoef for typing and Mrs I Seeger for reading the English text.

References

- 1 Ward M. The pathogenesis of Crohn's disease. *Lancet* 1977; **2**: 903-6.
- 2 Ward M. Pathocytic function in Crohn's disease. *Z Gastroenterol* 1979; **17** suppl: 116-24.
- 3 Segal AW, Loewi G. Neutrophil dysfunction in Crohn's disease. *Lancet* 1976; **2**: 219-21.
- 4 Wandall JH, Binder V. Leucocyte function in Crohn's disease. *Gut* 1982; **23**: 173-80.
- 5 O'Morain C, Segal AA, Walker D, Levi AJ. Abnormalities of neutrophil function do not cause the migration defect in Crohn's disease. *Gut* 1981; **22**: 817-22.
- 6 Ament ME, Ochs HD. Gastrointestinal manifestations of chronic granulomatous disease. *N Engl J Med* 1973; **288**: 382-7.
- 7 Ward M, Eastwood MA. The nitro blue tetrazolium test in Crohn's disease and ulcerative colitis. *Digestion* 1976; **14**: 179-83.
- 8 Worsaae N, Staehr Johansen K, Christensen KC. Impaired in vitro function of neutrophils in Crohn's disease. *Scand J Gastroenterol* 1982; **17**: 91-7.
- 9 Kelleher D, Feighery C, Weir DG. Neutrophil luminescence in chronic inflammatory bowel disease (CIBD). [Abstract] *Gut* 1982; **23**: A895-T22.
- 10 Best WR, Beckett JM, Singleton JW, Kern F. Development of a Crohn's disease activity index (National cooperative Crohn's disease study). *Gastroenterology* 1976; **70**: 439-44.
- 11 Roos D, Loos JA. Changes in the carbohydrate metabolism of mitogenetically stimulated human peripheral lymphocytes. *Biochem Biophys Acta* 1970; **222**: 565-82.
- 12 Johnston RB, Keele BB, Misra HP *et al.* The role of superoxide anion generation in phagocytic bacterial activity. Studies with normal and chronic granulomatous disease leucocytes. *J Clin Invest* 1975; **55**: 1357-72.
- 13 DeChatelet LR, Shirley PS, Johnston RB. Effect of Phorbol myristate acetate on the oxidative metabolism of human polymorphonuclear leucocytes. *Blood* 1976; **47**: 545-55.
- 14 Zabucchi G, Berton G, Soranzo MR. Mechanism of the potentiating effect of cytochalasin B on the respiratory burst induced by Concanavalin A in leucocytes. *FEBS Lett* 1982; **125**: 165-70.
- 15 Nakagawara A, Nabi FBZ, Minakani S. An improved procedure for the diagnosis of chronic granulomatous disease, using concanavalin A and cytochalasin E. *Clin Chim Acta* 1977; **74**: 173-6.
- 16 Rossi F, Bellavite P, Dobrina A, Dri P, Zabucchi G. Oxidative metabolism of mononuclear phagocytes. In: van Furth R, ed. *Mononuclear phagocytes: functional aspects*. The Hague: Martinus Nijhoff, 1980: 1187-214.
- 17 Guilbault CG, Brignac P, Zimmer M. Homovanillic acid as a fluorometric substrate for oxidative enzymes. *Anal Chem* 1968; **40**: 190-7.
- 18 D'Amalio R, Rossi P, LeMoli S, Ricci R, Montano S, Pallone P. In vitro studies on cellular and humoral chemotaxis in Crohn's disease using the under agarose gel technique. *Gut* 1980; **22**: 566-70.
- 19 Nathan DG, Baehner RL, Weaver DK. Failure of nitro blue tetrazolium reduction in the phagocytic vacuoles of leucocytes in chronic granulomatous disease. *J Clin Invest* 1969; **48**: 1895-904.
- 20 Takeshige K, Matsumoto T, Shibata R, Minakani S. Simple and rapid method for the diagnosis of chronic granulomatous disease, measuring hydrogen peroxide and superoxide anions released from leukocytes in whole blood. *Clin Chim Acta* 1979; **92**: 329-35.
- 21 Weening RS, Roos D, Weemaes CMR, Homan-Müller JWT, van Schaik MLJ. Defective initiation of the metabolic stimulation in phagocytizing granulocytes: a new congenital defect. *J Lab Clin Med* 1976; **88**: 757-68.
- 22 Harvath L, Andersen BR. Defective initiation of oxidative metabolism in polymorphonuclear leucocytes. *N Engl J Med* 1979; **300**: 1130-5.
- 23 Nathan CF. The release of hydrogen peroxide from mononuclear phagocytes and its role in extracellular cytotoxicity. In: van Furth R, ed. *Mononuclear phagocytes: functional aspects*. The Hague: Martinus Nijhoff, 1980: 1166-83.
- 24 Rosen H, Klebanoff SJ. Bactericidal activity of a superoxide anion generating system. A model for the polymorphonuclear leukocyte. *J Exp Med* 1979; **149**: 27-39.