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Superoxide production by Crohn's disease neutrophils

F T Curran, R N Allan, M R B Keighley

Abstract

Neutrophil superoxide anion production was measured in healthy subjects and in patients with quiescent and active Crohn's disease using superoxide dismutase inhibitable cytochrome C reduction. Three stimuli were used: phorbol 12-myristate 13-acetate (PMA1), phorbol 20-oxo-20-deoxy 12-myristate 13-acetate (PMA2), and *Candida albicans* in serum. Normal neutrophils produced significantly more superoxide anion than Crohn's disease neutrophils with both PMA1 (mean (SD) 9·6 (2·2) *v* 8·6 (1·8) nmol/10⁶ cells/5 minutes, p=0·04) and PMA2 (1·8 (0·8) *v* 0·8 (0·77) nmol/10⁶ cells/5 minutes, p=0·00004). With *C albicans* in serum, normal and Crohn's disease neutrophils produced similar amounts of superoxide anion (4·4 (1·5) *v* 4·3 (1·7) nmol/10⁶ cells/30 minutes, not significant). Results were independent of disease activity. Superoxide anion production by PMA-stimulated Crohn's disease neutrophils is significantly lower than by normal neutrophils.

Although neutrophil metabolism is based largely on anaerobic glycolysis an increase in oxygen consumption can be shown during phagocytosis.[1-3] This respiratory burst is necessary for the subsequent killing of micro-organisms; it coincides with the activation of the oxidase enzyme system which transfers a single electron from reduced nicotinamide adenine dinucleotide to the oxygen molecules, forming superoxide anion (the NADPH oxidase system).[4] Superoxide anion production was measured to determine whether it could be the cause of the impaired killing ability by neutrophils in Crohn's disease, which we have previously reported.[5]

Details of healthy subjects and patients with Crohn's disease

	Healthy subjects	*Crohn's disease*
Superoxide production with PMA1:		
No	15	25
Mean (SD) age (years)	45 (14)	42 (19)
Sex	8M:7F	9M:16F
Disease activity	–	Quiescent 15 Active 10
Steroid treatment	–	4
Superoxide production with PMA2:		
No	15	25
Mean (SD) age (years)	52 (17)	40 (16)
Sex	7M:8F	10M:15F
Disease activity	–	Quiescent 15 Active 10
Steroid treatment	–	3
Superoxide production with C albicans in serum:		
No	15	25
Mean (SD) age (years)	42 (15)	43 (17)
Sex	9M:6F	9M:16F
Disease activity	–	Quiescent 14 Active 11
Steroid treatment	–	4

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Superoxide production was determined by the spectrophotometric measurement of superoxide dismutase inhibitable cytochrome C reduction. Superoxide reduces cytochrome C[6] but other electron donors are also produced during phagocytosis which can similarly reduce cytochrome C. Superoxide dismutase is a copper-containing enzyme occurring in red and white blood cells. It destroys superoxide by catalysing its conversion to oxygen and hydrogen peroxide. In vivo the enzyme prevents local tissue damage by toxic radicals.[7] The use of superoxide dismutase in the assay makes it specific for superoxide since any remaining cytochrome C reduction must be the result of other electron donor systems and can be deducted from assays performed simultaneously without superoxide dismutase.

Methods

Fifteen healthy volunteers and 25 patients with Crohn's disease were studied using three different stimuli of phagocytosis. Patient details are summarised in the Table. Disease activity was assessed using the method of Harvey and Bradshaw,[8] and patients taking sulphasalazine were excluded from the study.

PREPARATION OF NEUTROPHILS

Neutrophils were isolated from fresh venous blood using discontinuous density gradients of Percoll (Pharmacia) as follows. An iso-osmotic stock solution of Percoll was made by adding 1 part 1·5 M saline to 9 parts Percoll (resulting density 1·123 g/ml). Stock Percoll was further diluted with 0·15 M saline to densities of 1·070 g/ml[9] and 1·097 g/ml[10] for the separation of white cells. Into a sterile universal container were placed 6 ml Percoll of density 1·097 g/ml, then 4 ml Percoll of density 1·070 g/ml was carefully layered on top taking care not to cause mixing at the interphase. Five ml of heparinised blood was diluted with an equal volume of 0·15 M saline and carefully layered on top of the Percoll in the same way. The tube was centrifuged at 200 *g* for 25 minutes, resulting in the isopycnic banding of cells at the relevant interphases; granulocytes were suspended between the two layers of Percoll. They were removed with a pipette and washed twice in Dulbecco's B phosphate buffered saline, centrifuging at 1500 *g* for 10 minutes after each wash. Any contaminating erythrocytes were lysed by hypotonic shock treatment by resuspending the cells in tris-ammonium chloride for 10 minutes before the second wash. Neutrophils were counted in an improved Neubauer chamber and resuspended in PBS at a concentration of 5×10^6 cells per ml. All neutrophil suspensions were at least 95%

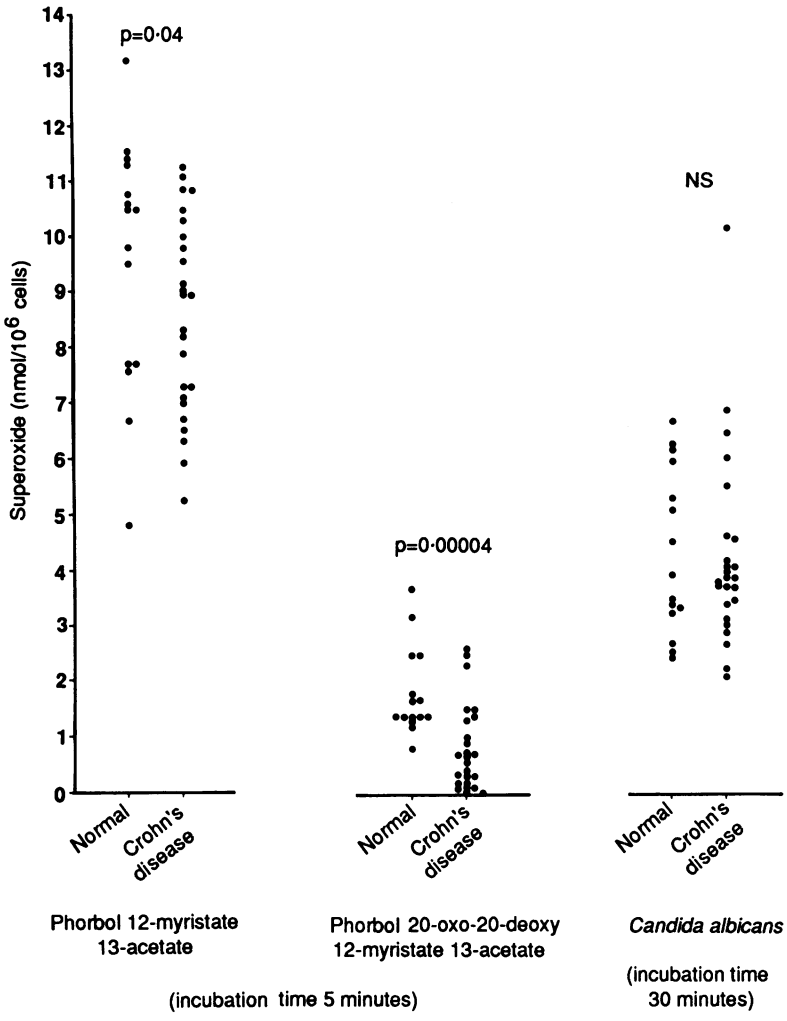

Figure 1: Neutrophil superoxide anion production.

pure, and their viability was 98% when assessed by trypan blue exclusion tests.[11]

PREPARATION OF ORGANISMS

Candida albicans from the same source was used in all experiments. The organisms were isolated from the faeces of a healthy woman receiving no medication and were maintained in stock culture on a slope of malt extract agar (Oxoid) at room temperature. When required, a sample of the organisms was inoculated into approximately 50 ml Sabouraud liquid medium (Oxoid) and grown to a stationary phase for three to five days at 30°C. They were washed twice in PBS, centrifuged at 1500 *g* for 10 minutes after each wash, counted in an improved Neubauer haemocytometer, and suspended in PBS at a concentration of 10^7 organisms per ml. Freshly cultured organisms were used in all tests.

MEASUREMENT OF SUPEROXIDE PRODUCTION

A 1·4 ml neutrophil suspension (7×10^6 cells) was placed in each of two tubes and 30 μg superoxide dismutase in 10 μl PBS was added to one tube. Both tubes were incubated at 37°C in a water bath for two minutes. To each tube was added 3 mg horse heart cytochrome C type VI (Sigma) in 0·1 ml PBS, followed immediately by a stimulus of phagocytosis. The stimuli used were: (a) 5 μg PMA1 in 1·5 ml PBS; (b) 5 μg PMA2 in 1·5 ml PBS; (c) 10^7 *C albicans* in 1 ml PBS+0·5 ml fresh normal serum. In addition, serum stimulated superoxide release – that is, without *Candida* – was assessed on two occasions, once with normal neutrophils and once with Crohn's disease neutrophils.

After mixing, 1·5 ml from each tube was reserved in melting ice (time 0) and the remaining 1·5 ml incubated at 37°C in a water bath for five minutes with PMA or 30 minutes with *Candida*. The reaction was stopped by placing the incubated tubes in melting ice. All four tubes were centrifuged at 4°C for 20 minutes to remove cells and the absorbances of the supernatants read in a single beam spectrophotometer (Cecil) using the unincubated (time 0) samples as blank. Thus for each assay two results were obtained, one with superoxide dismutase added and one without it.

Superoxide anion production was calculated from the absorbances using the formula:

$$\frac{A\times\text{reaction volume}}{E\times\text{number of cells}\times\text{light path}}$$

where A=(absorbance at 550 nm without superoxide dismutase)−(absorbance at 550 nm with superoxide dismutase), reaction volume=1·5 ml, E=extinction coefficient of cytochrome C=$2\cdot11\times10^4$/M/cm, number of cells=$3\cdot5\times10^6$ per assay, and light path=1 cm.

Results are expressed as superoxide production per incubation time rather than per minute because superoxide release from neutrophils is not linear with time. There is a lag time of approximately 60 seconds followed by a gradual rise until a linear rate of change is achieved which is dependent on the stimulus, and finally a termination phase.[11-13]

REPRODUCIBILITY OF THE ASSAY

This was measured by performing triplicate assays on three occasions, twice using normal neutrophils and once using Crohn's disease neutrophils. The stimulus used for these reproducibility studies was 5 μg PMA1. The coefficients of variance of the three assays were 0·8%, 7·3%, and 0·8%.

STATISTICAL ANALYSIS

Results were analysed using the Wilcoxon rank sum test and are expressed as means (SD).

Results

Superoxide production by normal neutrophils was significantly greater than Crohn's disease neutrophils when PMA1 was the stimulus (9·6 (2·2) *v* 8·6 (1·8) nmol/10^6 cells/5 minutes, p=0·04). PMA2 stimulated far less superoxide production than PMA1, but normal neutrophils still produced more than Crohn's disease neutrophils (1·8 (0·8) *v* 0·8 (0·77) nmol/10^6 cells/5 minutes, p=0·00004) (Fig 1).

When phagocytosis was stimulated by *C albicans* in serum, however, superoxide production by normal neutrophils (4·4 (1·5) nmol/10^6

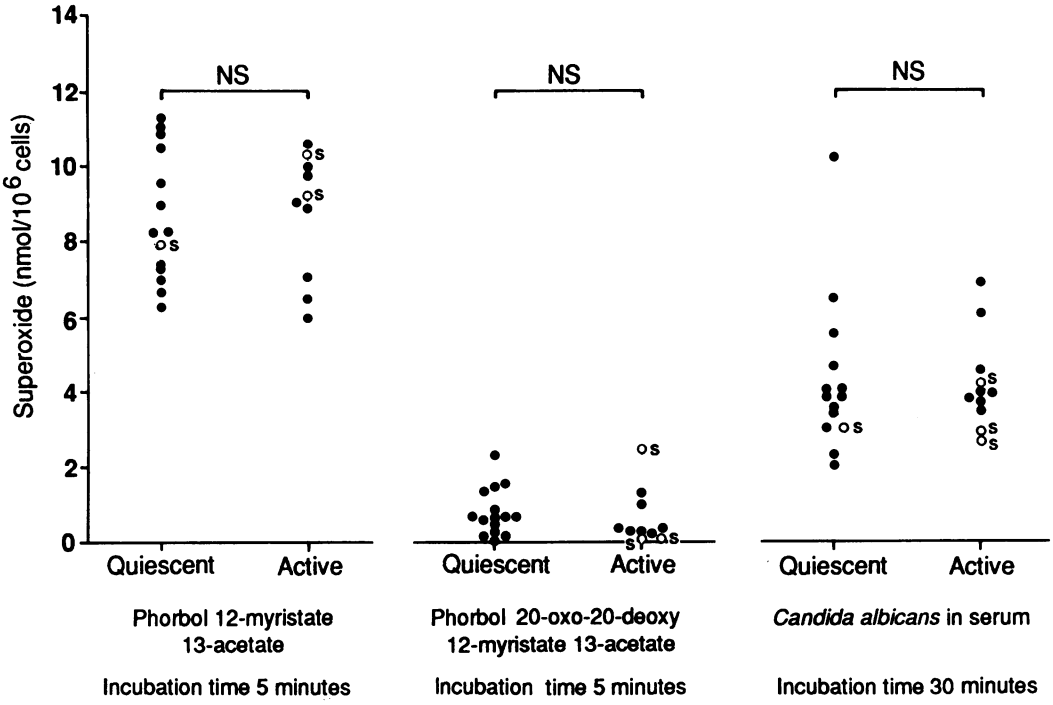

Figure 2: Neutrophil superoxide anion production related to disease activity. O=patient on steroids.

cells/30 minutes) did not differ significantly from that by Crohn's disease neutrophils (4·3 (1·7) nmol/10^6 cells/30 minutes). In serum alone both normal and Crohn's disease neutrophils produced superoxide at a rate of 1·2 nmol/10^6 cells/30 min (Fig 1).

The results were independent of disease activity (Fig 2).

Discussion

Superoxide anion production by neutrophils was first shown by Babior *et al* in 1973.[14] It is well established that oxygen radicals play an important part in killing phagocytosed micro-organisms.[1] The NADPH-oxidase system can be activated by several pathways.[15] Phorbol myristate acetate (PMA) is the active component of croton oil and the addition of microgram quantities to a suspension of resting human neutrophils causes a pronounced stimulation of all aspects of cellular oxidative metabolism which are normally associated with phagocytosis.[16]

The importance of an intact NADPH oxidase system is shown by patients with chronic granulomatous disease. They have inherited a defective oxidase which cannot generate sufficient superoxide and hydrogen peroxide, resulting in severe and recurrent bacterial infections.[17,18] The precise mechanism by which oxygen metabolites kill micro-organisms is uncertain but suggested methods include lipid peroxidation of the cell membrane, inactivation of intracellular enzyme systems, and DNA damage.[4]

Superoxide anions can also react with peroxidase; the aerobic oxidation of NADH by myeloperoxidase requires superoxide.[19] The killing of *C albicans* and some bacteria is dependent on this myeloperoxidase system and myeloperoxidase deficient neutrophils do not kill *Candida* in vitro.[20-22]

These results support the findings of Verspaget *et al* that superoxide production by Crohn's disease neutrophils is significantly lower than normal when phorbol myristate acetate is the phagocytic stimulus.[23-25] PMA2 stimulated far less superoxide production than PMA1, but the reasons for this are uncertain. Using a chemiluminescence assay, a technique not specific for superoxide anion, Suematsu *et al* found no difference between normal and Crohn's disease neutrophils.[26] Although it is possible that impaired oxidative metabolism accounts for the chronic inflammation, granuloma formation, and impaired microbial killing in Crohn's disease, we found that superoxide production during phagocytosis of *C albicans* in serum was normal. Thus we have no conclusive evidence that low superoxide production is the cause of the impaired candidacidal activity of Crohn's disease neutrophils which we previously reported.[5] It is of interest, however, that *C albicans* is reported to release a substance that selectively impairs the neutrophil respiratory burst without fully inhibiting the NADPH-oxidase system.[27]

Other factors, such as malnutrition or deficiency of micronutrients, can affect neutrophil function in patients with chronic disease. For this reason we investigated patients with quiescent as well as active Crohn's disease; malnutrition is unlikely to be present when patients with inflammatory bowel disease are in remission or in the early stages of an acute attack.[28] We found no relation between superoxide production and Crohn's disease activity, so it is unlikely that impaired neutrophil function is a phenomenon secondary to the disease itself. This could be clarified by studying patients with other chronic inflammatory diseases.

PMA-stimulated superoxide anion production by peripheral blood neutrophils is lower than normal in Crohn's disease. Whether this is of primary importance in the pathogenesis of the disease remains to be established.

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