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Monocyte superoxide anion production in rheumatoid arthritis: preliminary evidence for enhanced rates of superoxide anion production by monocytes from patients receiving penicillamine, sodium aurothiomalate and corticosteroids

N. P. HURST, B. BESSAC, AND G. NUKI

From the Rheumatic Diseases Unit, Department of Medicine (WGH), University of Edinburgh, Northern General Hospital, Edinburgh EH5 2DQ

SUMMARY In-vitro studies of superoxide (O_2^-) anion production by blood monocytes after stimulation with either serum treated zymosan (STZ), IgG treated zymosan (IgGTZ), or fluoride ion (F^-) were performed on cells from normal controls (n=22) and patients with classical or definite rheumatoid arthritis (RA) (n=35). Twenty-two of the patients were on nonsteroidal anti-inflammatory drugs (NSAID) alone and 13 were on either sodium aurothiomalate, penicillamine, corticosteroids, or a combination. Monocytes from RA patients on 'second-line therapy' showed significantly increased rates of O_2^- release in response to STZ compared with normal controls, but no increase was seen in monocytes from patients on NSAID alone. With IgGTZ as the stimulus, rates of O_2^- release were increased in monocytes from patients on NSAID alone compared with normal controls ($p<0{\cdot}02$), but were increased to a greater extent in monocytes from patients on second-line therapy ($p<0{\cdot}01$). There were no differences in basal unstimulated O_2^- production and no differences after stimulation with F^-. The enhanced release of O_2^- by monocytes from patients on second-line therapy could not be attributed to increased disease activity and may be an effect of therapy.

Mononuclear phagocytes, which include tissue macrophages and their precursors, blood monocytes, are thought to play an important role in chronic inflammation. Rheumatoid synovium contains numerous mononuclear phagocytes[1] which are a potential source of a variety of secretory products, including neutral proteases, lysosomal acid hydrolases, prostaglandins, leukotrienes, complement components, and oxygen-free radicals.[2] Oxygen-free radicals, which include superoxide anion (O_2^-), hydroxyl radical ($OH^\cdot$), and singlet oxygen ($^1O_2$), make an important contribution not only to host defence against infection[3] and tumour cells[4 5] but also against the tissue damage which may accompany inflammatory and immunological processes.[2 6–8] Furthermore, in RA there is biochemical evidence of free radical mediated oxidative damage, which includes reduced availability of serum-free thiol groups, reduced levels of superoxide dismutase in erythrocytes,[9] and the presence of free radical oxidation products of lipids.[10] In-vitro studies have also emphasised the potential role of oxygen-free radicals in damage to connective tissue constituents, including hyaluronic acid[11] and collagen.[12]

Phagocytosis of immune complexes by mononuclear phagocytes is a potential stimulus to oxygen-free radical release in RA, but the phagocytic event is not a necessary prerequisite for free radical generation, and the processes are functionally distinct.[13 14] Various soluble stimuli, including C5a,[14 15] can activate oxygen-free radical release, while others, including lymphokines[16] and proteases,[17 18] can enhance or 'prime' the free radical responses of phagocytes.

The capacity of mononuclear phagocytes from patients with RA to generate oxygen-free radicals has not been widely studied, and as part of an investigation of the role of the mononuclear phagocyte in

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Correspondence to Dr N. P. Hurst, Rheumatic Diseases Unit, Northern General Hospital, Ferry Road, Edinburgh EH5 2DQ.

RA we have compared O_2^- production by blood monocytes from normal controls and patients with classical or definite RA.

Materials and methods

Subjects. Twenty-two healthy hospital and laboratory employees (11 male, 11 female; mean age 40·2± (SD) 13·3 years and 35 patients with classical or definite RA were studied.[19] Twenty-two of these patients were taking NSAID alone (n=21) or no antirheumatic therapy (n=1) (11 male, 11 female; mean age 51·4± 16·6 years), and 13 patients (7 male, 6 female; mean age 50·9± 12·9 years) were taking either sodium aurothiomalate (n=3) (total dose 0·6–1·05 g), penicillamine (n=4) (125–500 mg per day), corticosteroids (n=4) (2·5–10 mg prednisolone per day or 8–10 units ACTH per day), or a combination (n=2) for at least 3 months. Eleven of the 13 patients receiving 'second-line therapy' (includes patients receiving corticosteroid therapy) were receiving NSAID in addition.

Assessment of RA disease activity. A composite score (0 to 6) to indicate increasing disease activity was assigned to each patient. The score was based on erythrocyte sedimentation rate (ESR, mm/1st hour), early morning stiffness (EMS), and a subjective global assessment with 3 categories—inactive, moderately active, and very active disease. Scores of 0, +1, and +2 were given for ESR <26, 26 to 50, and >50; EMS<1 hour, 1 to 2 hours, >2 hours; and inactive, moderately active, and very active disease respectively.

MONOCYTE SEPARATION

Indicator-free Hanks's balanced salt solution (HBSS) with 5 units/ml preservative-free heparin was used throughout.

Venous blood from each subject was anticoagulated with 10 mM EDTA. Mononuclear cells (lymphocytes and monocytes) were separated from blood according to the method of Boyum[20] on a density gradient (Lymphoprep, Nyegaard, Oslo). The mononuclear cell fraction was washed twice in HBSS and resuspended to give a final monocyte concentration of between 0·60 and $1·20\times10^9$/l. Exact concentrations of monocytes and lymphocytes in the final suspension were determined with a ZBI Coulter counter with a 70 μm aperture as previously described.[21] This method of counting numbers of monocytes and lymphocytes was validated by comparison with nonspecific esterase stains of cytocentrifuge preparations of mononuclear cells.[22] Control studies with naphthol AS–D chloroacetate esterase staining of mononuclear cells showed only insignificant contamination by neutrophil polymorphs in both normal controls and RA patients (mean 0·4%, range 0–4%).

CONTROL STUDIES ON LYMPHOCYTES

Since lymphocytes are present in large numbers in mononuclear cell preparations obtained from density gradients, control studies were carried out on enriched lymphocyte fractions to determine their contribution, if any, to O_2^- production. Lymphocytes were purified by removal of adherent cells (monocytes), and O_2^- generation was assayed as described below.

PREPARATION OF STIMULI

Zymosan was preopsonised in bulk as follows:

(i) *C3b/IgG coated zymosan (STZ).* Zymosan was incubated with fresh human serum obtained from a single donor in a ratio of 10 mg zymosan/ml serum.

(ii) *IgG coated Zymosan (IgTZ).* Zymosan was incubated in pooled human IgG (Blood Transfusion Service, Edinburgh) in a ratio of 10 mg zymosan/75 mg IgG/ml phosphate buffered saline (PBS).

Opsonisation was carried out at 37°C for 30 minutes, the zymosan washed 3 times with PBS and resuspended to 25 mg of zymosan/ml of PBS. After opsonisation the zymosan was stored in 0·25 ml aliquots in liquid nitrogen. Direct immunofluorescence staining of STZ showed the presence of IgG, IgM, and C3b, while IgGTZ showed the presence of IgG alone. Sodium fluoride (NaF)[23] was dissolved in PBS to give a 200 mM solution, with appropriate reduction of sodium chloride concentration to maintain isotonicity, and adjusted to pH 7·3.

ASSAY OF SUPEROXIDE ANION PRODUCTION

Superoxide anion (O_2^-) was measured by the reduction of horse heart ferricytochrome (type III Sigma) as previously described.[24] All experiments were performed in duplicate in plastic tubes (9×11 mm, RT 25, Sterilin). Aliquots (400 μl) of cell suspension were mixed with 50 μl cytochrome c in PBS (100 μM final) and 50 μl of the appropriate stimulus. Control tubes containing bovine superoxide dismutase (SOD) (type I, Sigma) at a final concentration of 200 μg/ml were included in each experiment. The tubes were capped and incubated at 37°C on a turntable for 60 minutes. The reaction was terminated by placing the tubes in iced water for 10 minutes and then spinning at 1000 *g* for 10 minutes. The extent of cytochrome reduction in the supernatant was measured as the change in absorbance at 550 nm after the addition of a grain of potassium ferricyanide, in a Cecil spectrometer using an extinction coefficient of 21·1 mM^{-1} cm^{-1}.[25] The total amount of cytochrome c present was obtained from the absorbance at 550 nm after the further addition of a few grains of sodium dithionite.

Control studies confirmed that for a given stimulus the reduction of cytochrome c followed first-order kinetics over 60 minutes and that the rate was directly proportional to the monocyte concentration. A rate constant K for reduction of cytochrome c by O_2^- could thus be obtained from the expression:

$$K = \frac{1}{t \times M\emptyset} \times \ln \left[\frac{\text{cyt } c^0}{\text{cyt } c^{60}} \right] \text{ ml minute}^{-1} \text{ monocyte}^{-1}$$

where t=time (minutes), Mø=monocyte concentration, and cyt c^0 and cyt c^{60} are the concentrations of oxidised cytochrome c initially and after 60 minutes incubation respectively. Rates of reduction of cytochrome c by O_2^- are expressed in terms of this rate constant.

Any cytochrome c reduction occurring in the presence of SOD was assumed to be due to reducing agents other than O_2^-, and these results are shown separately. In addition to the superoxide dismutase controls 2 additional tubes were always included, to which 50 μl PBS were added in place of stimulus. This provided a measure of basal O_2^- production by monocytes.

Statistical methods. Student's *t* test was used to make statistical comparisons.

Results

CONTROL STUDIES ON LYMPHOCYTES

Incubation of a lymphocyte-enriched cell preparation confirmed that lymphocytes make no significant contribution to O_2^- production. The rate of production of O_2^- was 1·5 nmoles of reduced cytochrome c per 10^6 lymphocytes per hour. This is equivalent to a rate constant of $0{\cdot}26 \times 10^{-9}$ ml/minute/lymphocyte and is probably due to the small numbers of contaminating monocytes in the lymphocyte-rich preparation.

PATIENT STUDIES

Basal superoxide anion production and SOD controls

There was no significant difference in mean (±SD) basal unstimulated rates of monocyte O_2^- production between normal controls (1·28±0·45 ml/min/monocyte), RA patients receiving NSAID alone (1·45±0·33 ml/min/monocyte), and RA patients on second-line therapy (1·33±0·26 ml/min/monocyte).

Similarly, in the SOD controls there was no significant difference in mean (±SD) 'non-dismutase inhibitable' cytochrome c reduction between normal controls (1·07±0·39 ml/min/monocyte), RA patients receiving NSAID alone (1·13±0·28 ml/min/monocyte), and patients on second-line therapy (1·06±0·24 ml/min/monocyte).

Stimulated superoxide anion production

In the presence of fluoride ion a 6-fold increase in rates of O_2^- production over basal levels occurred, but there were no differences between any of the groups studied (Fig. 1).

With IgTZ, rates of O_2^- production were significantly increased in monocytes from RA patients on 'second-line therapy' ($p<0{\cdot}01$) and to a lesser extent in monocytes from RA patients on NSAID alone ($p<0{\cdot}02$) compared with normal controls (Fig. 1).

With STZ significant enhancement of O_2^- generation was seen in monocytes from patients on second-line therapy ($p<0{\cdot}001$) compared with normal con-

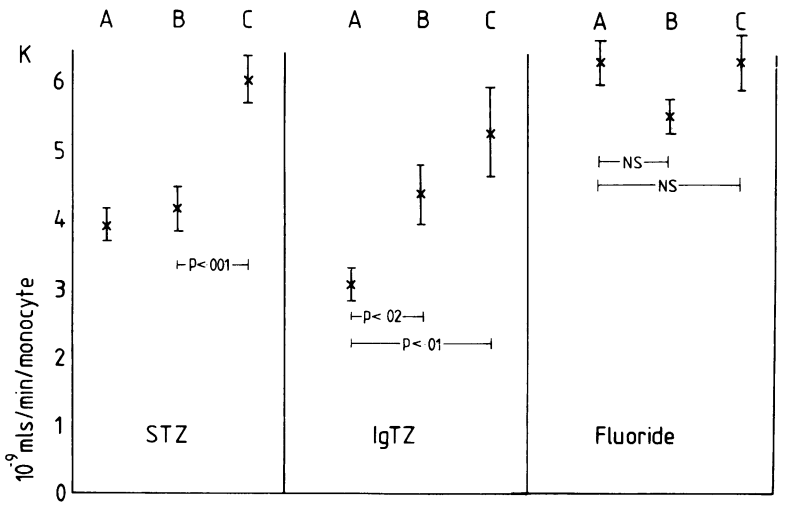

Fig. 1 *Rate constant K for cytochrome c reduction by O_2^-. Mean (±SEM) superoxide release after fluoride (F^-), STZ, or IgTZ stimulation of monocytes from* group A *normal controls (n=22),* group B *RA patients on NSAID only (n=22), and* group C *RA patients on second-line therapy (n=12).*

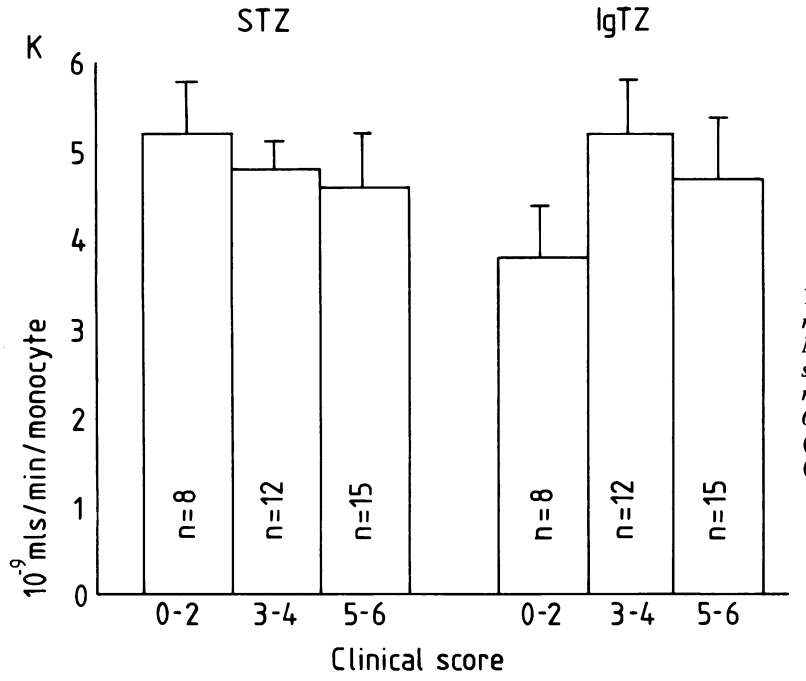

Fig. 2 *Disease activity and monocyte superoxide production. Mean (±SEM) rates of monocyte superoxide release in patients with mild or inactive disease (score 0—2), moderately active disease (score 3—4), or very active disease (score 5—6).*

trols, but no enhancement was seen in monocytes from patients on NSAID alone (Fig. 1).

Correlation of in vitro rates of O_2^- production with disease activity

No significant differences in monocyte O_2^- production were found between patients with mild or inactive disease, moderately active disease, or very active disease (Fig. 2). Furthermore, although patients or second-line therapy had higher rates of in-vitro monocyte O_2^- production, they had a lower mean clinical score (2·9± 2·5) than those on NSAID alone (4·3± 1·7). These differences are not statistically significant.

As there was no significant difference in mean values for superoxide production in patients under treatment with gold, penicillamine, or corticosteroid drugs, the results have been analysed as a combined 'second-line therapy' group in each case.

Discussion

These results suggest that monocytes from some RA patients have enhanced capacity to generate O_2^- in response to immunological stimuli but not to a soluble biochemical stimulus such as fluoride ion. An increase in rates of O_2^- release was found primarily with monocytes from patients on second-line therapy and was more marked after stimulation with STZ, a combined C3b/Fc receptor stimulus, than with IgTZ, which stimulates Fc receptors alone. No comparable increase in basal unstimulated release of O_2^- was seen, suggesting that these differences were not due to immune complexes binding to monocyte receptors and causing prior stimulation. Furthermore, the differences were entirely attributable to enhanced O_2^- release, since there was no change in levels of 'non-dismutase inhibitable' reducing activity.

Enhanced extracellular release of H_2O_2 and O_2^- by mononuclear phagocytes is seen both after stimulation in vitro with lymphokines and after antigenic stimulation in vivo with BCG or *Corynebacterium parvum*.[16 26] It has been suggested that such augmentation of oxidative metabolism is a feature of mononuclear phagocyte activation,[26] and it is therefore of considerable interest that this phenomenon should be identified in monocytes from patients with RA.

In our patients a number of factors might be responsible for enhancement of O_2^- release and include disease activity, an effect of second-line therapy or conceivably loss of inhibition of O_2^- production after *reduction* of NSAID intake by patients on second-line therapy. Mechanisms by which this could occur include changes in monocyte receptor expression, enhancement of intracellular NADPH production, or, by reduction of NSAID intake, removal of potential inhibitors of O_2^- production.[15] On the basis of the data presented and preliminary

data from a prospective study of patients before and after beginning second-line therapy[27] the most likely cause of monocyte activation in these patients is the introduction of second-line therapy. Our data do not suggest that enhanced O_2^- release is secondary to disease activity or to withdrawal of NSAID.

Altered receptor expression seems the most likely mechanism for the effect, since differences in O_2^- release were seen with opsonised zymosan but not with F^-, a biochemical stimulus. The observations of previous workers using rosetting techniques on monocytes from RA patients who found enhanced Fc receptor expression,[28 29] and others[30] who found increased antibody dependent cellular cytotoxicity (ADCC), lend support to this view. Furthermore, since ADCC may be mediated by oxygen free radicals,[31] our data provide a biochemical mechanism for such an increase in functional activity. The in-vivo effects of penicillamine and sodium aurothiomalate on monocyte receptor expression in RA do not appear to have been studied, so direct enhancement of receptor expression by these drugs cannot be excluded. On the other hand there is evidence that sodium aurothiomalate and penicillamine raise intracellular levels of glutathione and SOD,[32] and this might secondarily increase levels of NADPH, the key substrate for O_2^- production. Since we cannot preclude the possibility that stimulation with F^- was too insensitive to detect differences in rates of O_2^- generation, a biochemical rather than an immunological mechanism could also account for enhancement of O_2^- production.

In conclusion, our data provide evidence to suggest that monocytes from some RA patients have enhanced potential to generate oxygen-free radicals, but this should not be interpreted as evidence of increased release of O_2^- in vivo. The association of this phenomenon with second-line therapy may provide further insight into the mode of action of these drugs and suggests that they may cause monocyte activation. To explore this possibility further we have undertaken a prospective study of O_2^- production by monocytes from RA patients commencing second-line therapy.

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Book review

CBE Style Manual. 5th edn. Pp. 326. US$24·00. Council of Biology Editors, 9650 Rockville Pike, Bethesda, Maryland 20814, USA. 1983.

Success in medicine depends much on communicating through the written word. For those wishing to get ahead there is thus a strong case for adopting a professional approach to authorship. Facility in writing varies greatly, but, while practice is a great improver, there are also many publications offering helpful advice. What then should stand on the desk of the medical author and, for that matter, the editor, referee, and publisher? For those with the leisure to savour their reading and writing, volumes such as Fowler, Roget, and the Shorter OED will need to be supplemented with tables of SI units and microbial terminology. For others with less time to spare the increasingly comprehensive instructions to authors offered by some journals provides much useful advice. There remains, however, a need for a concise reference book dealing with all aspects of medical writing. This is admirably filled by the new edition of the *CBE Style Manual.*

The manual deals with writing in all the biological sciences, but most of it is relevant to medicine. It offers sound advice about the ethical aspects of authorship and publication; preparation of manuscripts, tables, and illustrations; prose style, including a list of common errors; technical style conventions; indexing; proof correction; refereeing ('Test the critique for fairness and objectivity by asking yourself if you would be willing to sign it and send it to the author'). The style of the manual itself is North American, but throughout it draws attention to differences between British and American usage, and these comparisons make interesting reading. The authors might have commented on how the simpler North American spelling avoids that most common error in British medical writing, the insertion of an inappropriate diphthong (as in *thrombocytopaenia* for *thrombocytopenia*). All in all, this is an excellent publication: concise, yet comprehensive and entertaining. I recommend it.

H. L. F. CURREY