

Inhibition of neutrophil oxidant secretion by D-penicillamine: scavenging of H₂O₂ and HOCl

Martin J Ledson, Roger C Bucknall, Steven W Edwards

Abstract

D-Penicillamine inhibited oxidant secretion from human neutrophils after activation by the chemotactic peptide *N*-formyl-methionyl-leucyl-phenylalanine (fMet-Leu-Phe), as assessed by luminol dependent chemiluminescence. In contrast, this drug had little effect on either intracellular oxidant production or lucigenin dependent chemiluminescence activated by the same agonist. The drug was shown to scavenge both H₂O₂ and HOCl in a cell free luminol chemiluminescence system, though its ability to scavenge HOCl was greater than that for H₂O₂. Both these oxidants could oxidise the drug, but again HOCl was more potent than H₂O₂. When D-penicillamine was oxidised by exposure to H₂O₂ it could no longer serve as a scavenger of secreted oxidants from neutrophils. These data suggest that in vivo the preferential scavenging of HOCl may be important under pathological conditions where secreted myeloperoxidase may be functional.

D-Penicillamine is a slow acting drug used in the treatment of rheumatoid arthritis, but despite its widespread use its biological mode of action remains obscure. Several mechanisms have been proposed to explain its efficacy, including effects of collagen metabolism,¹ immunopathological effects in the presence of copper,² superoxide dismutase activity,³ and ability to scavenge H₂O₂ and HOCl,⁴⁻⁶ both of which are products of activated phagocytes. Furthermore, a more direct inhibitory effect of this drug on the activity of the neutrophil enzyme myeloperoxidase has been proposed as it can promote the formation of compound III, which is inactive in the generation of HOCl.⁷

In view of the fact that neutrophils within inflamed joints of patients with rheumatoid arthritis have been activated to secrete reactive oxidants⁸⁻¹⁰ and myeloperoxidase,^{11,12} one explanation for the therapeutic activity of D-penicillamine may reside in its ability to scavenge the neutrophil products H₂O₂ and HOCl within such joints. However, only modest inhibitory effects of this drug on neutrophil dependent oxidant production have been described. For example, after stimulation of neutrophils by phorbol myristate acetate, D-penicillamine at 0.1 mmol/l only inhibited the activated chemiluminescence by 20%,¹³ and when opsonised zymosan was used as the stimulus the drug was reported to *enhance* the chemiluminescence response.¹⁴ These observations thus seem to contradict the view that D-penicillamine is an effective oxidant scavenger.

We have previously shown that activated neutrophils generate reactive oxidants both intra- and extracellularly,¹⁵ and that these two activities are dependent upon the nature of the stimulus used. For example, the chemotactic peptide *N*-formyl-methionyl-leucyl-phenylalanine (fMet-Leu-Phe) stimulates a rapid burst of oxidant secretion which is followed by a second phase of internally generated oxidants, whereas phagocytic stimuli, such as immune complexes or opsonised zymosan, activate oxidant production which is largely intracellular. Furthermore, the extent of myeloperoxidase secretion (and hence ability to constitute an extracellular myeloperoxidase-H₂O₂ system) is also dependent upon the nature of the neutrophil activating factor. Thus one explanation for the reports of an apparently low ability of D-penicillamine to scavenge neutrophil derived oxidants is that the experimental conditions used largely activated *intracellular* oxidant production, which may be inaccessible to the drug. This work aimed at establishing if D-penicillamine scavenged extracellularly released neutrophil oxidants (as these are likely to be of importance in inflammatory joint disease) and measuring the relative affinities of the drug for H₂O₂ and HOCl to determine its effects on oxidant production, dependent and independent of myeloperoxidase.

Materials and methods

ISOLATION AND PREPARATION OF NEUTROPHILS

Neutrophils were prepared from 20 ml heparinised venous blood from healthy volunteers using M-PRM (Flow Laboratories).¹⁶ After purification they were suspended in a Krebs/HEPES buffer containing (mmol/l): NaCl 120; KCl 4.8; KH₂PO₄ 1.2; CaCl₂ 1.3; MgSO₄ 1.2; HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid) 25 (pH 7.4); 0.1% bovine serum albumin. Cells were counted after a suitable dilution in the above buffer with a Fuchs-Rosenthal haemocytometer slide and used with four hours of preparation.

ANALYTICAL METHODS

Chemiluminescence

Neutrophils were suspended in buffer containing either 10 μmol/l luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) or 25 μmol/l lucigenin (bis-*N*-methylacridinium nitrate) at 10⁶ cells/ml, in a total volume of 1 ml. After the addition of 1 μmol/l fMet-Leu-Phe (final concentration) chemiluminescence was measured with an LKB Wallac 1250 luminometer.¹⁷ A cell

Department of
Biochemistry,
University of Liverpool,
PO Box 147,
Liverpool L69 3BX,
United Kingdom
M J Ledson
S W Edwards

Rheumatic Diseases
Unit, Royal Liverpool
Hospital, Prescott Road,
Liverpool L69 3BX,
United Kingdom
R C Bucknall

Correspondence to:
Dr Edwards.

Accepted for publication
16 April 1991

free $H_2O_2/HOCl$ /luminol chemiluminescence system¹⁸ was also used with concentrations of reagents as indicated in the text.

O_2^- generation

Superoxide generation was measured in a continuous assay by monitoring the rate of reduction of ferricytochrome *c* inhibitable by superoxide dismutase.¹⁹ The assay mixture (total volume 1 ml) contained 75 $\mu\text{mol/l}$ cytochrome *c* plus 5×10^5 cells and measurement was made with a Perkin-Elmer lambda 5 spectrophotometer.

H_2O_2 and $HOCl$ scavenging by D-penicillamine

Absorption spectra of D-penicillamine in the presence and absence of oxidising/reducing agents were recorded with a Perkin-Elmer lambda 5 spectrophotometer. The temperature of operation was 20°C, scanning speed 1 nm/s, and slit width 2 nm.

CHEMICALS

D-Penicillamine, luminol, lucigenin, fMet-Leu-Phe, and H_2O_2 were from Sigma, and $HOCl$ was from BDH. Before analyses freshly prepared solutions of H_2O_2 and $HOCl$ were quantified by measurement of their absorption at 240 and 290 nm, respectively, using molar absorption coefficients of 43.4 and 390, respectively.

Results

EFFECT OF D-PENICILLAMINE ON fMET-LEU-PHE STIMULATED NEUTROPHIL CHEMILUMINESCENCE
When neutrophil suspensions are stimulated by the chemotactic peptide fMet-Leu-Phe the luminol dependent chemiluminescence response, which requires the activities of both the O_2^-/H_2O_2 generating oxidase and myeloperoxidase,¹⁷ is biphasic. We have shown that the initial, rapid (within one minute) peak is

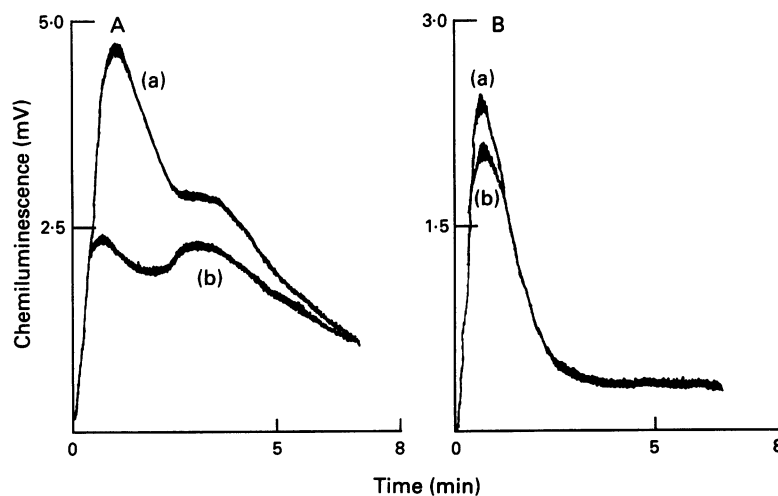


Figure 1 Effect of D-penicillamine on neutrophil chemiluminescence. Neutrophils ($10^6/\text{ml}$) were suspended in buffer containing either (A) 10 $\mu\text{mol/l}$ luminol or (B) 25 $\mu\text{mol/l}$ lucigenin at 37°C in (b) the presence and (a) the absence of 10^{-4} mol/l D-penicillamine. After a two minute preincubation period fMet-Leu-Phe was added (final concentration 1 $\mu\text{mol/l}$) and the chemiluminescence responses measured.

largely due to oxidant secretion, whereas the second, more sustained phase of oxidant production is largely intracellular.¹⁵ When neutrophil suspensions were preincubated with D-penicillamine for two min before addition of fMet-Leu-Phe the chemiluminescence responses generated were reduced in the presence of the drug (figs 1A and 2). The effects of the drug on intra- and extracellular luminol chemiluminescence were not identical, however: for example, at a drug concentration of 10^{-4} mol/l the extracellular (initial) oxidant production was inhibited by 72%, whereas the intracellular production (that detected between three and five minutes) was only inhibited by 31% (Fig 1A). There was little difference in the inhibitory effects of different concentrations of D-penicillamine on intracellular oxidant production, whereas a clear inhibition of extracellular secretion dependent on dose was seen (fig 2).

In contrast with its effects on luminol chemiluminescence, D-penicillamine had little effect on fMet-Leu-Phe stimulated lucigenin chemiluminescence (fig 1B). Whereas luminol measures intra- and extracellular, myeloperoxidase dependent oxidant production, lucigenin only measures myeloperoxidase independent oxidant secretion; it is believed that lucigenin detects O_2^- or H_2O_2 or both, though the precise oxidant species detected is uncertain. These observations lead to three conclusions: firstly, D-penicillamine has only a slight effect on intracellularly generated oxidants; secondly, the drug effectively scavenges one or more components of the myeloperoxidase- H_2O_2 system; and thirdly, the drug does not efficiently scavenge O_2^- or H_2O_2 , if these are the oxidant species detected by lucigenin.

OXIDANT SCAVENGING BY D-PENICILLAMINE

Attempts to determine whether D-penicillamine

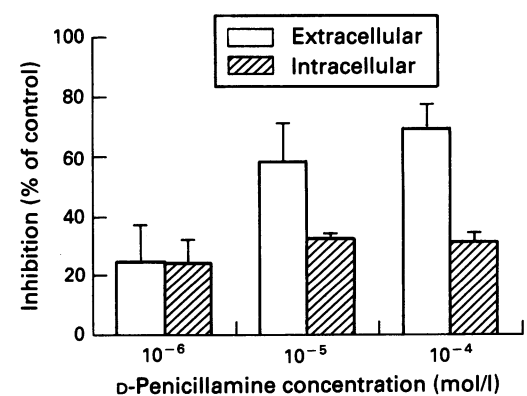


Figure 2 Effect of D-penicillamine on intra- and extracellular oxidant production. Neutrophils ($10^6/\text{ml}$) were suspended in buffer containing 10 $\mu\text{mol/l}$ luminol at 37°C in the presence and absence of D-penicillamine at the concentrations indicated. After a two minute preincubation period fMet-Leu-Phe (final concentration 1 $\mu\text{mol/l}$) was added and the chemiluminescence responses measured. Extracellular oxidant secretion was measured as the peak response seen at one minute after stimulation, whereas intracellular production was measured as the peak response at three to five minutes.¹⁵ Values presented are the mean response (with standard deviations) of five determinations on different preparations of neutrophils and shown as percentage inhibition of the responses noted in the absence of drug.

scavenged O_2^- either generated in a cell free xanthine/xanthine oxidase system or else secreted from activated neutrophils, by determining its effects on cytochrome *c* reduction were frustrated by the fact that the drug itself rapidly reduced the cytochrome *c*. The ability of the drug to scavenge H_2O_2 and HOCl was determined using a cell free chemiluminescence system.¹⁸ The system is based on the fact that when HOCl is added to mixtures of H_2O_2 and luminol a rapid chemiluminescence burst is initiated. Preincubation of the H_2O_2 /luminol mixture with D-penicillamine reduced the chemiluminescence response initiated by HOCl by about 50% (table): doubling the H_2O_2 concentration restored activity, indicating that (a) the drug itself had no effect on the assay and (b) that it could reduce the chemiluminescence response by scavenging the H_2O_2 . When the drug was added to the HOCl solution before its addition to the H_2O_2 /luminol mixture, however, the inhibitory effect was much greater. Again, doubling the HOCl concentration restored activity, but merely doubling the H_2O_2 concentration did not restore activity to the HOCl/D-penicillamine mixture. These results indicate that D-penicillamine can scavenge both oxidants, but that its ability to scavenge HOCl is much greater than its ability to scavenge H_2O_2 .

It seemed likely that when D-penicillamine scavenged these oxidants it might itself have become oxidised during the reaction. Absorption spectra of the native drug were recorded between 190 and 250 nm in the presence and absence of a chemical reductant (sodium dithionite) and oxidant (ammonium persulphate). These spectra indicated that the native drug was in a fully reduced state and that when oxidised its absorption maximum at 200 nm was lost (figs 3 (a) and (b)). Sequential additions of known concentrations of either H_2O_2 or HOCl were then added to samples of the native (reduced) drug and absorption spectra recorded. Both oxidants oxidised the drug as indicated by the decrease in absorbance at 200 nm (figs 3 (c) and (d)). On a molar basis, however, HOCl oxidised the drug more potently than did H_2O_2 (fig 4), being about twice as effective. This indicates that D-penicillamine is a more potent scavenger of HOCl than of H_2O_2 , confirming the data shown in the table.

EFFECT OF OXIDISED D-PENICILLAMINE ON NEUTROPHIL OXIDANT PRODUCTION

If the proposed effect of D-penicillamine on

Effect of D-penicillamine on H_2O_2 /HOCl dependent luminol chemiluminescence

Luminol mixed with:	Reaction initiated by:	Chemiluminescence (% of control)‡
H_2O_2	HOCl	100 (9) (n=23)
H_2O_2 +D-penicillamine	HOCl	47 (11) (n=9)*
2×† H_2O_2 +D-penicillamine	HOCl	100 (8) (n=8)
H_2O_2	HOCl+D-penicillamine	8.8 (6) (n=12)**
H_2O_2	2×†HOCl+D-penicillamine	105 (10) (n=5)
2×† H_2O_2	HOCl+D-penicillamine	8 (5) (n=8)**

H_2O_2 (8 μ mol/l) was mixed with luminol (10 μ mol/l) and the chemiluminescence generated upon injection of HOCl (8 μ mol/l) was measured and taken as 100%. Where indicated, D-penicillamine (10⁻⁶ mol/l) was added either to the luminol/ H_2O_2 mixture or else to the HOCl solution and its effect on the chemiluminescence response measured.

*p<0.05, **p<0.001, using Student's *t* test.

†2× represents experiments in which the concentration of the indicated oxidant was doubled.

‡Number of determinations is given (n) together with the standard deviation.

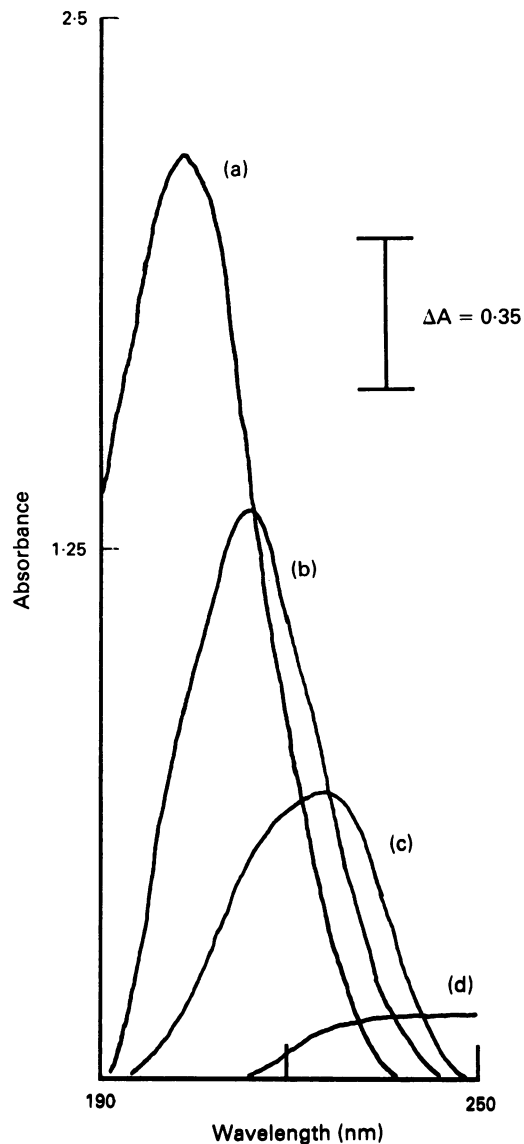


Figure 3 Effect of oxidants on D-penicillamine absorption spectra. The absorption spectrum of D-penicillamine (2×10^{-3} mol/l) was recorded between 190 and 250 nm with a Perkin-Elmer lambda 5 spectrophotometer. The absorption spectrum of the native drug (scan a) was unchanged after addition of the reductant, sodium dithionite (data not shown). Spectra were then recorded after the addition of a few crystals of ammonium persulphate (scan b), 9 mmol/l H_2O_2 (scan c), and 9 mmol/l HOCl (scan d).

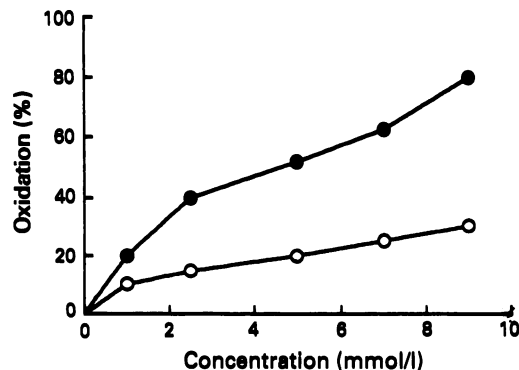


Figure 4 Oxidation of D-penicillamine by H_2O_2 and HOCl. D-Penicillamine was incubated in the absence and presence of varying concentrations of either H_2O_2 (○) or HOCl (●), as described in the legend to fig 3. The oxidation of the drug was then assessed as the decrease in absorption at 200 nm at a particular oxidant concentration and is expressed as a percentage of the untreated (native) drug absorbance.

neutrophil oxidant secretion was to scavenge extracellular oxidants (figs 1 and 2) and the drug itself became oxidised during this process (fig 4) then the oxidised drug should have little or no effect on neutrophil function. Thus a solution of D-penicillamine was mixed with just sufficient H_2O_2 for its complete oxidation, and its ability to scavenge neutrophil derived oxidants generated in response to fMet-Leu-Phe was determined. Whereas the native (reduced) drug inhibited oxidant secretion by over 60%, the oxidised form of the drug had no effect on oxidant secretion (fig 5). These data confirm that D-penicillamine scavenges secreted oxidants, thus reducing oxidant production by neutrophils, itself becoming oxidised (and non-functional) in the process.

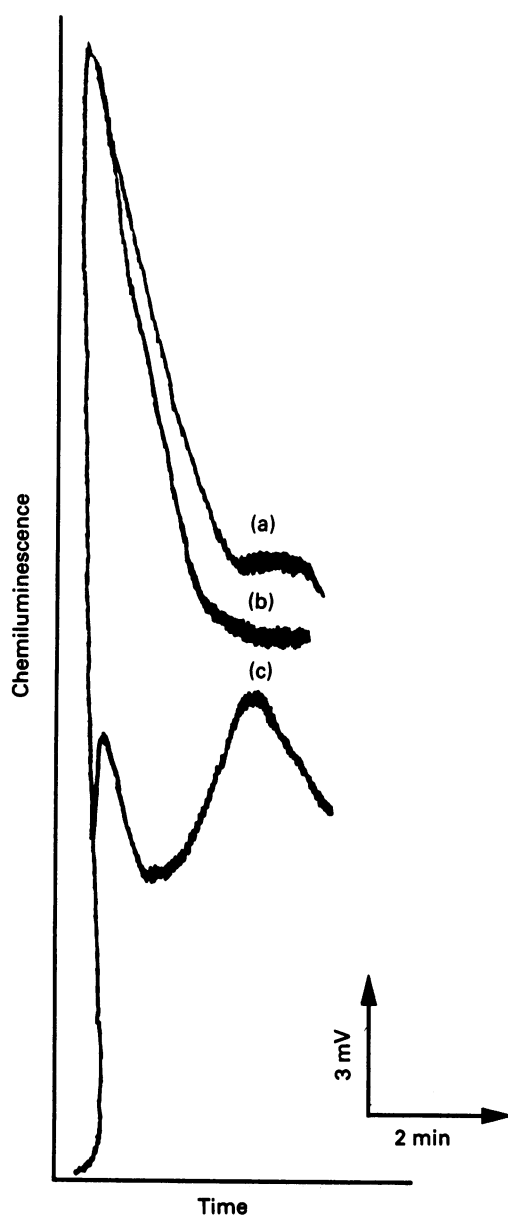


Figure 5 Effect of native and oxidised D-penicillamine on neutrophil chemiluminescence. Neutrophils were incubated in buffer containing $10 \mu\text{mol/l}$ luminol, as described in the legend to fig 1. Before addition of $1 \mu\text{mol/l}$ fMet-Leu-Phe (final concentration), suspensions contained (a) no further additions, (c) 10^{-4} mol/l native (reduced) D-penicillamine, or (b) 10^{-4} mol/l D-penicillamine which had previously been exposed to $4 \times 10^{-4} \text{ mol/l}$ H_2O_2 .

Discussion

The data presented in this paper confirm that D-penicillamine is a potent scavenger of both H_2O_2 and HOCl, two products of the respiratory burst of activated neutrophils. As the relative concentrations of these two oxidants at inflammatory sites will depend on the levels of extracellular activity of the O_2/H_2O_2 generating NADPH oxidase and the activity of secreted myeloperoxidase, it is important to identify the potency of D-penicillamine in scavenging both H_2O_2 and HOCl. We have shown here that on a molar basis D-penicillamine is at least twice as efficient at scavenging HOCl than at scavenging H_2O_2 . This may be of pathological importance as we have previously shown that although neutrophils isolated from the synovial fluid of patients with rheumatoid arthritis have been activated in vivo to secrete reactive oxidants,¹⁰ they have also been activated to secrete myeloperoxidase in these joints, and the myeloperoxidase is in a molecular form which indicates that it has reacted with H_2O_2 .¹¹ Furthermore, factor(s) present within synovial fluid can activate the myeloperoxidase- H_2O_2 system,¹² and hence the ability of D-penicillamine to scavenge HOCl within the inflamed joint may be of more importance than its ability to detoxify H_2O_2 .

Previous work has shown only a modest inhibitory effect of D-penicillamine on phorbol myristate acetate stimulated luminol chemiluminescence of neutrophils,¹³ and even a stimulatory effect on opsonised zymosan activated chemiluminescence,¹⁴ despite the fact that the drug efficiently scavenges oxidants in cell free systems. Neither of these experimental conditions, however, results in optimal secretion of reactive oxidants from neutrophils. In our study we used the chemotactic peptide fMet-Leu-Phe to stimulate neutrophils because this agonist activates clearly defined phases of oxidant secretion (detected within one minute of addition) followed by intracellular oxidant production.¹⁵ Under these conditions 10^{-4} mol/l D-penicillamine inhibited oxidant secretion by over 70% (fig 1), compared with only 20% inhibition of phorbol myristate acetate stimulated luminol chemiluminescence.¹³ The drug had little effect on intracellular oxidant production and this phenomenon was not dependent on concentration. The biological function of such intracellular oxidant generation and the significance of the effects of D-penicillamine on this phenomenon are unknown.

Luminol chemiluminescence requires the activity of the myeloperoxidase- H_2O_2 system, whereas lucigenin is thought to measure only O_2 or H_2O_2 secretion, or both,¹⁷ though the precise molecular species reacting with the latter lumigenic probe has not been unambiguously defined. Therefore, it is of considerable interest to note that although D-penicillamine scavenged extracellular luminol chemiluminescence, it had little effect on lucigenin chemiluminescence (fig 1). This implies that either lucigenin does not in fact measure O_2 or H_2O_2 , or else that the predominant effect of D-penicillamine in neutrophils is to scavenge a myeloperoxidase dependent product (that is, HOCl) rather than

H₂O₂. Therefore, we tested for the ability of D-penicillamine to scavenge both H₂O₂ and HOCl and found that it is a more potent scavenger of the latter oxidant.

The ability of D-penicillamine to scavenge H₂O₂ and HOCl resides in its thiol group. The drug becomes oxidised during its interaction with oxidants, and the oxidised form of the drug is incapable of interfering with the processes of neutrophil oxidant production. Although the serum concentration of this drug may be as high as 20 µmol/l,²⁰ its effective concentration within joints, which may be exposed to high levels of neutrophil derived oxidants, will be reduced owing to its oxidation, and this may be of pathological importance. In view of the emerging evidence indicating that an extracellular myeloperoxidase-H₂O₂ system is operational in the rheumatoid joint, scavenging of HOCl (and to a lesser extent H₂O₂) may be one means by which the efficacy of this drug is explained.

We thank the Arthritis and Rheumatism Council and Mersey Regional Health Authority for generous financial support.

- 1 Nimni M E, Deshmukh K, Gerth N, Bavetta L A. Changes in collagen metabolism associated with the administration of penicillamine and various amino and other thiol compounds. *Biochem Pharmacol* 1969; **18**: 469-72.
- 2 Staite N D, Messner R P, Zoschke D C. In vitro production and scavenging of hydrogen peroxide by D-penicillamine. *Arthritis Rheum* 1985; **28**: 914-21.
- 3 Lengfelder E, Fuchs C, Tounes M, Weser U. Functional aspects of the superoxide dismutase action of Cu-penicillamine. *Biochem Biophys Res Commun* 1979; **567**: 492-502.
- 4 Matheson N R. The effect of antiarthritic drugs and related compounds on the human neutrophil myeloperoxidase system. *Biochem Biophys Res Commun* 1982; **108**: 259-65.
- 5 Staite N D, Zoschke D C, Messner R P. Scavenging of hydrogen peroxide—a new mechanism of action for D-penicillamine in rheumatoid arthritis? *N Engl J Med* 1984; **311**: 538-9.
- 6 Wasil M, Halliwell B, Moorhouse C P, Hutchison D C S, Baum H. Biologically-significant scavenging of the myeloperoxidase-derived oxidant hypochlorous acid by some anti-inflammatory drugs. *Biochem Pharmacol* 1987; **36**: 3847-50.
- 7 Cuperus R A, Hoogland H, Wever R, Muijers A O. The effect of D-penicillamine on myeloperoxidase: formation of compound III and inhibition of the chlorinating ability. *Biochem Biophys Acta* 1987; **912**: 124-31.
- 8 Bender J G, Van Epps D E, Searles R, Williams R C Jr. Altered function of synovial fluid granulocytes in patients with acute inflammatory arthritis. Evidence for activation of neutrophils and its mediation by a factor present in synovial fluid. *Inflammation* 1986; **10**: 443-53.
- 9 Dularay B, Elson C J, Dieppe P A. Enhanced oxidative response of polymorphonuclear leukocytes from synovial fluids of patients with rheumatoid arthritis. *Autoimmunity* 1988; **1**: 159-69.
- 10 Nurcombe H L, Bucknall R C, Edwards S W. Neutrophils isolated from the synovial fluid of patients with rheumatoid arthritis: priming and activation in vivo. *Ann Rheum Dis* 1991; **50**: 147-53.
- 11 Edwards S W, Hughes V, Barlow J, Bucknall R. Immunological detection of myeloperoxidase in synovial fluid from patients with rheumatoid arthritis. *Biochem J* 1988; **250**: 81-5.
- 12 Nurcombe H L, Bucknall R C, Edwards S W. Activation of the neutrophil myeloperoxidase-H₂O₂ system by synovial fluid isolated from patients with rheumatoid arthritis. *Ann Rheum Dis* 1991; **50**: 237-42.
- 13 French R C, Herbert K E, Scott D L, Perrett D. Effect of penicillamine on luminol-dependent chemiluminescence in human leucocytes. *Biochem Soc Trans* 1988; **16**: 891.
- 14 Muller-Peddinghaus R, Wurl M. The amplified chemiluminescence test to characterise antiarthritic drugs as oxygen radical scavengers. *Biochem Pharmacol* 1987; **36**: 1125-32.
- 15 Nurcombe H L, Edwards S W. Role of myeloperoxidase in intracellular and extracellular chemiluminescence of neutrophils. *Ann Rheum Dis* 1989; **48**: 56-62.
- 16 Edwards S W, Say J E, Hart C A. Oxygen-dependent killing of *Staphylococcus aureus* by human neutrophils. *J Gen Microbiol* 1987; **133**: 3591-7.
- 17 Edwards S W. Luminol- and lucigenin-dependent chemiluminescence of neutrophils: role of degranulation. *J Clin Lab Immunol* 1987; **22**: 35-9.
- 18 Brestel E P. Co-oxidation of luminol by hypochlorite and hydrogen peroxide. Implications for neutrophil chemiluminescence. *Biochem Biophys Res Commun* 1985; **126**: 482-8.
- 19 Babior B M, Kipnes R S, Curnutte J T. Biological defense mechanisms. The production by leukocytes of superoxide, a potential bacteriocidal agent. *J Clin Invest* 1973; **52**: 741-4.
- 20 Saetre R, Rabenstein D L. Determination of penicillamine in blood and urine by high performance liquid chromatography. *Anal Chem* 1978; **50**: 276-80.