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Myeloperoxidase-dependent oxidative inactivation of neutrophil neutral proteinases and microbicidal enzymes

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The susceptibility of a number of human neutrophil granule enzymes to oxidative inactivation was investigated. Addition of H_2O_2 to the cell-free medium from stimulated neutrophils resulted in inactivation of all enzymes tested. This was inhibited by azide and methionine, indicating that inactivation was due to myeloperoxidase-derived oxidants. Lysozyme was more than 50% inactivated by one addition of 100 nmol of H_2O_2/ml, whereas myeloperoxidase, β-glucuronidase, gelatinase and collagenase were almost completely inactivated by three additions. Cathepsin G was slightly less susceptible, whereas elastase was extremely resistant to oxidative attack. Myeloperoxidase-dependent enzyme inactivation may be a means whereby the neutrophil can terminate the activity of its granule enzymes and control the release of degradative enzymes into the tissues.

INTRODUCTION

Activated oxygen species play a pivotal role in the microbicidal and cytotoxic action of neutrophils (Klebanoff, 1980; Babior, 1984). When stimulated, these cells produce large amounts of superoxide, which leads to the formation of H_2O_2 and, via the myeloperoxidase-catalysed reaction of H_2O_2 with chloride ions, of hypochlorous acid, HOCl (Klebanoff, 1980; Thomas *et al.*, 1982; Babior, 1984). In addition to being involved in the microbicidal and inflammatory reactions of the cells, oxidants can also attack the neutrophil itself. Inactivation of the superoxide-generating NADPH oxidase (Jandl *et al.*, 1978) and of some granule enzymes (Voetman *et al.*, 1981; Clark & Borregaard, 1985; Dri *et al.*, 1985) has been observed. Chronic-granulomatous-disease neutrophils, which lack a functional oxidase, and myeloperoxidase-deficient cells, secrete 2–3 times more active lysozyme and acid hydrolases than do control cells (Voetman *et al.*, 1981; Dri *et al.*, 1985), this difference being attributable to oxidative inactivation of the enzymes by normal neutrophils. Myeloperoxidase involvement was equivocal in these studies, but was clearly shown to be required for the inactivation of vitamin B_{12}-binding protein (Clark & Borregaard, 1985). Recently, stimulated neutrophils have been shown to activate their endogenous metalloenzymes, collagenase (Weiss *et al.*, 1985) and gelatinase (Peppin & Weiss, 1986), by an oxidative process dependent on myeloperoxidase.

These findings suggest that neutrophil oxidants can modulate the activity of granule enzymes, and this may be a means whereby the cells control their degradative capacity. We have, therefore, examined the susceptibility of a range of neutrophil enzymes, in particular the neutral proteinases, to oxidative inactivation. Neutrophils were stimulated to release a full complement of granule enzymes, and the inactivation of these enzymes by adding H_2O_2 to the cell-free medium was investigated.

METHODS AND MATERIALS

Neutrophils and cell-free medium

Human neutrophils, isolated by Ficoll/Hypaque-density-gradient centrifugation, dextran sedimentation and hypo-osmotic lysis of red cells (Boyum, 1968), were suspended at 10^7/ml in 10 mM-sodium phosphate buffer, pH 7.4, containing 138 mM-NaCl, 2.7 mM-KCl, 1 mM-$CaCl_2$, 0.5 mM-$MgCl_2$ and 1 mg of glucose/ml. Cell-free medium containing exocytosed neutrophil granule enzymes was prepared by stimulation for 20 min at 37 °C with 0.1 μM-fMet-Leu-Phe in the presence of cytochalasin B (5 μg/ml). The cells were pelleted by centrifugation at 1000 *g* for 10 min, and the supernatant always used on the same day.

Inactivation of neutrophil enzymes

Cell-free medium (1 ml) was incubated at 37 °C, and one to eight additions of 100 nmol of H_2O_2 were made at 5 min intervals. Control tubes did not receive H_2O_2 or contained 1280 units of catalase, 2 mM-azide or 4 mM-methionine. At 5 min after the last addition of H_2O_2, the tubes were removed from the water bath and enzyme activities were measured.

Enzyme assays

Lysozyme activity was measured against *Micrococcus lysodeikticus* (Worthington Enzyme Manual, 1979), myeloperoxidase with *o*-tolidine (Baggiolini *et al.*, 1969) and β-glucuronidase using phenolphthalein glucuronic acid (Baggiolini *et al.*, 1969). Elastase and cathepsin G were measured by monitoring cleavage of the specific synthetic peptide substrates methoxysuccinyl-Ala-Ala-

Abbreviations used: PMSF, phenylmethanesulphonyl fluoride; PAGE, polyacrylamide-gel electrophoresis; fMet-Leu-Phe, formylmethionyl-leucylphenylalanine.

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Pro-Val *p*-nitroanilide and succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide respectively at 400 nm. The cuvette (1 ml) contained 0.32 mM-substrate and 20 μl (elastase) or 400 μl (cathepsin G) of cell-free medium in phosphate-buffered saline, pH 7.4.

Gelatinase was measured by its ability to degrade NaB^3H_4-labelled heat-denatured Type I collagen (gelatin), essentially as described by Hibbs *et al.* (1985). Gelatin (100 μg) was incubated with 25 μl of neutrophil supernatant for 1 h at 37 °C. Solubilized radioactivity was determined in Triton/toluene scintillant after precipitation with 10% (w/v) trichloroacetic acid and centrifugation at 15000 *g* for 2 min. Gelatinase was measured with and without activation by 2 mM-phenylmercuric acetate.

Collagenase was measured by its ability to specifically cleave native soluble Type I collagen. Collagen (25 μg) was incubated overnight at 20 °C with 20 μl of cell-free medium, 2 mM-PMSF, 2 mM-phenylmercuric acetate, 0.2 M-NaCl, 5 mM-$CaCl_2$ in 200 μl of 0.1 M-Tris/HCl, pH 7.5, denatured under reducing conditions and analysed by SDS/PAGE in a 6% (w/v) slab gel (Laemmli, 1970).

Materials

Ficoll 400 was from Pharmacia Fine Chemicals, Uppsala, Sweden, and Hypaque from Sterling Pharmaceuticals. All other biochemicals were from Sigma Chemical Co., St. Louis, MO, U.S.A.

RESULTS

Neutrophil granule enzymes were released into the medium when the cells were stimulated with fMet-Leu-Phe in the presence of cytocholasin B (Table 1). Approx. 80% of the cell's total lysozyme, which is contained in azurophil and specific granules, and 40% of the β-glucuronidase, from azurophil granules and C-particles, was recovered in the medium. Total enzyme activities were measured in a sonicated cell sample.

The effects of adding H_2O_2 to the cell-free medium are shown in Figs. 1 and 2. Sequential additions of 100 nmol of H_2O_2/ml were made to simulate a continuous generation system and also to maintain a low enough H_2O_2 concentration for quantitative conversion into HOCl by myeloperoxidase (Winterbourn *et al.*, 1985). There was a loss in activity of all the enzymes measured. Lysozyme (Fig. 1*a*) was the most readily inactivated, with less than 50% of the original activity remaining after one addition of H_2O_2. Myeloperoxidase (Fig. 1*b*). β-glucuronidase (Fig. 1*c*), gelatinase (Fig. 1*d*), and collagenase (Fig. 2) were also readily inactivated, having lost most of their activity after three additions of H_2O_2. Cathepsin G (Fig. 1*e*) was less susceptible, with three additions of H_2O_2 causing only a 50% decrease in activity. In contrast with the other enzymes, elastase was almost resistant to concentrations of oxidant which gave complete loss of the other activities (Fig. 1*f*). With three additions of H_2O_2, 90% remained active, and even with eight additions elastase activity decreased by only 40%. Inactivation was fully inhibited by either azide or methionine, suggesting the involvement of the myeloperoxidase-derived oxidant HOCl. The activity of myeloperoxidase could not be assessed in the presence of azide, but, in contrast with the other enzymes, inactivation was only partially prevented by methionine (Fig. 1*b*).

Gelatinase and collagenase are metalloenzymes present in the neutrophil in latent form. We found that gelatinase present in the cell-free medium of cytochalasin B-treated neutrophils stimulated with fMet-Leu-Phe was mostly activated, having $64 \pm 18\%$ ($n = 5$) of its maximal activity, measured in the presence of 2 mM-phenylmercuric acetate. Activation was due to the action of serine proteinase also present in the medium, since the addition of PMSF fully inhibited this process (Vissers, 1986). The effect of oxidants on gelatinase was measured on partially and fully activated enzyme. The inactivation curves were identical in both cases (Fig. 1*d*), i.e. there was no evidence for further oxidative activation of the partially active gelatinase. Collagenase activity (Fig. 2) was measured only for phenylmercuric acetate-activated enzyme.

In the preparation of the cell-free medium the enzymes released from the neutrophils were exposed to oxidants produced by the cells. However, when fMet-Leu-Phe was the stimulus, neutrophils exhibited an attenuated respiratory burst (Tauber & Babior, 1985), and in our experiments only minimal inactivation occurred. When catalase or azide was included when the cells were stimulated, lysozyme activity was $121 \pm 4\%$, cathepsin G was $109 \pm 4\%$, and gelatinase $99 \pm 16\%$ of that measured in control cells ($n = 4$).

Table 1. Activities of granule enzymes released from neutrophils stimulated with fMet-Leu-Phe in the presence of cytochalasin B

For those enzymes measured spectrophotometrically (see the Materials and methods section), 1 unit of activity caused an absorbance change of 0.001/min. A unit of β-glucuronidase released 1 μg of phenolphthalein/18 h, and 1 unit of gelatinase degraded 10 μg of gelatin/h. Total activity was measured in a sonicated cell sample. Abbreviation used: n.d., not determined.

Enzyme	Activity released (units/10^6 cells)	Activity released (% of total activity)
Myeloperoxidase	444 ± 168	33 ± 10
Elastase	248 ± 65	n.d.
Cathepsin G	8 ± 3	n.d.
Lysozyme	47 ± 15	82 ± 24
β-Glucuronidase	18 ± 4	43 ± 15
Gelatinase	41 ± 6.3	n.d.

DISCUSSION

We have shown that the addition of H_2O_2 to neutrophil cell-free medium containing granule enzymes resulted in loss of activity of all the enzymes measured. That this was due to the reaction of myeloperoxidase present in the medium with H_2O_2 and most likely to the formation of HOCl, was shown by inhibition with azide and methionine, a scavenger of HOCl. Previous studies have shown that phagocytosing neutrophils can auto-inactivate lysozyme and β-glucuronidase (Voetman *et al.*, 1981; Dri *et al.*, 1985) and vitamin B_{12}-binding protein (Clark & Borregaard, 1985) by a myeloperoxidase-dependent process. We have now shown that the

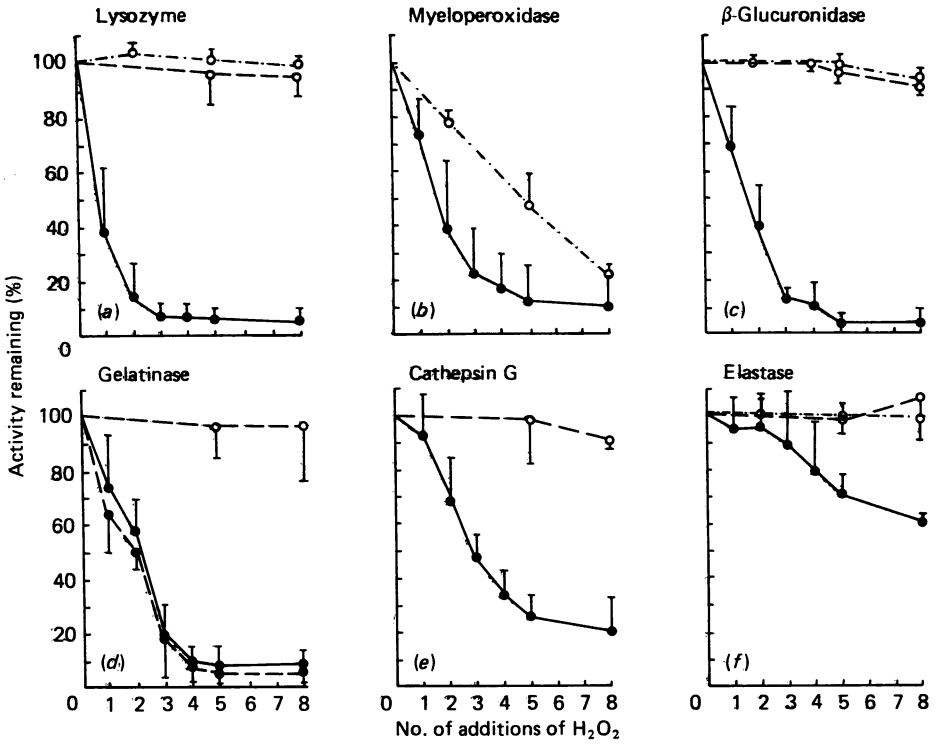

Fig. 1. Oxidative inactivation of neutrophil granule enzymes

Activities were measured before and after stepwise additions of 100 nmol of H_2O_2/ml (●——●). Controls contained 2 mM-azide (○----○) or 4 mM-methionine (○-·-·-○). Each point shown represents the mean ± S.D. for four to eight experiments. Gelatinase was measured both before (●——●) and after (●---●) activation with phenylmercuric acetate.

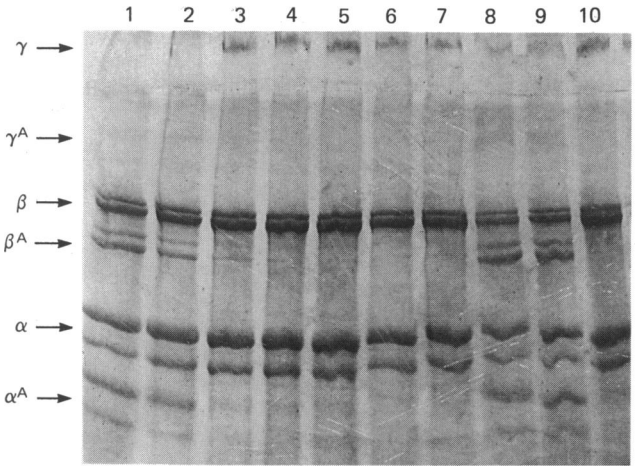

Fig. 2. Oxidative inactivation of neutrophil collagenase

Specific cleavage products of Type I collagen (α^A, β^A and γ^A) are shown. 1, Collagen after incubation with cell-free medium; 2–7, collagenase activity after one, two, three, four, five or eight additions respectively of H_2O_2; 8, eight additions of H_2O_2 plus 2 mM-azide; 9, eight additions of H_2O_2 plus catalase; 10, Type I collagen alone. Similar results were obtained on three other occasions.

neutral proteinases, and myeloperoxidase itself, are also susceptible to myeloperoxidase-dependent oxidative inactivation.

It was noteworthy that, of the seven enzymes monitored, elastase was largely resistant to oxidative inactivation. This is of particular interest, since α_1-antitrypsin, the major inhibitor of elastase *in vivo*, is readily inactivated by the myeloperoxidase system by the oxidation of a methionine residue at the active site (Clark *et al.*, 1981). Once oxidized, α_1-antitrypsin cannot prevent degradation of extracellular matrix by neutrophil elastase (George *et al.*, 1984). Thus it is conceivable that elastase released from neutrophils at a site of inflammation would remain active under conditions in which α_1-antitrypsin could be inactivated.

Activation of latent neutrophil collagenase and gelatinase by HOCl has been reported (Weiss *et al.*, 1985; Peppin & Weiss, 1986). We saw no oxidative activation of partially active gelatinase even when we added small amounts of H_2O_2. Peppin & Weiss (1986), however, could only achieve 25% maximal activation of gelatinase, and our preparation was already more active than this. They, too, observed inactivation of both enzymes at higher oxidant exposure (Weiss *et al.*, 1985; Peppin & Weiss, 1986). Although local concentrations of oxidants produced by stimulated neutrophils are not known, some predictions of their effects on metalloproteinases can be made. Where there is substantial β-glucuronidase or lysozyme inactivation, as is seen with phagocytosing cells (Dri *et al.*, 1985) and, in particular, when stimulation occurs on a surface and some inactivation of elastase occurs (Vissers & Winterbourn, 1986), inactivation rather than activation of gelatinase and collagenase would be expected. Although oxidation is one way by which neutrophil metalloproteinases can be activated, serine proteinases provide an alternative, and apparently more efficient, method. When released concurrently with gelatinase, they can cause up to 100% activation (M. C. M. Vissers & C. C. Winterbourn, unpublished work). Our present results also show that HOCl can

damage the latent form of gelatinase, decreasing the activity measurable after activation.

The sites in each protein oxidized by HOCl are not known. Cysteine and methionine are likely to be the most susceptible (Thomas *et al.*, 1982; Winterbourn, 1985), but HOCl also reacts readily with other protein groups (Winterbourn, 1985; Albrich & Hurst, 1982). It can cleave peptide bonds and react with free amino groups to form chloramines, which may enhance the oxidative capacity (Thomas, 1979; Albrich & Hurst, 1982; Thomas *et al.*, 1982). One factor which will influence the susceptibility of an enzyme to oxidative inactivation is the location of the more reactive amino acids at critical positions in the tertiary structure (e.g. at or near the active site, or within a stabilizing helix). Inactivation of myeloperoxidase, however, was only partially inhibited by methionine and is likely to be due to the formation of an inactive haem–H_2O_2 complex (Winterbourn *et al.*, 1985), as well as to oxidative attack by HOCl.

Although the oxidative burst of neutrophils is important for the microbicidal and cytotoxic function of neutrophils, the role of myeoloperoxidase is less clear (Segal, 1985). Although inactivation of the cell's own granule enzymes may seem to be self-defeating, this process, which is likely to occur mainly within the phagosome, may be beneficial to the host, since it would minimize the release of potentially harmful hydrolytic proteins into the tissues.

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