Regulation of superoxide generation by myeloperoxidase during the respiratory burst of human neutrophils

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The role of myeloperoxidase in the regulation of the respiratory burst of human neutrophils activated by the chemotactic peptide (*N*-formyl-L-methionyl-L-leucyl-L-phenylalanine) plus cytochalasin B was determined by using anti-(human myeloperoxidase) antibody. The respiratory burst activated under these conditions consisted of an initial (1–2 min) phase with high rates of O₂ uptake, luminol-dependent chemiluminescence and superoxide radical (O₂^{•-}) generation and a second, more sustained, phase of lower magnitude of chemiluminescence and O₂ uptake: O₂^{•-} generation did not occur during this second phase. In cell suspensions stimulated in the presence of anti-(human myeloperoxidase) antibody, the magnitude of the initial phase of both O₂ uptake and O₂^{•-} generation was unaffected, but these high rates were maintained over much longer periods than in control suspensions. It is therefore proposed that a product of myeloperoxidase normally regulates the duration of O₂^{•-} generation during the respiratory burst, possibly by inhibition of NADPH oxidase.

INTRODUCTION

Activation of polymorphonuclear leucocytes (neutrophils) results in the formation of a series of reactive oxygen metabolites during a respiratory burst of non-mitochondrial O₂ uptake (Babior, 1978; Klebanoff & Clark, 1978; Karnovsky & Bolis, 1982). These metabolites are utilized for microbial killing during phagocytosis and are also thought to be involved in the tissue damage associated with inflammatory diseases such as rheumatoid arthritis (Halliwell & Gutteridge, 1985). Two key enzyme systems of neutrophils are involved in this production. Firstly, O₂^{•-} is generated via a multi-enzyme NADPH oxidase complex whose terminal components are yet to be fully defined (Lutter *et al.*, 1984; Bellavite *et al.*, 1984; Cross *et al.*, 1984; Morel *et al.*, 1985; Ohno *et al.*, 1985). Secondly, H₂O₂, which is also generated during the respiratory burst, is utilized by myeloperoxidase, a haem-containing enzyme found within azurophilic granules of neutrophils, that, in the presence of a suitable halide ion such as Cl⁻, produces HOCl and related compounds (Stelmazynska & Zgliczynski, 1974; Harrison & Schultz, 1976; Winterbourn, 1985). Products of the myeloperoxidase-H₂O₂-halide system have wide biological reactivities and can mediate a number of important physiological effects, including microbial killing, tumour-cell lysis, and inactivation of toxins and inflammatory regulators (Klebanoff & Clark, 1978).

In addition to these effects, it has been proposed that myeloperoxidase also plays a role in the regulation of the respiratory burst itself. The two lines of evidence which support these conclusions are from experiments using haem inhibitors such as azide and cyanide (Sagone *et al.*, 1977; Jandl *et al.*, 1978; Nauseef *et al.*, 1983) and by measuring oxidase activity in myeloperoxidase-deficient neutrophils (Rosen & Klebanoff, 1976; Cech *et al.*, 1979; Dri *et al.*, 1985). There are, however, a number of

limitations to each approach. Firstly, no haem inhibitor exists that is specific for myeloperoxidase; other haemoproteins involved in the respiratory burst, such as catalase, will also be affected. Secondly, when comparisons are made of respiratory-burst activities in normal and myeloperoxidase-deficient neutrophils, the heterogeneities in oxidase activities that exist within the population of control and test subjects must be accounted for by strict statistical analyses. Thirdly, it is always assumed that myeloperoxidase deficiency is restricted to a single enzymic defect.

Therefore, to overcome these limitations and to fully establish the role of myeloperoxidase in the regulation of the respiratory burst, we have measured the rates of O₂^{•-} generation and O₂ uptake in preparations of human neutrophils incubated in the presence or absence of anti-(human myeloperoxidase) antibody. Since the period of O₂^{•-} generation was extended in cell suspensions incubated in the presence of anti-myeloperoxidase antibody, it is therefore proposed that a product of the myeloperoxidase system inhibits the NADPH oxidase responsible for O₂^{•-} formation.

EXPERIMENTAL

Preparation of neutrophils

Human polymorphonuclear leucocytes (neutrophils) were isolated from buffy-coat preparations or whole blood from healthy donors by utilizing a combined dextran (Dextran T-500)/Ficoll-Paque (both from Pharmacia Fine Chemicals, Uppsala, Sweden) separation procedure. After sedimentation of erythrocytes in 1.5% (w/v) dextran for 45 min at 1 g, the supernatant was centrifuged at 2000 g for 5 min to pellet the leucocytes. Residual erythrocytes were lysed by adding 9 vol. of distilled water to this pellet, followed by vigorous

Abbreviations used: O₂^{•-}, superoxide radical; fMet-Leu-Phe, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; OH[•], hydroxyl radical.

shaking for 30 s; osmoticity was restored by adding 1 vol. of 9% (w/v) NaCl before centrifugation at 1000 *g* for 5 min. The leucocyte pellet was suspended in a buffer containing (mM) NaCl (12), KCl (4.8), KH_2PO_4 (1.2), CaCl_2 (1.3), MgSO_4 (1.2), Hepes (25) (pH 7.4) and 0.1% bovine serum albumin before centrifugation in Ficoll-Paque at 1000 *g* for 30 min. The neutrophil-enriched (> 97% neutrophils) pellet was washed twice and suspended in the above buffer. Cells were used within 4 h of preparation.

Analytical methods

$\text{O}_2^{\cdot-}$ generation after cell activation was measured in a Cecil CE 595 spectrophotometer by using a continuous assay modified from that described by Babior *et al.* (1973). The incubation mixture (total volume 1 ml) contained 75 μM -cytochrome *c*. O_2 uptake was measured with a Clark-type oxygen electrode, whereas luminol-dependent chemiluminescence was monitored in an LKB Wallac 1250 luminometer. Rabbit anti-(human myeloperoxidase) antibody was raised after subcutaneous injections of human myeloperoxidase purified by the method of Pember *et al.* (1983). The titre of this antiserum was 290 $\mu\text{g}/\text{ml}$, calculated by rocket immunoelectrophoresis using purified enzyme. In all experiments the stimulus used was the chemotactic peptide fMet-Leu-Phe (final concn. 1 μM) plus cytochalasin B (final concn. 1 $\mu\text{g}/\text{ml}$).

Chemicals

fMet-Leu-Phe, cytochalasin B, cytochrome *c* and luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) were from Sigma, whereas superoxide dismutase was from Boehringer. All other chemicals were of the highest purity available.

RESULTS

Kinetics of the respiratory burst

Resting (unstimulated) neutrophils had low levels of luminol-dependent chemiluminescence and O_2 uptake and immeasurable rates of $\text{O}_2^{\cdot-}$ generation (Fig. 1). After activation, maximal rates of all three parameters measured occurred within 45–60 s of the addition of stimulus (Fig. 1). The rate of luminol-dependent chemiluminescence decreased to a low level by 5–6 min and then a second increase occurred that was sustained over the following 7–12 min (Fig. 1*b*). The rate of O_2 uptake also declined after the initial rapid increase and was then maintained, at a rate elevated 5-fold above the unstimulated level (Fig. 1*b*), for periods in excess of 30 min (results not shown). Similarly, the rate of $\text{O}_2^{\cdot-}$ generation declined after 1–2 min, but, in contrast with the other two measurements of oxidative metabolism, was undetectable after 6–7 min (Fig. 1*b*). Suitable control experiments ensured that this decrease in $\text{O}_2^{\cdot-}$ generation was not due to substrate (O_2 or cytochrome *c*) depletion or to cell sedimentation (results not shown). Therefore, as reported for rat neutrophils (Edwards *et al.*, 1983), the respiratory burst activated under these conditions may be separated in two phases: (a) the initial 1–2 min of high respiratory activity and (b) a second more prolonged phase of activity of lower magnitude. $\text{O}_2^{\cdot-}$ generation, however, essentially only occurred during the initial phase.

Fig. 1. Respiratory-burst kinetics of human neutrophils

In (a), neutrophils were suspended in buffer containing 10 μM -luminol to a concentration of $2.4 \times 10^6/\text{ml}$ (total volume 1 ml), whereas in (b), the cell concentrations were $1.5 \times 10^6/\text{ml}$ (total volume 2 ml) for O_2 uptake ($\circ$) and $6 \times 10^5/\text{ml}$ (total volume 1 ml) for $\text{O}_2^{\cdot-}$ measurements ($\bullet$). At zero time, the cell suspensions were stimulated by the addition of 1 μM (final concn.)-fMet-Leu-Phe plus cytochalasin B (1 $\mu\text{g}/\text{ml}$ final concn.). O_2 uptake and $\text{O}_2^{\cdot-}$ generation were measured continuously and rates were calculated at suitable time intervals. The result is typical for at least five separate experiments.

Effect of myeloperoxidase on O_2 uptake and $\text{O}_2^{\cdot-}$ generation

Maximal release of myeloperoxidase from human neutrophils activated under these conditions occurs within 1 min after stimulation (S. W. Edwards & T. F. Swan, unpublished observations). Therefore, to evaluate a possible role of myeloperoxidase in the regulation of the respiratory burst, rates of O_2 uptake and $\text{O}_2^{\cdot-}$ generation were measured after activation of cell suspensions incubated in the absence or presence of rabbit anti-(human myeloperoxidase) antibody. Although the maximal rate of $\text{O}_2^{\cdot-}$ generation was not significantly altered, the duration of production was markedly prolonged in suspensions incubated with antibody

Fig. 2. Effect of anti-(human myeloperoxidase) antibody on $O_2^{\cdot -}$ generation

The cell suspensions contained 5×10^6 cells/ml (total volume 1 ml) and at zero time were stimulated as described in the legend to Fig. 1. ●, Control; ■, $O_2^{\cdot -}$ production in the presence of $5 \mu\text{l}$ of anti-(human myeloperoxidase) antiserum; ○, $O_2^{\cdot -}$ production measured in the presence of $5 \mu\text{l}$ of pre-immune rabbit serum. Results are typical of at least five separate experiments.

(Fig. 2). For example, 7 min after stimulation the rates in the presence or absence of anti-myeloperoxidase antibody were 43% and 12% respectively of the maximal rate measured at 1 min, i.e. elevated 3.6-fold (mean = 3.16 ± 0.48 , $n = 9$). Rates of $O_2^{\cdot -}$ generation in the presence of pre-immune rabbit serum were essentially the same as in control suspensions (Fig. 2), indicating that non-specific radical scavenging by serum proteins was negligible under these conditions. In a similar series

Fig. 3. Effect of anti-(human myeloperoxidase) antibody on O_2 uptake

The cell suspensions contained 1.5×10^6 cells/ml (total volume 2 ml) and at zero time were stimulated as described in the legend to Fig. 1. ●, Control rate of O_2 uptake; ■, O_2 uptake measured in the presence of $40 \mu\text{l}$ of anti-(human myeloperoxidase) antiserum; ○, O_2 uptake measured in the presence of $40 \mu\text{l}$ of pre-immune rabbit serum. Results are typical for at least five separate experiments.

of experiments measuring O_2 uptake, the duration of the rapid period of respiration was prolonged in suspensions incubated with anti-(human myeloperoxidase) antibody (Fig. 3). For example, 7 min after stimulation, the rates of uptake in the presence or absence of anti-myeloperoxidase antibody were 94% and 60% respectively of the maximal rate, i.e. elevated 1.6-fold (mean = 1.77 ± 0.26 , $n = 6$). The kinetics and magnitude of the respiratory burst stimulated in the presence of pre-immune serum were essentially the same as those in control suspensions (Fig. 3).

DISCUSSION

The respiratory burst of human neutrophils activated by the chemotactic peptide plus cytochalasin B, in common with that of rat neutrophils (Edwards *et al.*, 1983), is separated into two discrete phases. In the initial phase, rates of O_2 uptake, $O_2^{\cdot -}$ generation and luminol-dependent chemiluminescence reached maximal values within 1–2 min after stimulation (Fig. 1); after this period, rates of all three parameters decreased and, by 6–7 min, rates of O_2 uptake and luminol-dependent chemiluminescence became maintained at levels elevated above the resting rate. However, this latter phase of oxidative metabolism was not accompanied by $O_2^{\cdot -}$ generation, as assayed by cytochrome *c* reduction. This observation raises the possibility that this second phase of oxygen uptake is mediated by an oxidase system, alternative to the $O_2^{\cdot -}$ -generating NADPH oxidase complex, whose product of oxygen reduction is H_2O_2 or water. However, it is possible that intracellular $O_2^{\cdot -}$ generation occurs during this second phase, but is inaccessible to the substrate used to monitor $O_2^{\cdot -}$ (cytochrome *c*).

Whereas a number of reports have suggested a role for myeloperoxidase in regulating the respiratory burst (see the Introduction section), our approach was to use anti-(human myeloperoxidase) antibody as a specific inhibitor of this enzyme and to measure its effect on oxidase activity. Another advantage of our approach was that we were able to directly compare these effects with control experiments on the same preparations of neutrophils. This allowed us to overcome the problems that result from the heterogeneities of respiratory-burst responses that exist within the population. The results conclusively show that myeloperoxidase, which is released from the cells in parallel with the activation of the respiratory burst, regulates the duration, but not the magnitude, of $O_2^{\cdot -}$ generation; presumably a product of the myeloperoxidase system (e.g. HOCl) inactivates the NADPH oxidase system responsible for $O_2^{\cdot -}$ production. The reported increase in $O_2^{\cdot -}$ production by myeloperoxidase-deficient neutrophils therefore represents an increase in the duration of oxidase activity without an intrinsic increase in the rate of generation.

These results are of importance for our understanding of the events that occur at inflammatory sites and are implicated in the mechanisms responsible for host tissue damage. Oxidizing compounds produced by neutrophils which have been implicated in this tissue damage are HOCl and 1O_2 , both presumed products of myeloperoxidase, and $OH^{\cdot}$, believed to be generated from $O_2^{\cdot -}$ and H_2O_2 in a metal-catalysed Haber-Weiss reaction (Halliwell & Gutteridge, 1984). Since the cellular processes of oxidase activation and degranulation may

be regulated by separate controls (Goldstein, 1984; Edwards & Lloyd, 1986), it is the extent of degranulation that will regulate the degree of formation of these products. For example, if myeloperoxidase is not released during activation, then the duration of NADPH oxidase activity is extended, resulting in the formation of large amounts of $O_2^{\cdot -}$. After dismutation to H_2O_2 , substantial quantities of $OH^{\cdot}$ may be formed, since conditions exist in synovial fluid to promote this reaction (Gutteridge *et al.*, 1981). However, if myeloperoxidase is released, then $O_2^{\cdot -}$ generation (and consequently the potential to form $OH^{\cdot}$ are reduced, whereas an alternative tissue-damaging system ($HOC1$ and 1O_2) will become activated. It is therefore essential that neutrophils from synovial fluid of rheumatoid joints are carefully characterized with respect to their levels of oxidase activation and myeloperoxidase release, in order that we can understand the mechanisms underlying tissue damage and hence design effective chemotherapy.

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