THE PRODUCTION OF HYDROXYL AND SUPEROXIDE RADICALS BY STIMULATED HUMAN NEUTROPHILS – MEASUREMENTS BY EPR SPECTROSCOPY

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1. Introduction

Neutrophilic polymorphonuclear leukocytes (neutrophils) consume considerably more oxygen in association with phagocytosis than in the resting state. This extra respiration of phagocytosis [1] is not affected by inhibitors of mitochondrial electron transport and is distinct from the process of phagocytosis itself, which proceeds [2] normally under anaerobic conditions. The oxidase system appears to involve a novel cytochrome *b* [3,4] and is important [5] for the microbicidal processes responsible for the killing of certain bacteria.

Stimulated neutrophils generate [6,7] hydrogen peroxide and reduce cytochrome *c* in the medium, a process that can be inhibited [8] by superoxide dismutase. These cells have also been shown [9] to convert methional to ethylene, an oxidation thought to be mediated by hydroxyl radicals. The postulated series of events following stimulation of the cell has been suggested to involve the production of superoxide which dismutates spontaneously to H_2O_2, and that these two compounds then interact [10] in the Haber-Weiss reaction to generate [11] hydroxyl radicals. The mechanism by which the generation of these reduced oxygen species is related to bacterial killing has not been established.

This study describes the use of EPR spectroscopy in conjunction with 'spin-traps' [12,13] which react readily with short-lived radical species to yield longer-lived nitroxide derivatives.

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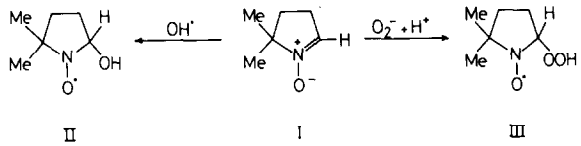

II I III

Scheme 1

2. Methods

Neutrophils were prepared [3] from fresh human blood by dextran sedimentation, centrifugation through a gradient of ficoll/sodium metrizoate and contaminating erthrocytes were removed by haemolytic lysis. Cell suspension (1 ml) containing 4×10^7 cells in RPMI 1640 medium and 100 mmol/l 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) I, was stirred rapidly in the chamber of a Clark oxygen electrode at 37°C. The cells were stimulated by either the addition of 1×10^9 latex particles (0.81 Difco) coated with IgG, 1 mg serum opsonised zymosan (Sigma) or 10 μg phorbol myristate acetate (Sigma) in 10 μl dimethyl sulphoxide. At various intervals after the addition of the stimulus a portion (0.5 ml) of the cell suspension was transferred to the chamber of a 0.2 ml flat cell using a simple syringe technique [14].

The DMPO was prepared according to [15], redistilled, diluted with water and further purified by the method in [16]. The concentration of the stock solution was determined spectrophotometrically ($\epsilon_{234} = 7700\ M^{-1}\ cm^{-1}$) in ethanol.

EPR spectra were recorded using a Varian E 109

spectrometer operating with 100 kHz field modulation in the first derivative mode. Superoxide dismutase (SOD) derived from baker's yeast was the kind gift of Dr J. Johansen. Catalase was obtained from Sigma; all other compounds were of Analar grade.

3. Results

Unstimulated cells consumed oxygen at ~4 nmol/4×10^7 cells/min. After stimulation with latex, zymosan and phorbol the rates rose to 75, 29 ånd 47 nmol/min, respectively. The addition of DMPO (I) to the incubation chamber had no effect on the oxygen consumption and hence is not a stimulant on its own. However it increased the rate of latex-induced oxygen consumption to 125 nmol/min and this was unaltered in the presence of catalase or superoxide dismutase.

The EPR spectrum of a sample containing resting unstimulated neutrophils and the spin-trap, DMPO, is shown in fig.1a. The absence of resonances suggests that either no radicals are produced by the unstimulated neutrophils, any produced do not react with the spin-trap or the product is rapidly consumed by the neutrophils. In contrast the addition of (I) to neutrophils stimulated by IgG-coated latex particles results in a sample, taken 1 min after stimulation, having the EPR spectrum shown in fig.1b. The spectrum is that expected [16–18] from the product (II) formed by the reaction of hydroxyl radicals with DMPO. Samples taken after 1 min also had EPR spectra consistent with the formation of (II), though the signal intensity diminished with time. Stimulation of the neutrophils by zymosan gave rise to essentially the same results. However, when phorbol was used as a stimulant, in the presence of (I), the EPR spectrum of resultant solution, taken 1 min after addition of the stimulant (fig.1c) is that of a mixture of the products [16,17,19] of the reaction of (I) with $OH^{\cdot}$ (II) and O_2^- (III). The addition of yeast superoxide dismutase to neutrophils, activated by either IgG-coated latex particles, zymosan or phorbol in the presence of (I) has a dramatic effect on the subsequently measured EPR spectrum (fig.2a). No resonances are observed. In contrast the addition of catalase under the same conditions has little effect on the resultant EPR spectrum (fig.2b).

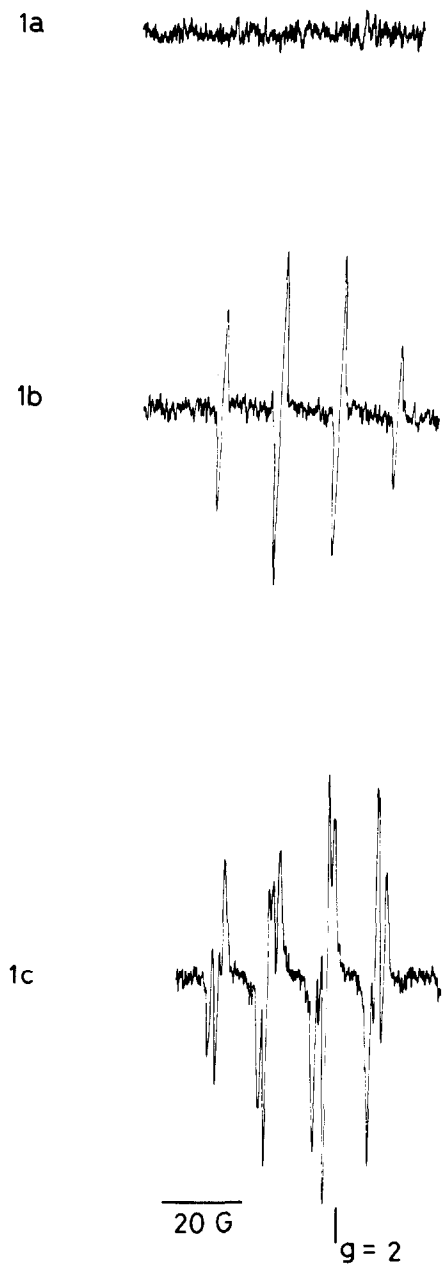

Fig.1. EPR spectra obtained after incubation at 37°C of human neutrophils (4×10^7 cells/ml) I (100 mM) with latex (1×10^9 particles) or phorbol (10 μg/ml). Samples were removed from the thermostated chamber and transferred to an EPR flat cell after 1 min. Neutrophils (Ia) + DMPO; (Ib) + latex particles; (Ic) + phorbol. (Field 3385 G, frequency 9.462 GHz, power 30 mW, modulation 1 G, time constant 0.128 s, scan rate 0.8 Gs^{-1}, gain 5×10^4.)

Of the known [20] hydroxyl radical scavengers, formate ion, ethanol and mannitol (140 mM) only the last of these had a marked effect on the formation of (II) reducing the signal intensity by half. In order to investigate the involvement of haem proteins, inhibitors of such enzymes, cyanide ion and azide ion, were added to neutrophils stimulated by IgG-coated latex particles in the presence of (I). Both anions lead to an increased yield of (II) (fig.2c) and particularly with azide, the formation of (III).

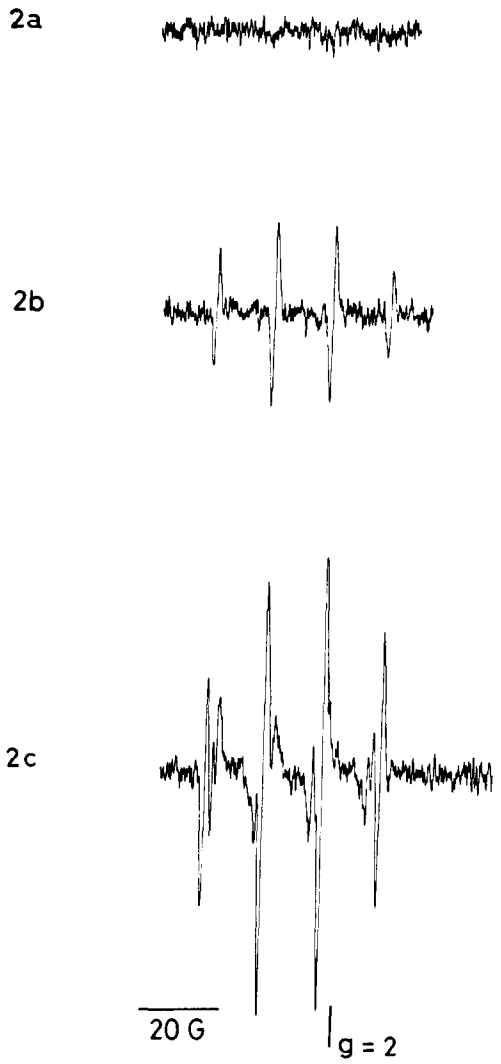

Fig.2. EPR spectra obtained after incubation at 37°C of human neutrophils (4 × 10^7 cells/ml), I (100 mM) with latex (1 × 10^9 particles) in the presence of SOD (100 μg/ml), catalase (100 μg/ml) or sodium azide (1 mM). Samples were removed from the thermostated chamber and transferred to the flat cell. Neutrophils + DMPO + latex in the presence of (a) SOD (after 1 min); (b) catalase (after 4 min); (c) sodium azide (after 1 min). EPR conditions as in fig.1.

4. Discussion

The formation, by stimulated neutrophils, of superoxide ions and hydroxyl radicals has been proposed [9] on the basis of somewhat more indirect methods of detection than those used here. Our results are consistent with these proposals. The formation of (II) reveals the presence of hydroxyl radicals and the inhibition of their formation by superoxide dismutase, an enzyme for which the superoxide ion is the only [21] known substrate, suggests that the hydroxyl radicals are formed by a process which involves the superoxide ion. The formation of (III), derived from O_2^-, in the experiments using phorbol as the stimulant and in those which include cyanide or particularly azide, is additional evidence for the implication of the superoxide ion as a product formed from the stimulated neutrophils. However, it is likely that, under normal circumstances, this radical is not released free as such, but acts as an intermediate in the generation of the hydroxyl radical.

How is the hydroxyl radical derived from the superoxide ion? A favoured [22] mechanism of formation was the so-called Haber-Weiss reaction:

$$O_2^- + H_2O_2 \rightarrow OH^{\cdot} + O_2 + OH^-$$

but recent work [23–25] has indicated that the rate of this reaction is very slow. There remains the possibility [20,26,27] that a metal-catalysed variant may lead to the formation of hydroxyl radicals, namely:

$$O_2^- + Fe^{3+} \rightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^-$$

The lack of inhibition by catalase in the experiments carried out in this study would, at first sight, suggest that hydrogen peroxide is not a precursor of the hydroxyl radical. However, if the hydrogen peroxide formed was not 'free', i.e., it remained coordinated to

a metal ion, catalase may not act as an effective scavenger. It is possible that the hydroxyl radical is formed from the superoxide ion via disproportionation reaction within the locus of a reducing metal ion, that is:

$$2\ O_2^- + Fe^{2+} + 2\ H^+ \rightarrow OH^{\cdot} + OH^- + O_2 + Fe^{3+}$$

It is possible that these metal ions are contained within the haem groups of myeloperoxidase which can react with both [28], O_2^- and [29] H_2O_2. This protein has [30] two haem protein subunits with different properties, including different reactivities for hydrogen peroxide and cyanide and these subgroups might interact. The reaction of myeloperoxidase with CN^- and N_3^-, is consistent with this role, although these inhibitors could of course react with other metalloenzymes [31]. Of the known hydroxyl radical scavengers, only mannitol was effective in these experiments in inhibiting the formation of (II). This may reflect the relative rates of reaction of $OH^{\cdot}$ with [I] and the other three hydroxyl radical scavengers or it is possible that ethanol and formate ion are excluded from the hydroxyl-generating site.

These studies confirm the results obtained with indirect assays which indicated that superoxide and hydroxyl radicals are generated by stimulated neutrophils. Whether these compounds are the natural microbicidal product of the oxidase process, or simply intermediates in a complex series of reactions, has yet to be determined.

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