PRODUCTION OF SUPEROXIDE BY NEUTROPHILS: A REAPPRAISAL

Anthony W. SEGAL and Tova MESHULAM+

Division of Cell Pathology, Clinical Research Centre, Watford Road, Harrow, Middlesex, England and *+Department of Microbiology, Technion Medical School, Haifa, Israel*

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1. Introduction

It is generally accepted that the burst of oxygen consumption that accompanies phagocytosis by polymorphonuclear leukocytes (neutrophils) [1] generates superoxide ($O_2^{\cdot-}$) [2] which then spontaneously dismutates to form hydrogen peroxide. It is thought that the $O_2^{\cdot-}$ is itself bactericidal [3] and that the hydrogen peroxide may act as substrate for a killing system involving myeloperoxide and a halide [4]. It is also thought that when neutrophils are stimulated they release these superoxide anions from their external surface and that these radicals may then cause oxidative damage to surrounding tissues [5]. Superoxide release from neutrophils has been proposed as an important promoter of tissue damage at sites of inflammation [5] and superoxide dismutase (SOD), an enzyme that accelerates the dismutation of $O_2^{\cdot-}$ to H_2O_2 [6], has been suggested as a therapeutic tool to inactivate the $O_2^{\cdot-}$ [5]. Clinical trials of the efficacy of SOD as an anti-inflammatory drug have already been initiated in patients with arthritis and with urinary tract infections [7,8].

The evidence that $O_2^{\cdot-}$ is produced by stimulated neutrophils rests predominantly on the indirect evidence that these cells will reduce cytochrome *c* if this is added to the medium, and that this effect can be inhibited by superoxide dismutase [2,9]. We have investigated the effect of SOD on oxygen consumption and cytochrome *c* reduction, and out studies indicate that although $O_2^{\cdot-}$ may be an intermediate in the oxidase system, it does not appear to be the final product, and that little, if any, is discharged into the extracellular environment. The addition of cytochrome *c* to stimulated neutrophils appears to induce an unphysiological diversion of electrons through oxygen to the cytochrome *c* itself.

2. Materials and methods

Cytochrome *c* (horse heart type VI) superoxide dismutase (from bovine blood, 2950 IU/mg protein), xanthine oxidase (from buttermilk, 0.66 IU/mg protein) and Hepes were obtained from Sigma, hypoxanthine from British Drug Houses and RPMI 1640 medium from Flow Labs. $Cu(Tyr)_2$ complexes were a gift from Dr H. A. O. Hill, Department of Inorganic Chemistry, Oxford.

Neutrophils were separated from fresh human blood taken from normal volunteers into heparin (5 IU/ml Paines and Byrne, Greenford) by sedimentation of erythrocytes with dextran (Dextran 500, Sigma), centrifugation through a gradient of ficoll/sodium metrizoate (Ficoll-paque, Pharmacia, density 1.077) and hypotonic lysis with distilled water for 30 s after which isotonicity was restored with 0.3 mol/l NaCl containing heparin (5 IU/ml).

Oxygen consumption was measured polarographically in a Clark-type oxygen electrode (Rank Brothers, Bottisham). The cells were incubated in a rapidly stirred, thermostatically controlled (37°C) chamber above the electrode and were stimulated by the addition of latex particles (0.81 μm diam., Difco, particle : cell ratio 50:1) opsonised with IgG [10], through a vent in the stopper.

Table 1
To show the effect of cytochrome *c* (80 μmol/l) and superoxide dismutase (33 μg/ml) on oxygen consumption by stimulated human neutrophils and by xanthine oxidase

Additive		Maximum rate (nmol/10^7 cells/min)				Total (nmol/10^7 cells/2 min)			
Oxidase system	Cytochrome *c*	–	–	+	+	–	–	+	+
	Superoxide dismutase	–	+	–	+	–	+	–	+
Neutrophils	Oxygen consumption	29.8 ± 3.6	31.2 ± 4.0	30.9 ± 3.3	28.4 ± 13.5	46.5 ± 5.5	45.6 ± 4.7	40.2 ± 4.6	36.7 ± 4.7
	Cytochrome *c* reduction							21.2 ± 1.3	0
Xanthine oxidase	Oxygen consumption			13.3 ± 1.6	23.2 ± 1.1			29.1 ± 4.0	47.4 ± 1.3
	Cytochrome *c* reduction							38.0 ± 7.7	0

Experimental details as in fig.1,3. Xanthine oxidase (33 mU/ml, Sigma) replaced the neutrophils and oxygen consumption was initiated by the addition of hypoxanthine (1 mmol/l, British Drug Houses). Results are expressed as the mean (± SE) of 4 studies

3. Results and discussion

Figure 1 shows the lack of any demonstrable effect of SOD and/or cytochrome *c* on oxygen consumption by stimulated neutrophils. Table 1 shows that this is a consistent observation, that cytochrome *c* is reduced by stimulated neutrophils and that this reduction is completely inhibited by SOD. Copper tyrosine complexes ($Cu(Tyr)_2$) [11] had a similar effect. Cytochrome *c* reduction was inhibited 90, 76 and 68% at 1 mmol/l and 100 and 10 μmol/l respectively, but oxygen consumption was unaffected. In experiments in which the neutrophils are replaced with a xanthine oxidase and hypoxanthine system that generates $O_2^{\cdot-}$ [12], cytochrome *c* is reduced and this reduction is inhibited by SOD, but in contradistinction to the experiments with neutrophils, the inhibition of cytochrome *c* reduction is associated with increased oxygen consumption (table 1).

Figure 2 shows the reproducibility of the polarographic measurement of oxygen consumption and the consistent lack of enhancement of oxygen consumption by SOD when the cells were suspended in RMPI 1640 medium containing a higher concentration of cytochrome *c* (270 μmol/l) instead of Hanks BSS. The rates of oxygen consumption, in consecutive experiments, performed alternately, in the presence and in the absence of SOD were 25.68 ± 1.37 ($n = 4$) and 25.63 ± 1.02 ($n = 4$) nmol/10^7 cells/min, respectively.

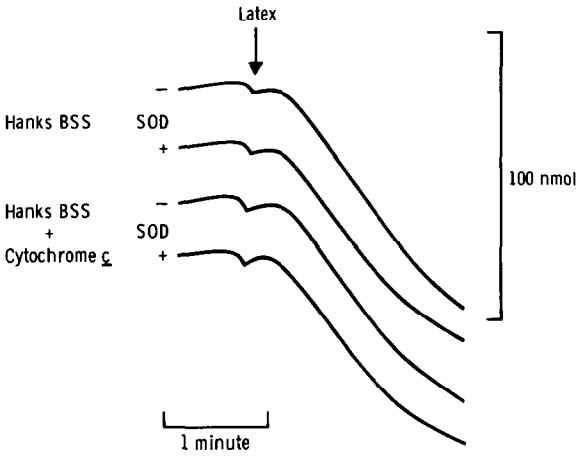


Fig.1. Polarographic tracings in a typical experiment to show the absence of a measurable effect of cytochrome *c* and of superoxide dismutase on oxygen consumption by human neutrophils. Neutrophils were suspended (2×10^7/ml) in Hanks balanced salt solution (BSS) containing Hepes (15 mmol/l, pH 7.4) and (5 IU/ml) heparin. Hanks BSS (1.0 ml) alone or containing cytochrome *c* (240 μmol/l) and/or superoxide dismutase (150 μg/ml) was mixed with cell suspension (2.0 ml) in a chamber attached to an oxygen electrode. An aliquot (1.5 ml) of the cell suspension was removed for control measurements prior to the addition of latex particles (50/cell) opsonised with human IgG.

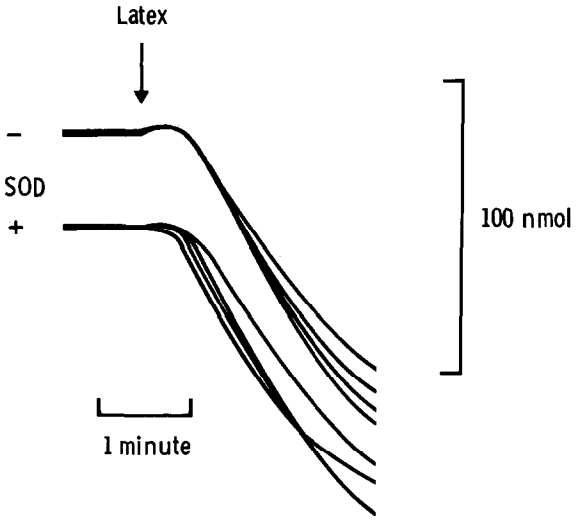


Fig.2. To show the absence of a measurable effect of SOD on oxygen consumption by neutrophils suspended in RPMI 1640 medium containing (5 IU/ml) heparin and (270 μmol/l) cytochrome *c*. Experimental details as in fig.1.

Figure 3 shows the time course of cytochrome *c* reduction and of oxygen consumption after the addition of particles to the incubation chamber. Cytochrome *c* reduction is almost complete by 30 s when oxygen consumption is only just commencing [10].

4. Discussion

It is accepted that superoxide is generated by the respiratory burst of neutrophils [9,13–20] and that this superoxide then dismutates to form H_2O_2 [2,21,22], a possible substrate for the proposed myeloperoxidase mediated microbicidal system [4].

The basis of the proposal that stimulated neutrophils generate superoxide has been the observation that they reduce cytochrome *c* in solution in the suspending medium and that this reduction is inhibited by superoxide dismutase. The data presented in this

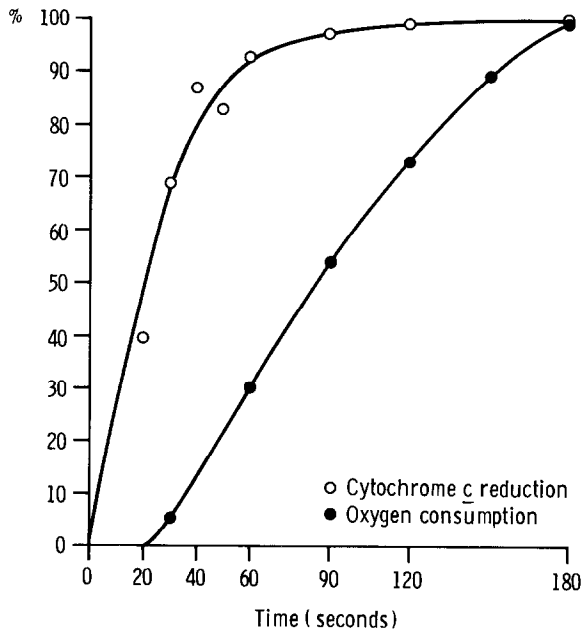
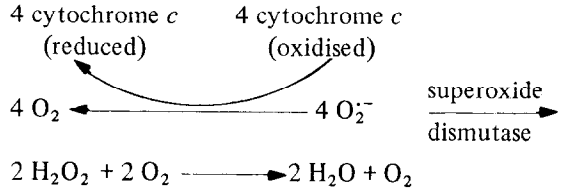


Fig.3. To show the temporal relationship between oxygen consumption and cytochrome *c* reduction by stimulated human neutrophils. Incubation conditions as in fig.1 were terminated by the addition of 5.0 ml ice-cold Hanks BSS containing 1 mmol/l tetrasodium EDTA to the chamber after which the mixture was placed on ice. An aliquot was then centrifuged at 15 000 × *g* for 10 min at 4°C in a Sorval SS3 centrifuge and reduced cytochrome *c* in the supernatant fluid was determined in comparison with control suspensions in a dual beam spectrophotometer at 550 nm (E = 21.0 mM^{-1} cm^{-1}) [32]. The mean results from 3 studies are shown and expressed as a % of the cytochrome *c* reduction (16.1 ± 3.8 (SE) nmol/10^7 cells) and oxygen consumption (34.0 ± 8.0 nmol/10^7 cells) 3 min after the addition of the particles.

paper calls into question the validity of this indirect assay and the conclusions drawn from it.

In the reduction of cytochrome *c* by $O_2^{\cdot-}$, the $O_2^{\cdot-}$ loses its electron, regenerating O_2. SOD is thought to prevent the reduction of cytochrome *c* by causing the dismutation of $O_2^{\cdot-}$ to H_2O_2.



Thus in these experiments, if the cytochrome *c* is in fact being reduced by $O_2^{\cdot-}$ then the inhibition of its reduction should be accompanied by an increase in the consumption of oxygen. Stoichiometrically, the inhibition of the reduction of 4 molecules of cytochrome *c* by SOD should incorporate 2 molecules of O_2 into H_2O_2. The H_2O_2 could remain in that state or be catabolised by a peroxidase [23], in which case the stoichiometry would remain unchanged, or it could be broken down to H_2O and O_2, either spontaneously or by catalase, incorporating 1 molecule of O_2 into H_2O for each 4 molecules of cytochrome *c* that would have been reduced.

In this study it was found that although SOD completely inhibited cytochrome *c* reduction, the rate and extent of oxygen consumption remained unchanged (fig.1,2, table 1). Failure to detect a change in oxygen consumption was not due to inadequate sensitivity of the methodology because the predicted change in oxygen consumption was observed when superoxide was generated by xanthine oxidase [12] under exactly the same conditions (table 1). The reproducibility of the polarographic techniques is shown in fig.2.

Two other observations argue against the generation and release of $O_2^{\cdot-}$. The first is the difference in the time course of oxygen consumption and cytochrome *c* reduction after the addition of particles (fig.3). Oxygen is only consumed after a lag of 30 s whereas cytochrome *c* reduction is almost complete by this time. If the addition of SOD inhibits cytochrome *c* reduction by producing H_2O_2 then this should occur at the time that the cytochrome *c* is reduced and should be reflected by a decrease in the lag period before oxygen consumption. In fact there was no significant difference in the lag time of cell suspensions in the presence (19 ± 1.3 s, n = 4) or absence (20 ± 1.5 s, n = 4) of SOD which abolished cytochrome *c* reduction from 21.2 ± 1.3 nmol/10^7 cells in 1 min to immeasurably low levels. Few other studies of a similar nature have been done. The time course of cytochrome *c* reduction of neutrophils exposed to zymosan particles has been measured [15]. They found that it only commenced after 5 min and continued for at least 35 min. They did not however relate cytochrome *c* reduction to particle uptake, and this is obviously crucial because cytochrome *c* is reduced only very slowly by unstimulated cells. The incubation conditions they used were very

different from those in our study in which the conditions have been standardised and the rates of particle uptake and their relationship to oxygen consumption characterised. They used a cell concentration that was 2 orders of magnitude lower, particles unopsonised, or coated with a less efficient opsonin, and less efficient mixing. A similar criticism can be made of the study [24] in which latex particles were coated with either SOD or bovine serum albumin (BSA) and the effect on oxygen consumption observed. They found that cells exposed to particles coated with SOD demonstrated a greater burst of oxidative metabolism than those coated with BSA. They measured the rate of phagocytosis of the two particles and found them to be the same, however, the incubation conditions for the metabolic studies and particle uptake were entirely different. They also investigated the effect of SOD and BSA on the reduction of nitroblue tetrazolium (NBT) (which was inhibited ~60% by the SOD), but surprisingly in this case they used soluble rather than latex-adsorbed proteins and did not assess the effect of these proteins on phagocytosis. NBT reduction is not a good measure of superoxide production by neutrophils as the dye is toxic to the cells [25]. It has been used by other investigators who found that SOD only inhibited NBT reduction by ~30% [26].

Cytochrome *c*, acting as a scavenger of $O_2^{\cdot-}$, should inhibit oxygen consumption by stimulated cells. In this study it did not do so (fig.1, table 1). Using very similar techniques an inhibition of oxygen consumption by cytochrome *c* was observed [17] and this was partially reversed by SOD. However, their experiments were performed in the presence of 1 mM azide which limits the physiological relevance of the observations.

The results of this study indicate that SOD does not appear to be inhibiting the reduction of cytochrome *c* simply by the dismutation of $O_2^{\cdot-}$ to H_2O_2. This indicates that either the $O_2^{\cdot-}$ is not produced by neutrophils and that the reduction of cytochrome *c* is inhibited by SOD by another, non-specific mechanism such as the blocking of an active site of cytochrome *c* reduction on the cell surface, or that the $O_2^{\cdot-}$ is produced by the cell but is not normally released into the suspending medium and that the reduction of cytochrome *c* by this bound $O_2^{\cdot-}$ is inhibited by a mechanism other than dismutation. The inhibition of cytochrome *c* reduction by $Cu(Tyr)_2$ complexes suggests that it is not an effect of the protein moiety of the SOD. It has recently been shown that the neutrophil oxidase system included a cytochrome *b* [27]. Superoxide can be generated by, and may in fact be an essential intermediate in electron transport by cytochromes of the mitochondrial [28], photosynthetic [29], and P450 [30] systems. It is possible that the $O_2^{\cdot-}$ could be retained as a $Fe^{3+}.O_2^{\cdot-}$ adduct [31] of the cytochrome *b* which is relatively stable and only dissociates in the presence of another acceptor containing a haem group such as myeloperoxidase, under physiological conditions, or cytochrome *c* in the test system. Similarly it is also possible that $O_2^{\cdot-}$ is an intermediate in, or minor byproduct of the neutrophil oxidase system. The addition of cytochrome *c* could create an artificial electron sink trapping electrons through an intermediate $O_2^{\cdot-}$ radical.

The extensive literature on the generation of $O_2^{\cdot-}$ by stimulated neutrophils, and its possible relationship to bactericidal mechanisms and tissue damage, should be reassessed in the light of the present studies, and the role of free $O_2^{\cdot-}$ as the major product of the respiratory burst of stimulated neutrophils should be regarded with caution until the production of this radical can be quantitated by more direct methods.

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