Activated polymorphonuclear leucocytes consume vitamin C

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Polymorphonuclear leucocytes (PMN) are known to produce superoxide and other oxygen derivatives upon activation as part of their microbicidal armory. Here we report that extracellular ascorbate is effectively oxidised by activated but not by resting human PMN in vitro. The oxidation of ascorbate is mainly caused by the superoxide that is generated by the activated cells, as shown by its effective inhibition by superoxide dismutase. However, myeloperoxidase, which may generate hypochlorite, also contributes to a significant extent. Ascorbate reduces superoxide to peroxide, as indicated by measurements of the stoichiometry of ascorbate and oxygen consumption. These results support the notion that extracellular ascorbate may serve as an important physiological protecting agent against oxygen radical damage in inflammation.

Ascorbate *Superoxide* *Myeloperoxidase* *Neutrophil* *Inflammation* *Oxygen radical*

1. INTRODUCTION

Activation of polymorphonuclear leucocytes (PMN) and other phagocytic cells is associated with an 'oxygen burst' [1,2]. This is primarily due to the activation of a membranous NADPH oxidase which catalyses reaction (1):

$$2O_2 + NADPH \longrightarrow 2O_2^{\cdot -} + NADP^+ + H^+ \quad (1)$$

The formed superoxide may disproportionate to dioxygen and hydrogen peroxide (reaction 2), either spontaneously or catalysed by superoxide dismutase (SOD):

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \longrightarrow O_2 + H_2O_2 \quad (2)$$

The formed hydrogen peroxide serves as a substrate for the myeloperoxidase reaction in the PMN, where chloride is converted to hypochlorite (reaction 3 [3–5]).

$$H_2O_2 + Cl^- \longrightarrow ClO^- + H_2O \quad (3)$$

Alternatively, the hydrogen peroxide may be further reduced to the hydroxyl radical [1], or consumed by the action of glutathione peroxidase or catalase.

The physiological role of the oxygen burst and the associated production of toxic oxygen derivatives and hypochlorite in phagocytic cells is to cause killing and destruction of foreign particles such as bacteria and viruses, and apparently of tumour cells as well [1,2]. However, the production of superoxide has also been suggested to contribute to the development and maintenance of some inflammatory states [6–9]. Moreover, injections of SOD have been found to suppress inflammation in three animal model systems [10], and in humans [11]. Also, hypochlorite has been implicated in inflammatory processes [12–14]. More generally, oxygen radicals have been proposed to be a major cause of cancer, heart disease and aging (review [15]).

The extracellular defense against 'oxygen radicals' appears to depend on the rather low activities of SOD [16], and possibly on ascorbate [17] and urate [15]. Here we have evaluated the effectivity of extracellular ascorbate in the protection against the production of oxygen radicals by activated PMN. The results are discussed in relation to the possible role of vitamin C in inflammation.

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2. MATERIALS AND METHODS

Human polymorphonuclear leucocytes (PMN) from peripheral blood were separated from other blood cells essentially as in [18]. Contaminating erythrocytes were haemolysed by a 20–30 s hypotonic treatment (pure water), and the cells were suspended in PBS medium (138 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4 and 5 mM glucose) at 20×10^6 cells/ml, and kept at room temperature. The trypan blue exclusion test indicated that >98% of the cells were intact.

Oxygen consumption was measured polarographically with a Clark-type electrode in a closed thermostatted glass vessel with magnetic stirring. The final volume was 1.6 ml.

Consumption of ascorbate was measured using two independent methods. In the first, oxidation of ascorbate was monitored spectrophotometrically at 265 nm using an Aminco DW-2 spectrophotometer. The thermostatted cuvette had a light path of 1 cm and the absorption scale was calibrated using known amounts of ascorbate. The second method was based on the reaction catalysed by ascorbate oxidase (EC 1.10.3.3), i.e., 2 ascorbate + O_2 ⟶ 2 dehydroascorbate + 2 H_2O. The measurement was carried out in the oxygraph. At various times, 12 μg/ml of ascorbate oxidase was added, which caused very rapid and stoicheiometric oxidation of the ascorbate that remained in the reaction mixture. This amount was determined on the basis of the extent of rapid O_2 consumption.

All measurements were carried out at 37°C and pH 7.4. The reagents used were all commercial products of the highest purity available.

3. RESULTS

Spectrophotometric measurements revealed that ascorbate is consumed by activated but not by resting human PMN (fig.1). PMN may be activated by several different agents in vitro. We have tested the tumour promoter phorbol-myristate-acetate (PMA; fig.1), the lectin concanavalin A, the calcium ionophore A-23187, and the bacterial chemotactic tripeptide formylmethionyl-leucyl-phenylalanine. In all cases, cell activation initiated the consumption of ascorbate. The

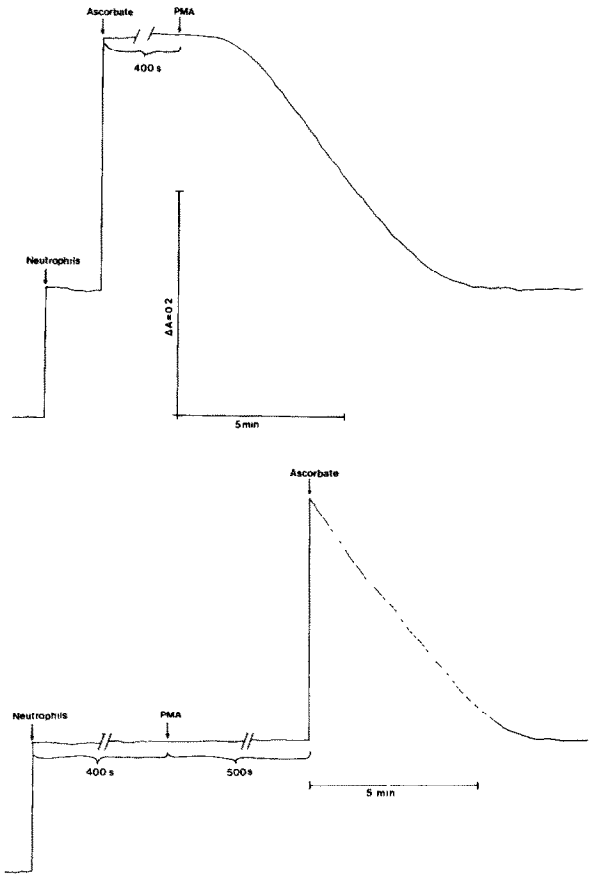

Fig.1. Oxidation of ascorbate by activated PMN. The ascorbate concentration was followed spectrophotometrically as described in section 2; 2×10^6 cells were suspended in 3 ml PBS with 0.5 mM $CaCl_2$. The initial concentration of ascorbate was 17 μM and that of PMA 10 ng/ml.

activation of O_2 consumption and $O_2^{\cdot-}$ formation by these agents is typically preceded by a brief lag (fig.2). This lag is also seen in the consumption of ascorbate (fig.1A), but is lacking when the ascorbate is added to preactivated cells (fig.1B). These results were confirmed (not shown) using the ascorbate assay with ascorbate oxidase (see section 2).

To characterise the cause of ascorbate consumption, the effects of added SOD, azide and catalase were tested. SOD catalyses reaction 2, azide inhibits myeloperoxidase ([19], reaction 3) and catalase accelerates breakdown of hydrogen peroxide.

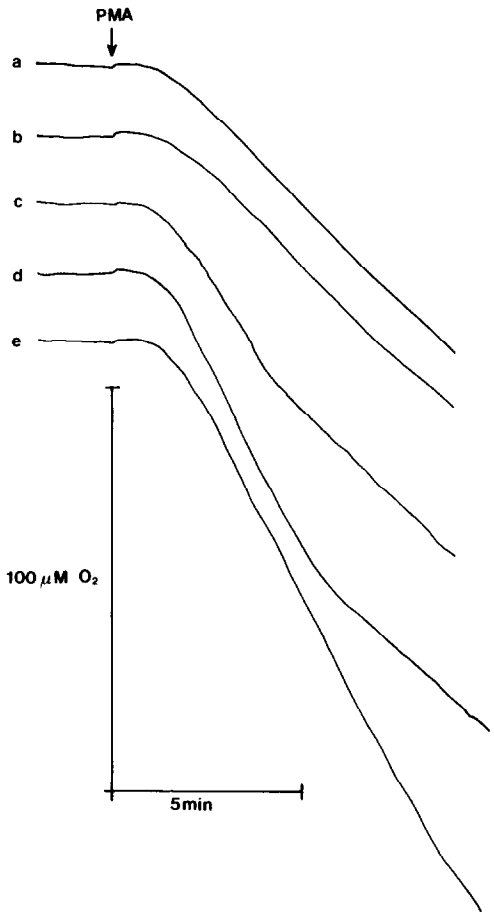

Fig.2. Stimulation of net oxygen consumption by ascorbate; 2×10^6 cells were suspended in 1.6 ml PBS + 0.5 mM $CaCl_2$. The cells were activated by addition of 16 ng/ml PMA. (a) Control; (b) 3 μg/ml of SOD; (c) 25 μM ascorbate; (d) 50 μM ascorbate; (e) 250 μM ascorbate.

SOD effectively inhibited the consumption of ascorbate, whereas catalase and azide were much less effective (table 1, expt.1). This suggests that generated superoxide is the main species responsible for ascorbate consumption, causing its oxidation to dehydroascorbate [17] according to reaction 4 (at pH 7.4):

$$\text{Ascorbate}^- + 2O_2^{\cdot -} + 3H^+ \longrightarrow \text{dehydroascorbate} + 2H_2O_2 \quad (4)$$

The small inhibitory effect of azide and catalase (table 1) suggests that also myeloperoxidase may be partially involved in the oxidation of ascorbate due to production of hypochlorite. On the other hand, hydroxyl radicals are apparently not involved in these conditions. If they were, an increase in the peroxide concentration by blockage of catalase and myeloperoxidase should enhance ascorbate oxidation, and in contrast, added catalase should be inhibitory. However, no difference in the effects of azide and catalase was noted (table 1, expt 1). To further assess the possible role of myeloperoxidase in ascorbate oxidation, we tested the effects of azide and catalase in the presence of SOD. When $O_2^{\cdot -}$ is efficiently disproportionated by SOD, it would be expected that a larger proportion of ascorbate breakdown would depend on myeloperoxidase. Indeed, in this situation the inhibition by catalase or azide reached about 80% (table 1, expts 2 and 3).

Disproportionation of superoxide (reaction 2) regenerates half of the O_2 that is reduced to superoxide in reaction 1. In such conditions the net oxygen consumption underestimates the true flux through NADPH oxidase. SOD did not significantly change the rate of net oxygen consumption by activated PMN (fig.2, cf. [20]). This may be expected since reaction 2 takes place spontaneously, though with a much lower rate constant than when catalyzed by SOD. SOD hence causes a drastic decrease in the steady-state concentration of superoxide, which explains the inhibition of ascorbate oxidation (table 1, expt 1). When SOD is absent, ascorbate effectively reduces superoxide to peroxide (reaction 4) thus preventing the spontaneous regeneration of dioxygen from superoxide by reaction 2. In agreement with this, ascorbate caused an approximately 2-fold stimulation of net O_2 consumption (fig.2). This is expected if reaction 4 replaces reaction 2. With cell concentrations of about 2×10^6/ml only about 15–20 μM of ascorbate was required for full stimulation of net oxygen uptake (not shown).

Fig.2 also shows that when limited amounts of ascorbate were present, the rate of oxygen consumption returned to control values after consumption of the added ascorbate. From the consumption of ascorbate and total O_2, the ascorbate/O_2 ratio was found to be 0.48 and 0.65 in the presence and absence of azide (fig.3). The former value is in harmony with reactions 1 plus 4, i.e., in conditions where superoxide is the only oxidant of

Table 1

The effect of superoxide dismutase, azide and catalase on the rate of oxidation of ascorbate

	Additions	Velocity (μM/min)	%
Expt 1	none	2.92	100
	azide, 0.1 mM	2.53	87
	catalase, 8 μg/ml	2.62	90
	SOD, 80 ng/ml	1.94	66
	SOD, 800 ng/ml	1.01	35
	SOD, 4 μg/ml	0.81	28
Expt 2			
(SOD, 1.6 μg/ml)	none	1.02	100
	azide, 0.1 mM	0.23	23
Expt 3			
(SOD, 1.6 μg/ml)	none	1.01	100
	catalase, 8 μg/ml	0.23	23

Ascorbate oxidation by 2×10^6 granulocytes was followed spectrophotometrically as in fig.1. The rate was determined from the linear part of the absorbance change. In experiment 1, the additions were made prior to the activator, which was 10 ng/ml PMA. In experiments 2 and 3 the SOD was added prior to the activator (10 ng/ml PMA), but azide and catalase were added when the ascorbate oxidation was linear. Both decreased the rate, which remained linear. The rate prior to addition of azide or catalase was used as control

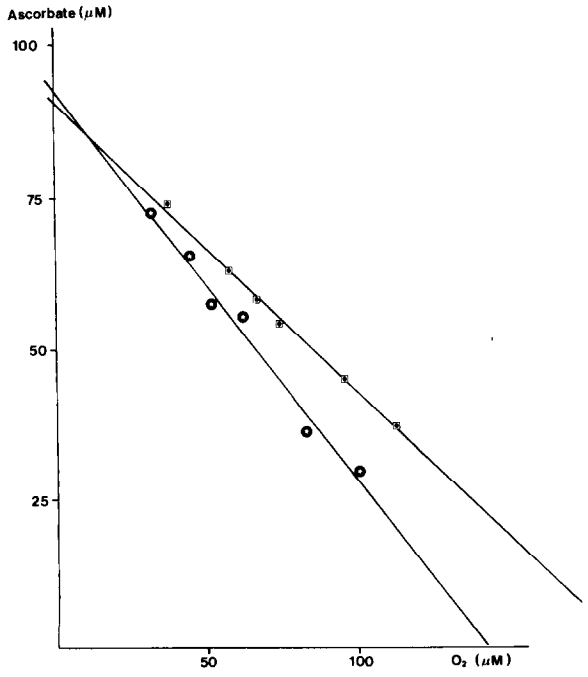

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Fig.3. Stoicheiometry between ascorbate oxidation and oxygen consumption in PMN. Basic conditions as in fig.2, but in the presence of 93 μM ascorbate. At various times after activation with PMA, the remaining ascorbate was determined using the ascorbate oxidase assay (section 2). The total oxygen consumed from addition of PMA to addition of ascorbate oxidase was plotted against consumed ascorbate. Circles, no additions; squares, +0.1 mM azide. The slopes were calculated by linear regression analysis ($r = -0.991$ and -0.998, respectively).

ascorbate. The 35% larger stoicheiometry in the absence of azide agrees with the notion that part of ascorbate may be oxidised in a myeloperoxidase-dependent fashion (e.g., by produced hypochlorite).

4. DISCUSSION

Our data show that extracellular ascorbate is effectively oxidized by activated, but not by resting PMN, and that this occurs mainly as a reaction between ascorbate and superoxide, and to a lesser, but significant extent as ascorbate oxidation through myeloperoxidase action. The oxidation of ascorbate by superoxide is prominent at physiological extracellular concentrations of the vitamin (~50 μM in humans). The fact that oxidation of extracellular ascorbate by the activated PMN is stoicheiometric with consumption of O_2 shows that there is, in our conditions, no mechanism of sufficient kinetic capacity in these cells for re-reduction of extracellular dehydroascorbate or monodehydroascorbate to extracellular ascorbate. In agreement with this, addition of the former (not shown) did not simulate the effects of ascorbate described here. In [21,22] it was shown that PMN take up dehydroascorbate, reduce it to ascorbate, and concentrate it in this form. The lack of any effect of dehydroascorbate in our experiments may be due to the absence of a kinetically significant efflux of ascorbate from the cells.

Authors in [23] reported a slight oxidation of intracellular ascorbate during phagocytosis of latex particles. In preliminary experiments we did not find any significant oxidation of intracellular ascorbate when the cells were stimulated by agents (such as PMA) which do not induce phagocytosis. This, together with the stoicheiometric consumption of extracellular ascorbate with respect to consumed oxygen, supports the notion that NADPH oxidase functions vectorially with production of superoxide on the outside of the cell membrane. This would imply that during phagocytosis superoxide is produced into the primary phagosome rather than into the cytoplasm. Yet, a considerable fraction of superoxide is probably released extracellularly as well during phagocytosis (see [24]).

The concentration of superoxide dismutase in human extracellular fluids is found to be low when care is taken to prevent leakage of the enzyme from cells. In serum a special high molecular mass isoenzyme accounts for most of the superoxide dismutase activity [16]. At normal extracellular ascorbate concentrations the vitamin would be expected to react more effectively with superoxide than extracellular SOD [16]. On the basis of our results it seems likely, therefore, that one of the functions of extracellular vitamin C is to minimize tissue damage caused by expulsion of superoxide and hypochlorite from activated PMN during an inflammatory response. Since ascorbate reduces $O_2^{\cdot-}$ to hydrogen peroxide, the latter important substrate for the myeloperoxidase reaction would still be produced. The combination of reactions 1 and 4 implies, in fact, that ascorbate can be a co-reductant with NADPH in the reduction of O_2 to peroxide by the activated cells. The proposed antioxidant function of urate [15] may complement rather than exclude that suggested here for ascorbate, since the former is ineffective against superoxide (unpublished), and all toxic oxygen metabolites are produced via superoxide in phagocytic cells [1].

Enhanced rates of oxidation of ascorbate may, according to our data, be expected in connection with inflammation due to activation of a large number of PMN. This could result in a decrease of the steady-state concentration of the reduced vitamin, at least locally. Enhanced oxidation of ascorbate would be expected to favour depletion of the vitamin because dehydroascorbate is much less stable than ascorbate, and is effectively degraded and excreted [25]. A site of inflammation could therefore also constitute a drain of vitamin C from the whole organism. Leucocyte and plasma levels of ascorbate are significantly decreased in many different disorders [25], for example, the common cold [26,27], rheumatoid arthritis [28,29], and myocardial failure [30]. The mechanism proposed here could contribute to such changes. A decrease in ascorbate concentration may, in turn, result in diminished protection against toxic oxygen metabolites derived from superoxide, but also other ascorbate-dependent reactions, such as the biosynthesis of collagen [31], may tend to proceed less efficiently.

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