CONTINUOUS MONITORING OF OXYGEN CONSUMPTION AND SUPEROXIDE PRODUCTION BY PARTICLE-STIMULATED HUMAN POLYMORPHONUCLEAR LEUKOCYTES

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1. Introduction

Since Baldrige and Gerard [1] who first described the extra oxygen consumption of phagocytosing leukocytes, a great deal of new information has accumulated regarding the respiratory burst. A reduced pyridine nucleotide oxidase seems to be responsible for the reduction of molecular O_2 [2] to superoxide anions (O_2^-) [3] which then dismutate to form hydrogen peroxide (H_2O_2) [4]. The interaction of these latter compounds results in the production of hydroxyl radicals ($OH^\cdot$) [5].

By measuring oxygen consumption and superoxide anion production continuously and simultaneously under identical conditions, it is possible to show that all the initial O_2 consumed can be accounted for as O_2^-. The continuous monitoring of both parameters allows accurate observations of the time course of activation.

2. Materials and methods

Cytochrome *c* (from horse heart type VI), superoxide dismutase (SOD) (EC 1.15.1.1) (from bovine blood, 3000 IU/mg protein) and zymosan were obtained from Sigma Chemical Co., Saint Louis, MO; xanthine, xanthine oxidase (XOD) (EC 1.2.3.2) (from cow's milk , 0.3 IU/mg protein) were purchased from Calbiochem-Behring Corp., and catalase (EC 1.11.1.6) (from beef liver, 65 000 IU/mg) from Boehringer, Mannheim.

The polymorphonuclear leukocytes (PMN) were separated according to the method described previously [6], which combines a centrifugation on Ficoll-Hypaque (Ficoll-Paque, Pharmacia) with a dextran sedimentation (Dextran T-500, Pharmacia). The final cell pellet containing more than 95% PMN was suspended in Dulbecco's buffer pH 7.4 without calcium and magnesium to a concentration of $1-2.2 \times 10^{11}$ PMN/l. Zymosan particles were prepared and preopsonised as in [7] by incubating in human autologous serum at 37°C for 30 min.

Oxygen consumption was measured polarographically with a Clark type electrode (M. Dansoreanu, Department of Biophysics, Medical and Pharmaceutical Institute, 3400 Cluj-Napoca, Rumania) fitted to the side of a stirred, thermostatically controlled (37°C), glass cuvette of 0.42 ml and 0.6 cm light path length. The whole device is fitted onto an eppendorf photometer (Eppendorf Gerätebau, Hamburg, FRG) [8] and allows simultaneous polarographic and photometric recordings.

The production of superoxide anions was measured in the same cuvette by following continuously the reduction of cytochrome *c* at 546 nm (filter) in the presence or absence of SOD, using an extinction coefficient of 8.1 $cm^2 \cdot \mu mol^{-1}$ [9] (reduced minus oxidised).

3. Results

Fig.1 shows the time course of simultaneous and continuous monitoring of oxygen uptake and cytochrome *c* reduction after the addition of opsonised zymosan. The reduction of cytochrome *c* shows the same lag period of 30 to 40 s as that of oxygen consumption. From the slope of the curves, the maximal rate of both parameters can be measured. Cyto-

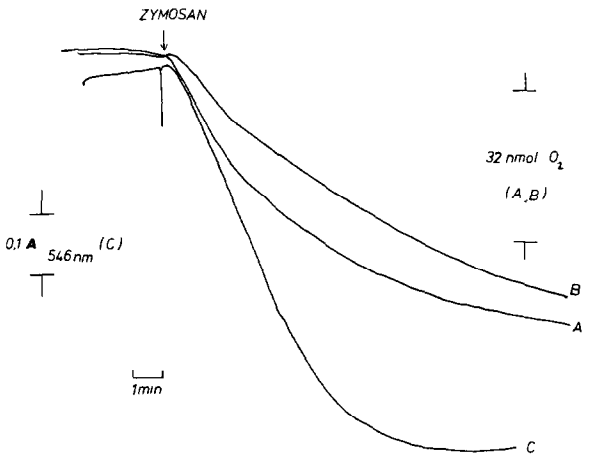

Fig.1. Continuous assay for O_2 consumption (A,B) and O_2^- production (C). PMN were suspended (2.4–3 × 10^6/ml) in Dulbecco's buffer pH 7.4 containing $CaCl_2$ (0.7 mmol . l^{-1}), $MgCl_2$ (0.85 mmol . l^{-1}) and glucose (12.8 mmol . l^{-1}). After 3 min, 2 mg zymosan particles were injected through a vent in the stopper. (A) no addition; (B,C) + cytochrome *c* (160 μmol . l^{-1}).

chrome *c* reduction stops after 5 to 7 min, where there is still a low rate of oxygen consumption. Addition of SOD does not affect the rate of oxygen uptake nor the total oxygen consumed at 7 min, the time point which corresponds to the end of reaction with cytochrome *c*. The rates of O_2 uptake in the absence or presence of SOD are 8.46 (SD 0.96) and 7.57 nmol . min^{-1} . 10^{-6} PMN (SD 1.00), respectively for 5 paired experiments. The oxygen consumption measured after 7 min is 35.39 nmol . 7 min^{-1} . 10^{-6} PMN (SD 4.42) for the controls and 34.21 (SD 4.67) for the assays containing SOD.

The addition of cytochrome *c* causes a slight inhibition of the rate and total oxygen consumption, which is fully restored by the addition of SOD. SOD, on the other hand, completely abolishes cytochrome *c* reduction (table 1). A similar effect is found with $MnCl_2$. A concentration of 0.7 mmol . l^{-1} completely inhibits cytochrome *c* reduction without significant effect on O_2 consumption (data not shown).

From the maximal rates of oxygen consumption in the presence of cytochrome *c* and the maximal rates of cytochrome *c* reduction obtained from 7 simultaneous recordings, it can be demonstrated that all the O_2 consumed is recovered as O_2^-. Indeed the mean value obtained for O_2 uptake is 7.21 nmol . min^{-1} . 10^{-6} PMN (SD 1.27) and 6.67 (SD 1.86) for cytochrome *c* reduction. But when considering the total amount of oxygen consumed, only 57% is accounted for as O_2^- as measured by cytochrome *c* reduction, the mean values being 31.97 nmol . 7 min^{-1} . 10^{-6} PMN (SD 6.46) for O_2 uptake and 18.30 (SD 5.77) for cytochrome *c* (n = 5).

As the stimulation reaction proceeds, the cytochrome *c* is very slowly reoxidized (fig.2, curve A). This reoxidation can be prevented by adding catalase to the system (fig.2, curve B). Addition of catalase plus ethanol is even more effective in protecting ferrocytochrome *c* reoxidation as shown by curve C in fig.2.

Table 1
Effect of cytochrome *c* and SOD on maximal rate and total oxygen consumption by zymosan stimulated PMN

– cytochrome *c*				+ cytochrome *c*							
O_2 consumption				O_2 consumption				O_2^- production			
Maximal rate (±SOD)		Total		Maximal rate (±SOD)		Total		Maximal rate (±SOD)		Total	
–	+	–	+	–	+	–	+	–	+	–	+
8.33	6.40	32.86	29.74	5.24	5.45	25.24	25.82	4.12	0	11.36	0
9.73	8.28	38.61	38.14	7.68	7.85	36.63	41.46	5.59	0	22.14	0
9.78	8.74	41.53	40.18	7.52	8.65	40.57	44.12	7.50	0	24.62	0
7.37	6.69	32.41	30.59	5.92	6.48	30.21	33.74	4.62	0	20.25	0

Experimental conditions as in fig.1. SOD when added 30 μg in the reaction medium. Results are expressed in nmol O_2 . min^{-1} . 10^{-6} PMN or nmol O_2^- . min^{-1} . 10^{-6} PMN for maximal rate and nmol O_2 or O_2^- . 7 min^{-1} . 10^{-6} PMN for the total. 4 paired experiments are shown

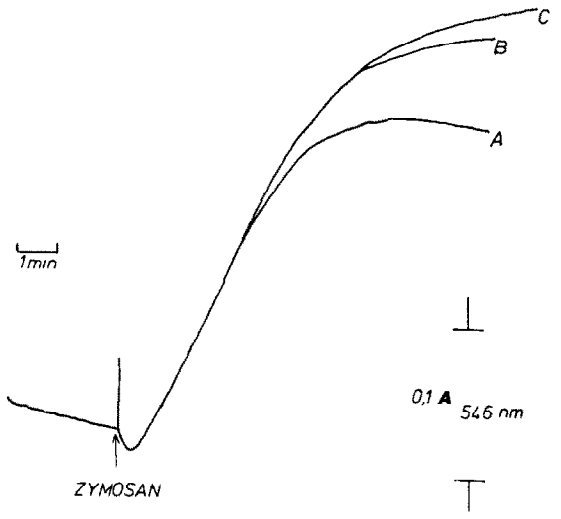

Fig.2. Continuous monitoring of cytochrome *c* reduction. For experimental conditions, see legend to fig.1. (A) no addition; (B) + catalase (290 U . ml^{-1}); (C) + catalase (290 U . ml^{-1}) and ethanol (40 mmol . l^{-1}).

When NaN_3 is added to the medium in order to inhibit the haemcontaining enzymes of the system, only a small portion of the total cytochrome *c* is reduced (fig.3a), while the maximal rate is only slightly affected. The effect on O_2 consumption measured simultaneously is shown in fig.3b. The initial rate is not affected by NaN_3, but the total O_2 consumed is increased, which is an indication that no O_2 can be regenerated in the system. The same results can be demonstrated when the PMN are replaced by the system xanthine-xanthine oxidase which generates O_2^- and H_2O_2. Fig.4b shows the polarographic traces and the effect of cytochrome *c* and SOD on oxygen consumption. The addition of cytochrome *c* alone inhibits the rate and total amount of oxygen consumed. By adding SOD, the inhibition is prevented and the rate and total O_2 uptake are restored to the initial level. The reduction of cytochrome *c* measured simultaneously in the same cuvette is shown by trace A in fig.4a. It can be seen that cytochrome *c* is completely reoxidized when the oxygen is half consumed. In the presence of SOD no reduction occurs (fig.4a, curve C). Catalase and ethanol when present in the medium prevent the reoxidation of ferrocytochrome *c*, thereby increasing the total amount of cytochrome *c* reduced (fig.4a, curve B). Catalase stops reoxidation when injected while the reaction takes place. These effects of cytochrome *c* and SOD

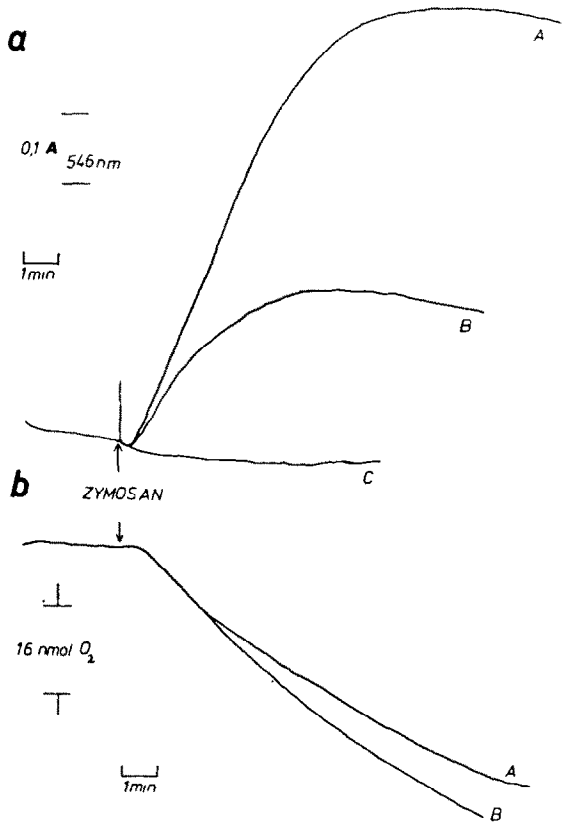

Fig.3. (a) The effect of NaN_3 and SOD on the production of O_2^-. The conditions are those as described in the legend to fig.1. (A) no addition; (B) + NaN_3 (2 mmol . l^{-1}); (C) + SOD (68 μg . ml^{-1}). (b) The effect of NaN_3 on O_2 consumption run simultaneously with O_2^- production shown in (a). (A) no addition; (B) + NaN_3 (2 mmol . l^{-1}).

on O_2 consumption and O_2^- production are shown in table 2. Under all conditions used, cytochrome *c* has an inhibitory effect on oxygen consumption, which is prevented by the addition of SOD.

NaN_3 has no effect in the xanthine-XOD system whereas $MnCl_2$ has the same superoxide dismutative effect as SOD without affecting the oxygen consumption.

4. Discussion

A number of reports have appeared concerning the production of O_2^- by activated neutrophils [3,10–16]. All the assays were discontinuous except for [14], where a detergent was used instead of particles as the activating agent. Therefore no accurate observation

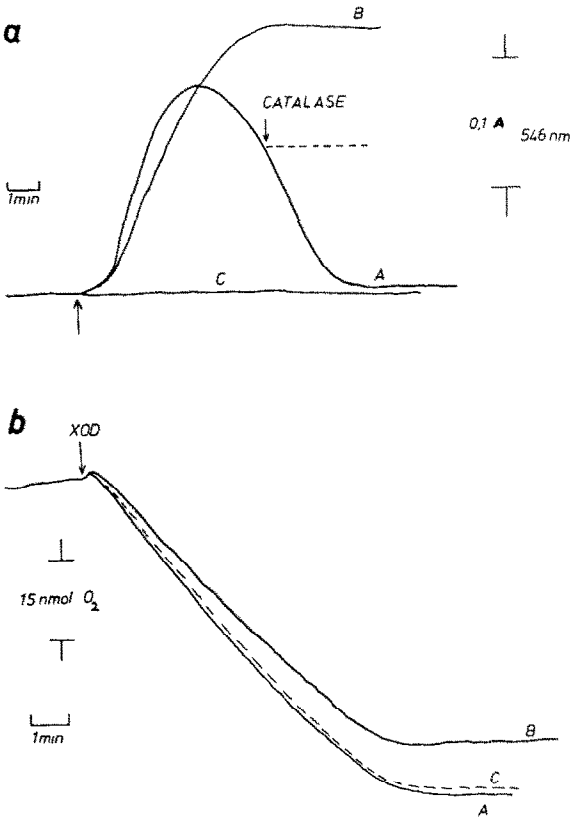

Fig.4. (a) Cytochrome *c* reduction in the xanthine-XOD system. The experimental conditions as in fig.1, except that the PMN were replaced by xanthine (167 μmol . l^{-1}). Cytochrome *c* added at a concentration of 34 μmol . l^{-1}. The reaction is started by injecting 17 mU XOD. The enzymatic activity of XOD was previously assayed spectrophotometrically at 292 nm in the same conditions as the experiment. (A) no addition; (B) + catalase (57 μg . ml^{-1}) and ethanol (40 mmol . l^{-1}); (C) + SOD (68 μg . ml^{-1}). (b) The effect of cytochrome *c* and SOD on O_2 consumption. Conditions as in (a). (A) no addition; (B) + cytochrome *c*; (C) + cytochrome *c* and SOD.

on the activation of the system could be made from these experiments.

The continuous and simultaneous monitoring of both parameters, O_2 consumption and O_2^- production, by zymosan activated PMN allows a better characterisation of the time course of the respiratory burst. A lag period of 30–40 s can be demonstrated for both kinetic curves (fig.1) which is in agreement with the lag of 44 s found for guinea pig PMN's continuous assay [14]. It is therefore not possible to extrapolate discontinuous curves to zero time. Rosen and Klebanoff [11], using zymosan particles which were not preopsonised, observed a lag of 5 min for superoxide production. This is the time required for zymosan

Table 2
Effect of cytochrome *c* and SOD on maximal rate and total oxygen consumption by xanthine-XOD system

– cytochrome *c*				+ cytochrome *c*							
O_2 consumption				O_2 consumption				O_2^- production			
maximal rate ±SOD		Total		maximal rate ±SOD		Total		maximal rate ±SOD		Total	
–	+	–	+	–	+	–	+	–	+	–	+
(1) 18.93	16.91	44.46	42.80	13.11	17.14	33.28	40.73	4.15	0	4.75	0
(2)				15.72	18.55	36.08	43.82	8.38	0	7.99	0
(3)				16.00	17.97	39.46	42.60	9.29	0	7.20	0
(4) 16.88	17.32	61.38	58.28	15.88	18.44	53.12	59.69	7.10	0	6.91	0
(5) 46.62	50.93	71.93	67.95	35.78	52.90	57.72	64.98	7.24	0	4.97	0

Experimental conditions: (1) xanthine 111 μmol . l^{-1}, cytochrome *c* 57 μmol . l^{-1}. (2) xanthine as in 1, cytochrome *c* 34 μmol . l^{-1}. (3) xanthine as in 1, cytochrome *c* 23 μmol . l^{-1}. (4) xanthine 167 μmol . l^{-1}, cytochrome *c* as in 2. (5) xanthine 250 μmol . l^{-1}, cytochrome *c* 100 μmol . l^{-1}. (1,2,3,4) XOD 40 mU . ml^{-1}. (5) XOD 100 mU . ml^{-1}. SOD 68 μg . ml^{-1} when added. Results are expressed as nmol O_2 or O_2^- . min^{-1} . 0.42 ml^{-1} for the rate and nmol O_2 or O_2^-. 0.42 ml^{-1} for the total

opsonisation when the serum is added together with the particles to the reaction mixture. Indeed it has recently been demonstrated that the lag time for O_2 consumption is dependent on the serum concentration used [7]. At optimal serum concentration a mean lag period of 4.5 min was found. The same can be shown for O_2^- production and conditions which alter the oxygen consumption affect the superoxide production in the same manner. These data suggest that the processes, O_2 consumption and generation of the one-electron reduction product O_2^- are accomplished by the same enzyme.

Addition of the superoxide scavenger ferricytochrome *c* inhibits, but only slightly, the O_2 consumption in the PMN system, whereas it is more striking in the xanthine-XOD assay. This inhibitory effect on the rate and total O_2 consumption is completely reversed with SOD (table 1 and 2). Segal and Meshulam [16] did not find a change in oxygen consumption upon addition of cytochrome *c* in their assay with latex stimulated neutrophils, but observed it in the xanthine-XOD system. A recent report by Babior [17] demonstrates that when the cell concentration is high, the efficiency with which the O_2^- is trapped by the cytochrome *c* is not optimum. The doubling of cytochrome *c* concentration in our experimental conditions did neither change the cytochrome *c* reduction, nor further diminish the oxygen uptake.

Roos et al. [18] have shown that the degradation of H_2O_2 was induced by cytochrome *c* in a sodium azide insensitive reaction. Hence the inhibiting effect of NaN_3 on cytochrome *c* reduction could be explained by a greater release of H_2O_2 into the medium. As expected no effect of NaN_3 could be demonstrated in the xanthine-XOD assay.

Hydroxyl radicals have been shown to be produced by activated PMN [5] and by the xanthine-XOD system [19]. As a powerful oxidant, the hydroxyl radical causes oxidation of ferrocytochrome *c* [19]. Since the amount of $OH^\cdot$ generated depends on the concentration of H_2O_2, the plateau of reduced cytochrome *c* will be lowered when the concentration of H_2O_2 is increased [19]. This happens to be the case with NaN_3 in the PMN medium (fig.3a curve B). Conversely the presence of catalase raises the plateau of reduced cytochrome *c*. The oxidation of ferrocytochrome *c* can be stopped by injecting catalase into the reaction mixture. The same results have been obtained by Goldstein and Weissmann [20] with the xanthine-XOD system.

The inhibition of cytochrome *c* reduction by Mn^{2+} in both systems can be compared to that described for the complex $Cu(Tyr)_2$ [16]. These metals are both contained in PMN SOD, the Cu in cyanide-sensitive SOD and the Mn in cyanide-insensitive SOD [21].

The formation of Mn^{3+} in the xanthine-XOD system and in chloroplasts producing O_2^- on illumination, and the concomitant inhibition of cytochrome *c* reduction by Mn^{2+} have been measured. The use of a continuous and simultaneous assay for O_2 consumption and O_2^- production demonstrated an identical lag time and kinetics of the burst of oxygen metabolism upon stimulation with particles. These results provided further evidence for a similar triggering mechanism initiating the burst, in any case for a given stimulus.

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