The enzymic reduction and kinetics of oxidation of cytochrome b_{-245} of neutrophils

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1. The absorption coefficient of human neutrophil plasma-membrane reduced-minus-oxidized cytochrome b_{-245} was determined [$\Delta\epsilon$ (nm; 559–540 nm) = 21.6 cm⁻¹]. 2. Neutrophil polymorphonuclear leucocytes (neutrophils) were prepared from human, ox, horse and pig blood. In each case plasma-membrane fractions were found to contain low-potential cytochrome b . When membranes from horse neutrophils were incubated anaerobically with either NADH or NADPH the cytochrome b became reduced. Prior stimulation of the cells with phorbol myristate acetate did not increase the rate or extent of cytochrome b reduction in isolated membranes, but did increase both the rate and extent of reduction by NADPH in Triton-treated cells. 3. A cytochrome b was present also in the specific granule fraction of human neutrophils. Its E_m (pH 7.0) was found to be -248 mV, very similar to that of the plasma-membrane cytochrome b . 4. The rate of oxidation of reduced cytochrome b_{-245} by air-saturated buffer, was determined by using stopped-flow techniques. In intact membranes $t_{\frac{1}{2}}$ for oxidation was 4.7 ms. This rate is sufficiently rapid to support the view that cytochrome b_{-245} is the oxidase in the respiratory burst of neutrophils. 5. Plasma-membrane cytochrome b of human neutrophils formed a complex with CO. At room temperature and 1 atm of CO approx. 40% of the cytochrome formed a complex; approx. 60% binding was measured at the increased concentration of dissolved CO achieved at 5°C. The concentration of CO giving 50% binding was 1.18 mM.

Neutrophil polymorphonuclear leucocytes (neutrophils) form part of the mammalian host defence system against bacterial infection. They greatly increase their O₂ consumption as they phagocytose and kill microbes. This increased respiration of phagocytosis (Baldridge & Gerard, 1933) is not mitochondrial, as it is not prevented by inhibitors of mitochondrial respiration, such as cyanide and azide (Sbarra & Karnovsky, 1959), and the products of O₂ uptake and reduction are superoxide, peroxide and hydroxyl radicals (for review, see Badwey & Karnovsky, 1980). It has been proposed that these reduced products of O₂ are directly or indirectly responsible for bacterial killing.

In a series of papers we have shown that human neutrophils contain a cytochrome b that is located both in the plasma membrane and in the specific granule fraction of the cells (Segal & Jones, 1979a; 1980a) and that is incorporated into the phagocytic vacuoles (Segal & Jones, 1978). The involvement

of this cytochrome in the oxidase system is suggested by its observed reduction when the cells are stimulated under anaerobic conditions with phorbol myristate acetate (Segal & Jones, 1979b), a potent activator of the oxidase system, and by abnormalities observed in cells from patients with chronic granulomatous disease. This is a syndrome in which a severe, often fatal, predisposition to bacterial and fungal infection is associated with complete absence from the neutrophils of any evidence of oxidase activity after stimulation. Absorption spectra show that the cytochrome b is absent from the cells in the commonest sub-group of these patients in whom it is inherited in an X-chromosome-linked manner (Segal & Jones, 1980b). Although this cytochrome is present in cells from the rarer patients with an autosomal recessive mode of inheritance, it is not reduced after stimulation with phorbol myristate acetate (Segal & Jones, 1980b). Thus it appears that there are at least two possible genetic defects in the special

oxidase of neutrophils: a specific cytochrome *b* component may be absent or a reductase or activator system for the cytochrome *b* may be defective.

We have found that this cytochrome *b* has the unusually low oxidation–reduction potential at pH 7.0 ($E_{m7.0}$) of -245 mV (Cross *et al.*, 1981), sufficiently low to reduce O_2 to $\text{O}_2^{\cdot-}$ at a rapid rate. The reduced cytochrome b_{-245} formed a complex with CO which was photo-dissociable and it was oxidized by air-saturated buffer with a half-time faster than 1 s at 20°C , a resolution limited by mixing time. These results are compatible with cytochrome b_{-245} acting as an oxidase, leading directly to the formation of $\text{O}_2^{\cdot-}$. We have also found that this cytochrome is present in other phagocytic leucocytes, namely monocytes, macrophages and eosinophils (Segal *et al.*, 1981), and probably plays an important part in the specialized activities of these cells, which also demonstrate phagocytosis-induced respiration.

In the work that we have described previously we have commented on our inability to reduce cytochrome b_{-245} *in vitro* using NADPH or NADH as reductant, under anaerobic conditions, although both have been suggested as electron donors in the respiratory burst (see Badwey & Karnovsky, 1980). Similarly, in a recent report that confirmed the presence of cytochrome b_{-245} in human polymorphonuclear leucocytes (Light *et al.*, 1981) it was noted that the cytochrome was not reduced by NADPH *in vitro* and this failure was used to argue against its participation in the $\text{O}_2^{\cdot-}$ -generation system. This was not supported by the report in an early Japanese paper, of the presence of a cytochrome *b* in animal neutrophils. It was proposed, but not established, as a component of this oxidase system, since NADH-dependent reduction of the cytochrome was reported (Shinagawa *et al.*, 1966). In the present paper we show that under suitable conditions cytochrome b_{-245} is reduced by both NADPH and NADH *in vitro* and that its rate of oxidation by air-saturated buffer is sufficient to account for the rate of O_2 consumption in the respiratory burst. We believe that these results implicate cytochrome b_{-245} as a component in the microbicidal oxidase system. We also report that cytochrome b_{-245} is present in neutrophils from horse, pig and ox blood and is likely to be of widespread distribution in mammals.

Materials and methods

Isolation of neutrophils

Human neutrophils were isolated from buffy-coat residues by dextran sedimentation of erythrocytes, centrifugation through Ficoll/sodium metrizoate and haemolysis of residual erythrocytes as described previously (Segal & Jones, 1979a). Neutrophils were

prepared from fresh horse and pig blood, taken into EDTA [0.15% (w/v) in 0.9% (w/v) NaCl] to prevent coagulation, and using the fractionation procedure described above for human blood. Neutrophils were prepared from fresh ox blood by using the procedures of Carlson & Kaneko (1973).

Activation of neutrophils and preparation of sub-cellular particles

Suspensions of neutrophils (approx. 10^9 cells in Krebs Ringer solution, previously gassed with CO_2 and O_2) were stirred and $25\text{ }\mu\text{g}$ of phorbol myristate acetate was added in $50\text{ }\mu\text{l}$ of dimethyl sulphoxide. After 5 min the suspension was sonicated ($3 \times 20\text{ s}$; MSE Ultrasonic Disintegrator; 150 W). The total homogenate was used in some experiments, or the homogenate was centrifuged for 10 min at 500 g at 4°C . This supernatant was taken and a membrane pellet was prepared by centrifugation at 27000 g for 20 min.

Spectroscopy

Reduced-minus-oxidized difference spectra were recorded by using a split-beam spectrophotometer as described previously (Jones & Saunders, 1972). Anaerobic spectra were recorded in Thunberg cuvettes where the atmosphere above the suspension was repeatedly evacuated and flushed with O_2 -free N_2 . In some experiments glucose (1 mM), glucose oxidase (1 mg/ml) and catalase ($50\text{ }\mu\text{g}$) were added to the suspension to scavenge residual dissolved O_2 ; NADH (1 mM final concentration) or NADPH recycling mixture (to give 0.2 mM-NADPH final concentration) was tipped from the side arm to initiate reduction.

Potentiometric titration

The method, based on measurement of absorption spectra at known oxidation–reduction potentials, was as described by Cross *et al.* (1981).

Measurements of kinetics of oxidation of cytochrome b_{-245}

A suspension of neutrophil plasma membranes was 80% reduced with sodium dithionite in an anaerobic cuvette with a side arm fitted with a rubber septum. Samples were withdrawn in a glass syringe, which was attached to one flow line of a stopped-flow spectrophotometer fitted with two photomultipliers, designed by Dr. D. W. Yates of the Department of Biochemistry, University of Bristol. The second syringe of the stopped-flow spectrophotometer was filled with air-saturated buffer. After rapid mixing, measurements were made in the flow cell of the change in absorbance of 559 minus 540 nm, due to the disappearance of the α -band of the reduced *b* cytochrome (cf. Cross *et al.*, 1981). Changes in absorbance were fed to a linear differ-

ential amplifier and the output stored in a transient recorder (Datalab 905)

Determination of $O_2^{\cdot -}$ production

Samples of neutrophil homogenate or crude membrane suspension were added to a cuvette containing 1 mg of oxidized cytochrome *c* (horse heart; Sigma, Poole, Dorset, U.K.) dissolved in 2 ml of 50 mM-Mops (4-morpholinepropanesulphonic acid), pH 7.0, NADPH or NADH was added. The rate of cytochrome *c* reduction was measured at 30°C in a dual-wavelength spectrophotometer at 550 minus 540 nm before and after the addition of 40 µg of superoxide dismutase (Sigma).

Preparation of extracts enriched in membranes and specific granules

Neutrophil cytochrome *b* was found, by analytical subcellular fractionation, to have dual location, with the plasma membrane and specific granule fractions. These two fractions were isolated on discontinuous sucrose gradients as described previously (Segal & Jones, 1979a).

Results and discussion

Determination of reduced minus oxidized absorption coefficient for cytochrome b_{-245}

To calculate accurately the concentrations of cytochrome used in various experiments, an accurate determination of the absorption coefficient was necessary. A plasma-membrane fraction, almost completely free from contaminating myeloperoxidase, was prepared by sucrose-density-gradient centrifugation of a homogenate of human neutrophils (Segal & Jones, 1979a). The dithionite-reduced-minus-air-oxidized difference spectrum of a sample of this membrane was recorded in aqueous buffer and the height of the maximum at 559 nm relative to the trough at 540 nm was determined. A second sample of the purified membrane was suspended in alkaline pyridine (see Smith, 1975) to dissociate protohaem from its protein and the reduced-minus-oxidized spectrum of the pyridine haemochrome was recorded. By using the $\Delta\epsilon$ (mM; 557–541 nm) (Porra & Jones, 1963) for the protohaem pyridine haemochrome, the difference absorption coefficient of the original cytochrome b_{-245} at 559–540 nm was calculated to be $21.6 \text{ mM}^{-1} \cdot \text{cm}^{-1}$.

Reduction of cytochrome b_{-245}

In previous experiments we found that cytochrome b_{-245} of human neutrophils was not reduced when isolated plasma membranes or total homogenates were treated with NAD(P)H, even under anaerobic conditions. Both NADH and NADPH have frequently been suggested as likely donors of electrons for superoxide generation (see review by

Badwey & Karnovsky, 1980). It was possible that this resistance to reduction was associated with the age of the cells used in our experiments. The human neutrophils were prepared from blood-bank material and techniques of membrane fractionation took several hours. To obtain fresh neutrophils we separated the cells from animal blood obtained at the local abattoir. In preliminary experiments we showed that plasma membranes from a variety of animal species contain low-potential cytochrome *b*. The results of a potentiometric titration of horse neutrophil plasma membranes given in Fig. 1 show

Fig. 1. Potentiometric titration of horse neutrophil plasma-membrane cytochrome *b*

Sucrose-density-gradient purified plasma membranes (1.08 mg of protein/ml) were suspended in 40 mM-Mops/100 mM-KCl, pH 7.0, in an anaerobic stirred cuvette fitted with platinum and calomel electrodes. The following mediators were present: phenazine methosulphate (12.5 µM); phenazine ethosulphate (12.5 µM) (both from Sigma); pyocyanine (6 µM) (K & K Laboratories; supplied by Kodak Fine Chemicals, Kirkby, Liverpool, U.K.); 2-hydroxy-1,4 naphthoquinone (12.5 µM); anthroquinone 2,6-bis-sulphonate (12.5 µM); anthroquinone (10 µl of a saturated solution in ethanol) (all from Aldrich Chemical Co., Gillingham, Dorset, U.K.); 3,6-diaminodurene (12.5 µM) (Fluka A.G. Chemische Fabrik; supplied by Fluorochem, Glossop, Derbys., U.K.); duroquinone (12.5 µM) (Sigma). The extent of reduction of the cytochrome *b* at different oxidation-reduction potentials (E_h) was calculated by measuring the height of the absorption band at 559 nm above a line joining the two troughs on each side of this band (see Cross *et al.*, 1981). ●, Reductive titration with sodium dithionite; ○, oxidative titration with potassium ferricyanide. The line through the points is the theoretical curve for a one-electron-transfer reaction.

Fig. 2. Reduction of cytochrome *b* after stimulation of intact pig neutrophils with phorbol myristate acetate (a) Neutrophils (6.5 mg of protein/ml) were suspended in an O_2 -free medium containing KCl (4.75 mM), $MgCl_2$ (1.19 mM), KH_2PO_4 (25 mM), Ficoll 400 (57 g/litre) and sodium diatrizoate (78.2 g/litre), pH 7.4. Glucose (10 mg/ml) and glucose oxidase were added and the suspension was divided between two stoppered cuvettes. Phorbol myristate acetate ($1 \mu\text{g/ml}$ final concentration) was added to the sample cuvette from a stock solution (10 mg/ml in dimethyl sulphoxide) and difference spectrum A was recorded after 20 min. Difference spectrum B was recorded after the addition of a few grains of sodium dithionite to the sample cuvette. (b) Incubation conditions were as described for (a). Spectra were recorded at regular intervals after addition of phorbol myristate acetate and the extent

that the $E_{m7.0}$ of the cytochrome *b* was -255 mV and that it behaved as a one-electron donor. Similar results were obtained for ox and pig neutrophil membranes and the results are shown in Table 1, together with a determination of the content of the *b* cytochrome. In the interest of clarity we apply the name cytochrome *b*₋₂₄₅ to these cytochromes from different species, although there may be some small differences in the oxidation-reduction midpoint potential. Fresh neutrophil preparations from horse and pig blood were used in measurements of cytochrome *b* reduction by NAD(P)H.

We have previously reported (Segal & Jones, 1979b) that cytochrome *b* becomes reduced when intact neutrophils were stimulated with phorbol myristate acetate. We have found that this reduction is relatively slow and incomplete in cells from human, horse and pig blood (Figs. 2a and 2b) with a stable level of about 50% reduction achieved some 10 min after stimulation. Care was taken to exclude O_2 from the cuvettes by gassing with argon and by adding glucose oxidase and glucose as O_2 scavengers. It is therefore unlikely that this relatively slow and incomplete reduction is caused by reaction with O_2 . It is noteworthy that reduction *in vivo* is accomplished without reduction of myeloperoxidase, seen as a band at 475 nm on dithionite reduction.

It was possible that partial reduction of cytochrome *b* occurs because it is present in two cellular locations; the plasma membrane and the specific granule fraction (Segal & Jones, 1979a). We investigated the possibility that granule cytochrome *b* might differ in its oxidation-reduction properties from the plasma-membrane cytochrome and thus be non-reducible in the stimulated cell. A potentiometric titration (Fig. 3) showed, however, that its midpoint potential ($E_{m7.0} = -248 \text{ mV}$) was almost identical with that of the plasma-membrane cytochrome. The reason for incomplete reduction of cytochrome in stimulated whole cells is therefore still uncertain.

Freshly prepared neutrophils from horse or pig blood were sonicated (to 95% disruption) and the entire suspension was incubated anaerobically in cuvettes. Baseline spectra were run at regular intervals and then NADPH was added to the sample cuvette. Cytochrome *b* became partly reduced, the extent of reduction remaining stable after 10 min, at about 25% reduction (Fig. 4). The time

of reduction of cytochrome *b* calculated from the height of the absorption band at 559 nm above a line joining the troughs at 570 and 540 nm. The total cytochrome *b* was calculated from the dithionite-reduced difference spectrum.

Table 1. Concentration and oxidation–reduction mid-point potential of cytochrome *b* of neutrophil plasma membranes from different mammalian species

Neutrophils were prepared from whole blood as described in the Materials and methods section. The plasma-membrane fraction were isolated after homogenization and density-gradient centrifugation. Conditions for the potentiometric titrations were as described in the legend to Fig. 1. In each case the titrations fitted the curve for a one-electron transfer.

Source of neutrophils	$E_{m7.0}$ of cytochrome <i>b</i> (mV)	Concn. of cytochrome b_{-245} in plasma membrane (pmol/mg of protein)
Man	–245	417
Ox	–259	498
Pig	–253	600
Horse	–255	260

Fig. 3. Potentiometric titration of the cytochrome *b* of the specific granule fraction of human neutrophils
Conditions were exactly as described in the legend to Fig. 1, except that human specific granules (0.71 mg of protein/ml) were used in place of horse neutrophil plasma membranes.

course of cytochrome *b* reduction by NADH was similar but the extent of reduction at 10min was greater, near 50%. Repeated experiments failed to demonstrate a substantial increase in the rate or

Fig. 4. The reduction of crude membranes from horse neutrophils by NADPH

A suspension of crude membranes (15 mg of protein/ml) in a buffer containing NaCl (97mM), KCl (5 mM), MgCl₂ (1 mM), Hepes (50mM), KH₂PO₄ (1 mM), pH 7.0, was placed in the main well of a Thunberg cuvette. NADPH (to give a final concentration of 0.18 mM) was placed in the side arm and the cuvette was repeatedly evacuated and gassed with O₂-free argon. Difference spectra were recorded: (a) before tipping; (b) after tipping NADPH; (c) after opening cuvette and stirring in air; and (d) after adding a few grains of sodium dithionite.

extent of cytochrome *b* reduction in suspensions of phorbol-stimulated cells. It appeared possible that exposure to sonication was itself stimulating the neutrophils and measurement of O₂^{•-} production confirmed this (results not shown). A more gentle method of disruption was therefore attempted. Cell suspensions were treated with the detergent Triton X-100 to make membranes permeable to added nucleotides and the rate of NADPH-dependent reduction of cytochrome *b* was measured (Fig. 5). An increase in the rate and extent of reduction was observed after phorbol treatment.

Our experience, over many experiments, is that neutrophils may be stimulated during a variety of manipulations, such as sonication, detergent treatment, homogenization or stirring in glass vessels. We have also found that the stimulated state has a short lifetime (measured by superoxide production) in disrupted cells, in agreement with the observations of Light *et al.* (1981), who found a half-time of inactivation of 1.9 min in particles and 0.75 min in detergent-treated particles. This sensitivity of the activation system of the respiratory burst may contribute to the relatively slow and incomplete reduc-

Fig. 5. Kinetics of reduction of cytochrome b_{-245} in detergent-disrupted pig neutrophils by added NADPH under anaerobic conditions

Pig neutrophils (6.62 mg of protein/ml) were suspended in a medium containing Hepes (50 mM, pH 7.4), KCl (4.75 mM), $MgCl_2$ (1.19 mM), NaH_2PO_4 (1.19 mM) and NaCl (97 mM). Cells were treated either with 5 μ l of phorbol myristate acetate solution in dimethyl sulphoxide (1 mg/ml) or with 5 μ l of dimethyl sulphoxide. They were then treated with Triton X-100 (0.05%, v/v, final concentration). Suspensions were divided between two cuvettes of a split-beam spectrophotometer and glucose (10 mg/ml) and glucose oxidase (100 units) and NADPH (1 mM, maintained using a glucose 6-phosphate dehydrogenase-based regenerating system) was added to the sample. Difference spectra were recorded at 20°C over the indicated time and the extent of reduction was assayed by measuring the extent of absorption at 559 nm relative to the trough at 540 nm. Total reduction was assayed by adding sodium dithionite to the sample. ▲, Phorbol myristate acetate-treated cells; ■, control cells.

tion of cytochrome b by NADPH that we have found in neutrophil extracts. It is also possible that reduced cytochrome b , or a molecule in redox equilibrium with it, is an inhibitor of its reduction. Since the $E^{\circ'}$ of the couple $O_2^- \cdot / O_2^{2-}$ is +900 mV (James, 1978) and the $E_{m7.0}$ of the b cytochrome is -245 mV, the b cytochrome must be highly oxidized during the respiratory burst in whole cells if it is the donor of electrons to O_2 . It is therefore highly unlikely that reduced cytochrome b is present at a significant concentration when O_2 is present, as it must be during the respiratory burst.

Oxidation of reduced cytochrome b_{-245}

In a previous paper (Cross *et al.*, 1981) we have shown that cytochrome b_{-245} has many of the properties of an oxidase. It binds CO and is oxidized with a half-time of less than 1 s. Our measurements

Fig. 6. The kinetics of oxidation by air-saturated buffer of membrane-bound cytochrome b_{-245} from human neutrophil plasma membranes

Membranes (2.5 mg of protein/ml before mixing) were suspended in 50 mM-Mops, pH 7.0, in an anaerobic stirred cuvette under an atmosphere of argon. The cytochrome was approx. 90% reduced by the addition of sodium dithionite and was transferred anaerobically to one flow line of a stopped-flow spectrophotometer (see the Materials and methods section). The oxidation of cytochrome b was measured at 558 nm minus 539 nm, after mixing with equal volumes of air-saturated buffer. The broken line shows a computer-calculated fit to the trace, giving a $t_{1/2}$ for oxidation of 4.7 ms.

of the kinetics of cytochrome oxidation were limited by the rate of stirring in O_2 in the cuvette we used. We have therefore measured the rate of oxidation of reduced cytochrome b_{-245} in intact membranes, by using a stopped-flow spectrophotometer. Oxidation was 50% completed in 4.7 ms after mixing with aerated buffer (Fig. 6).

The kinetics of cytochrome b_{-245} oxidation are sufficiently rapid for it to act as an oxidase in the respiratory burst. Stimulated neutrophils respire at a variable rate, around 40 nmol of O_2 /min per 10^7 cells (see Segal & Coade, 1978) and the cytochrome b reduced on stimulating anaerobic cells is approx. 0.1 nmol/ 10^7 cells, giving a rate of 400 nmol of O_2 /min per nmol of cytochrome b .

Cytochrome b_{-245} does not react completely with CO at room temperature to form a CO-complex. The extents of the trough at 559 nm and the peak at 419 nm in the CO-difference spectrum are only about 40% of the extents of the corresponding absorption bands of the reduced cytochrome. Many oxidases form CO-complexes, although it is not a necessary property of oxidases, and this apparently incomplete CO binding encouraged us to examine the reaction further at increasing concentrations of CO. These were achieved by lowering the temperature of the cuvette and contents so that the solubility of the CO increased. At a temperature of 5°C approx. 60% of the cytochrome b formed a CO-complex (Fig. 7) and a plot of the change in frac-

Fig. 7. The reaction of cytochrome b_{-245} with CO. A suspension of sodium dithionite-reduced human neutrophil plasma membranes (3.1 mg of protein/ml) was diluted 1:5 with a mixture of buffer (containing sodium dithionite) and CO-saturated buffer (containing sodium dithionite) to give the desired CO concentration, and the difference spectrum recorded against a 1:5 dilution of sodium dithionite-reduced membranes in the reference cuvette. The temperature was maintained at 5°C throughout. The proportion of the cytochrome that bound CO was determined by using ϵ (575–559 nm) = $21.6 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ for the reduced plus CO-reduced spectrum. This absorption value was derived from Chance (1961). —, dithionite reduced – oxidized spectrum; ----, dithionite-reduced plus CO-reduced (saturated 5°C) minus reduced spectrum.

Fig. 8. Variation in extent of formation of cytochrome b_{-245} -CO complex with increasing $p\text{CO}$

The proportion of human neutrophil plasma-membrane cytochrome *b* that bound CO was determined as described in the legend to Fig. 7. CO-saturated buffer at 5°C (760 mm Hg) was assumed to contain 1.4 mm-CO.

tional saturation of the cytochrome with CO with increasing $p\text{CO}$ (Fig. 8) indicates that all the cytochrome would form a complex at high partial pressures of CO. This suggests that all the cytochrome binds CO, not a minor component. This is further evidence of its oxidase-like properties. The

relatively low affinity of the cytochrome for CO may arise from steric hindrance, which is known to affect the binding of CO to haemoglobin. In high-affinity CO-complexes, Fe, C and O atoms are in a linear array, whereas in O_2 -complexes the O_2 axis is at an angle to the Fe–O bond. An amino acid residue in the vicinity of the haem site could interfere with CO binding without affecting O_2 binding.

In conclusion, these studies confirm that cytochrome b_{-245} can be reduced by the postulated electron donors to the neutrophil microbicidal oxidase system and that its rapid oxidation rate and CO-binding properties are compatible with its participation as the terminal donor of electrons to O_2 .

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