The Subcellular Distribution and some Properties of the Cytochrome *b* Component of the Microbicidal Oxidase System of Human Neutrophils

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A novel cytochrome *b* has recently been described in human neutrophils. The subcellular distribution of this cytochrome was investigated by analytical fractionation on continuous sucrose gradients and it was found to have a dual localization, the major component having a similar distribution to the plasma-membrane marker [³H]concanavalin A, and a denser peak located with the specific granules. The two components were separated on discontinuous gradients and studied independently.

Phagocytosis by neutrophilic polymorphonuclear leucocytes (neutrophils) is associated with a markedly enhanced respiratory rate. This extra respiration of phagocytosis (Baldridge & Gerard, 1933) is not depressed by the mitochondrial inhibitors azide or cyanide (Sbarra & Karnovsky, 1959) and is important for the killing of certain bacteria (Mandell, 1974). The importance of this O₂-dependent microbicidal system is demonstrated by the rare condition of chronic granulomatous disease (Holmes *et al.*, 1967), in which absence of this burst of respiration is accompanied by a severe, often fatal, predisposition to bacterial infection. A previously undescribed cytochrome *b* has been identified in human neutrophils (Segal & Jones, 1978*a,b*), and the association of this cytochrome with the phagocytic vacuoles and its absence from, or gross abnormality in, cells from patients with chronic granulomatous disease (Segal *et al.*, 1978) implicate it as a component of the microbicidal oxidase system. We now report the results of studies conducted to determine the subcellular distribution of this *b* cytochrome, and to investigate some of its properties in fractionated tissue enriched in this component.

Methods

Isolation of neutrophils

Neutrophils were isolated from fresh human blood or buffy-coat residues (North West London Blood Transfusion Service, Edgware, Middx., U.K.) by dextran sedimentation of erythrocytes, centrifugation

through Ficoll/sodium metrizoate, and haemolytic lysis of residual erythrocytes as described previously (Segal & Peters, 1977).

Opsonization of latex

To 5 ml of an aqueous suspension of latex particles (0.81 μ m in diameter; Difco Laboratories, Detroit, MI, U.S.A.; 1×10^{10} /ml) was added 1.0 ml of 100 mM-Tricine (Sigma) adjusted to pH 8.5 with NaOH, and 1.0 ml of human IgG (250 mg/ml; Lister Institute, Elstree, Herts., U.K.). The mixture was incubated at 37°C for 30 min and then centrifuged at 8000*g* for 2 min in an Eppendorf 3200 centrifuge. The supernatant was discarded and the latex particles in the pellet were resuspended in a similar volume of Hanks balanced-salt solution (Hanks BSS; Flow Laboratories, Irvine, Ayrshire, Scotland, U.K.), centrifuged under the same conditions, and finally resuspended in fresh Hanks balanced-salt solution, at 1×10^{10} particles/ml. The concentration of IgG on the surface of these latex particles was measured with ¹²⁵I-labelled rabbit anti-(human γ -chain) antibodies and amounted to approx. 3×10^7 molecules/particle (Segal & Coade, 1978).

Subcellular fractionation

Analytical subcellular fractionation of neutrophil homogenates was performed as described previously (Segal & Peters, 1977) with the following modifications.

(a) Centrifugation was conducted in a swinging-bucket rather than a Beaufay zonal rotor using an MSE 3×65 ml head. Linear sucrose gradients were constructed with an MSE gradient maker (Bock & Ling, 1954) by using 23 ml of each of 8.6 and 53%

Abbreviations used: Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; IgG, immunoglobulin G.

(w/w) sucrose on a 3ml cushion of 60% sucrose. This gradient was overlaid with 15ml of the whole-cell homogenate, instead of a postnuclear supernatant preparation, and centrifuged at 23000 rev./min in an MSE Super Speed 65 centrifuge for 11 h at 4°C (g values, max. = 1×10^5 , min. = 4×10^4 , av. = 7×10^4).

The gradient was unloaded from the top of the tube in 3ml fractions into tared tubes by piercing the tube with an MSE tube piercer and pumping (60ml/h) dense (60%, w/w) sucrose into the bottom of the tube with a peristaltic pump.

(b) Organelle markers were measured as described previously (Segal & Peters, 1977) with the following additions or variations. Cytochrome oxidase (EC 1.9.3.1) was measured by the oxidation of reduced cytochrome *c* (Cooperstein & Lazarow, 1951). Malate dehydrogenase (EC 1.1.1.37) was assayed by the decrease in absorbance at 340nm with NADH and α -oxoglutarate as substrate (Biochemica Test Combination catalogue no. 15981). γ -Glutamyl-transferase (EC 2.3.2.2) was measured by the absorbance change at 405nm due to the generation of 5-amino-2-nitrobenzoate from L- γ -glutamyl-3-carboxy-4-nitroanilide (Boehringer Mannheim γ -GT new L-system, catalogue no. 125938). β -Glucuronidase (EC 3.2.1.31) was assayed fluorimetrically with 4-methylumbelliferyl- β -D-glucuronide trihydrate as substrate, in 0.2M-sodium acetate buffer, pH 4.0, containing 0.1% Triton X-100 (Morgan *et al.*, 1978). Protein was measured by the method of Lowry *et al.* (1951), and lysozyme (EC 3.2.1.17) by the decrease in absorbance at 450nm, indicating lysis of the cell wall of *Micrococcus lysodeikticus* (Klass *et al.*, 1977). Lactoferrin was measured by the Laurell 'rocket' technique of immunoelectrophoresis (Laurell, 1965); 50 μ l of rabbit anti-(human lactoferrin) serum (Behringwerk, Marburg-Lahn, Germany) was incorporated into the 4ml of 1% agarose on each 5.5cm $\times$ 11cm plate. Then 4.0 μ l of sample was placed in each well and electrophoresed overnight at 15V in 0.03M-veronal buffer, pH 8.6. Lactoferrin prepared from human colostrum (Calbiochem, La Jolla, CA, U.S.A.) was used as standard.

The lectin [3 H]concanavalin A was used as a possible marker of the plasma membrane. The concanavalin A (*N*-acetyl- 3 H)acetylated; The Radiochemical Centre, Amersham, Bucks., U.K.; TRK 497, 16.9Ci/mmol) was dissolved in water and a portion of this solution (400 μ l containing 20 μ Ci) was added to 2×10^9 neutrophils in 50ml of RPMI 1640 medium (Flow Laboratories; containing 5i.u. of heparin/ml) kept at 4°C to inhibit endocytosis (Silverstein *et al.*, 1977). After 15 min at 4°C the cells were centrifuged at 400g for 5 min at 4°C. The pellet was washed with a further 50ml of the RPMI medium, next with 50ml of 0.34M-sucrose and the cells were then homogenized in 0.34M-sucrose with a Dounce homogenizer, using a tight-fitting pestle as described

previously (Segal & Peters, 1977). Radioactivity in 100 μ l samples of the fractions was counted for 100s in a liquid-scintillation counter, with 10ml of a Triton/toluene scintillant. (Turner, 1968).

Latex phagocytosis. In some experiments, cells that had been labelled with [3 H]concanavalin A and washed with RPMI medium as described above were allowed to phagocytose latex particles before fractionation: 4×10^8 cells in 5ml of RPMI medium were incubated in a rapidly stirred incubation chamber containing a Clark type of oxygen electrode (Rank Brothers, Bottisham, Cambs., U.K.) for 2min at 37°C. Then 1×10^{10} IgG-opsonized latex particles in 1ml of Hanks balanced-salt solution were added. After 75s the incubation mixture was rapidly transferred to 200ml of cold (4°C) Hanks balanced-salt solution containing 1mM-EDTA, pH 7.4. This procedure was repeated five times, and the bulked cells were then centrifuged at 200g for 5min at 4°C, resuspended in 50ml of cold 0.34M-sucrose and centrifuged at 200g for 5min. The pellet was then homogenized in 0.34M-sucrose as described above. Latex particles were counted microscopically. All sucrose solutions used in fractionation studies contained 1.0mM-EDTA, pH 7.4, and 5.0i.u. of heparin/ml (preservative-free; Paynes and Byrne, Greenford, Middx., U.K.).

Discontinuous fractionation. Neutrophil homogenates, prepared as described above, were fractionated on discontinuous sucrose gradients to isolate two fractions enriched in the cytochrome *b*. Samples (15ml) of the homogenate were layered on the surface of discontinuous gradients comprising layers of (14.0ml) sucrose of densities 1.10, 1.17 and 1.21 g/ml, in MSE 65ml centrifuge tubes and centrifuged in a swinging-bucket rotor as described above. The material at the interfaces between densities of 1.10 and 1.17 and 1.17 and 1.21 g/ml was collected by aspiration from above.

Spectroscopy. Samples of fractions from the gradient were diluted to 5ml with sucrose buffer and divided between two cuvettes. A few crumbs of sodium dithionite were added to the sample cuvette and a difference spectrum was recorded using a sensitive split-beam spectrophotometer (see Jones & Saunders, 1972). The addition of potassium ferricyanide (1mM) to the reference cuvette did not increase the absorption bands of any *b*-type cytochromes detected in the gradient fractions.

Determination of oxidation-reduction midpoint potentials

Oxidative and reductive titrations of the cytochrome component were carried out, under argon in a stirred cuvette fitted with platinum and calomel electrodes, as described by Dutton (1978); measurements of the extent of reduction were made in a dual-wavelength spectrophotometer at 559nm minus

541 nm. The lack of interference by any other components was shown by repeating the titrations in a scanning split-beam spectrophotometer, recording spectra at 10 mV intervals over the midpoint region.

Results

Fractionation studies to identify the subcellular location of the components of dithionite difference spectra

The markers of the organelles (Table 1) of both the resting and phagocytic neutrophils were well resolved on the sucrose gradients (Figs. 1–3). The peaks of 430 nm- and 560 nm-absorbing material in dithionite difference spectra, henceforth referred to as the *b* cytochrome, appeared to have a dual localization.

The denser peak with a modal density of approx. 1.19 g/ml coincided with the peak activity of the two markers from the specific granules, lactoferrin (Spitznagel *et al.*, 1974; Dewald *et al.*, 1975) and vitamin B₁₂-binding protein (Kane & Peters, 1975). The localization of the lighter peak of activity, with a modal density of 1.14 g/ml, is less clear. It is quite distinct from the cytosol (lactate dehydrogenase), mitochondria (cytochrome oxidase), specific and azurophil (myeloperoxidase) granules and lysosomes (β -glucuronidase), and falls into the ill-defined region of the gradient occupied by the light membranous organelles.

The marker [³H]concanavalin A used for the plasma membrane does not appear to have been completely satisfactory. It was used, at a concentration well below that which stimulates the metabolic activity of the cell (Romeo *et al.*, 1977), because it

binds to sugars on the external surface of the cell. In five experiments $18.4 \pm 3.4\%$ (mean $\pm$ s.e.m.) of the radioactivity became attached to the cells. However, the linkage is not covalent and the lectin can redistribute after homogenization of the cell. This appears to have taken place in these experiments, as a large proportion (37.8%) of the radioactivity was distributed with the dense organelles. It is highly unlikely that this was due to co-distribution with the plasma membrane, which is of relatively low density (Segal & Peters, 1977). It is also unlikely to have sedimented with intact cells, most of which were disrupted by homogenization, as observed by phase-contrast microscopy, because little activity of the lactate dehydrogenase activity, or other markers of light organelles was observed in these dense regions of the gradient. There was a quite distinct peak of radioactivity with a modal density of approx. 1.14 g/ml that corresponded to that of the light peak of the *b* cytochrome. Other markers with a similar distribution include NADH–Nitro Blue Tetrazolium diaphorase, which is probably located in the plasma membrane (Segal & Peters, 1977), γ -glutamyltransferase and alkaline phosphatase.

The distribution of the small amount of endoplasmic reticulum that is present in these cells is unclear. The activity of glucose 6-phosphatase, a classical marker of this structure (Hers & de Duve, 1950; Colbeau *et al.*, 1971), was insufficient to assay with [¹⁴C]glucose 6-phosphate as substrate (Hübscher & West, 1965; Meerov & Ryzhkova, 1969). NADPH–cytochrome *c* reductase had a slightly different distribution from that of the presumptive plasma-membrane markers. Although this enzyme has been

Table 1. *Specific activity of neutrophil enzymes and marker substances from resting and phagocytic cells*

Values are expressed in munits/mg of protein unless stated to the contrary, where 1 unit of activity is defined as 1 μ mol/min.

Marker	EC no.	Specific activity	
		Control cells	Phagocytic cells
Alkaline phosphatase	3.1.3.1	1.34	0.72
[³ H]Concanavalin A		6027 c.p.m./mg	6073 c.p.m./mg
Cytochrome <i>b</i>		4 pmol/mg*	4 pmol/mg
Cyanocobalamin-binding protein		393 pg/mg	526 pg/mg
NADH–Nitro Blue Tetrazolium reductase	1.6.99.–	5.5	3.7
NADPH–cytochrome <i>c</i> reductase	1.6.2.4	2.0	3.5
Cytochrome oxidase		2.6	1.6
β -Glucuronidase	3.2.1.31	4.5	5.2
<i>N</i> -Acetyl- β -glucosaminidase	3.2.1.30	8.1	6.2
γ -Glutamyltransferase	2.3.2.2	8.9	8.1
Lactate dehydrogenase	1.1.1.27	24.7	28
Lactoferrin		226 μ g/mg	236 μ g/mg
Lysozyme	3.2.1.17	466 Sigma units/mg	624 Sigma units/mg
Myeloperoxidase	1.11.1.7	1460	1412
5'-Nucleotidase	3.1.3.5	4.0	3.4
Protein (total)		123 mg	115 mg

* Taking an approximate ϵ_{max} value of 21 (Wikström, 1973).

Fig. 1. Isopycnic centrifugation of neutrophil homogenates from cells before (----) or after (—) the phagocytosis of latex particles

The percentages of recovered activity for the resting and phagocytic cells respectively are: protein 73, 94; cytochrome *b* 126, 115; ^3H radioactivity 83, 84; latex particles 86; cytochrome oxidase 120, 190; vitamin B_{12} -binding protein 86, 69; lactoferrin 52, 63; myeloperoxidase 45, 65; *N*-acetyl- β -glucosaminidase 64, 70.

classified as an endoplasmic-reticulum marker in rat liver (Omura *et al.*, 1967), its localization has not been clearly characterized in neutrophils, where difficulties arise from the presence of a number of different diaphorases (Segal & Peters, 1977; De Chatelet *et al.*, 1974).

The assay of $5'$ -nucleotidase activity in neutrophils gave inconsistent results because the activity is very low, and so poor localization of this marker was achieved.

Sedimentable malate dehydrogenase (not shown) was assayed to confirm that the cytochrome oxidase activity, which was localized between the two peaks of the cytochrome *b* spectral changes, was in fact a mitochondrial cytochrome oxidase because of the variety of 'oxidases' described in neutrophils. In fact the distribution of sedimentable malate dehydro-

genase activity corresponded exactly to that of cytochrome oxidase, with a peak of activity at a density of 1.16g/ml (Segal & Peters, 1977).

The latex particles were counted microscopically and were mostly distributed in lighter regions of the gradient than the *b* cytochrome. Fractionation studies on phagocytic neutrophils demonstrated similar marker-enzyme distribution patterns to those of unstimulated cells, except in that region of the gradient occupied by the latex particles. A small tail of activity of myeloperoxidase, lactoferrin and the *b* cytochrome was observed in this region. Although this could indicate adsorption of these proteins on the latex particles, the majority of these were probably within phagosomes (Segal & Jones, 1978a), and the change in the distribution of the cytochrome *b* probably identifies it as a component of the wall of

Fig. 2. Subcellular fractionation on continuous sucrose gradients of neutrophil homogenates from cells before (----) or after (—) the phagocytosis of latex particles

The percentages of recovered activity for the resting and phagocytic cells respectively are: cytochrome *b* 126, 115; NADPH-cytochrome *c* reductase 52, 69; cytochrome oxidase 120, 190; lactoferrin 52, 63; 5'-nucleotidase 51, 46; alkaline phosphatase 84, 86; γ -glutamyltransferase 78, 84; NADH-Nitro Blue Tetrazolium (NBT) reductase 32, 40; lactate dehydrogenase 139, 86; lysozyme 85, 63.

the phagosome, or as in the case of lactoferrin or myeloperoxidase, as a content of the granules that empty into the vacuole.

Spectroscopy

Reduced-minus-oxidized difference spectra of the fractions separated by gradient centrifugation enable the distribution of two absorbing species to be measured (Fig. 3). Myeloperoxidase had an absorption maximum near 475 nm and the cytochrome *b* has absorption bands at 559, 530 and 429 nm at room temperature. At a density near 1.150 g/ml a membrane fraction is obtained that contains cytochrome *b* with very little myeloperoxidase. At higher density,

near 1.220 g/ml, a fraction is found that contains both myeloperoxidase and cytochrome *b*.

The two cytochrome *b*-containing components were routinely separated by using discontinuous gradient centrifugation. When the fraction containing cytochrome *b* with very little myeloperoxidase (Fig. 4, curve C) is suspended in alkaline pyridine, the difference spectrum (Fig. 4, curve A) shows the sharp band at 556.5 nm characteristic of protohaem pyridine haemochrome (see Smith, 1975), as expected from a *b*-type cytochrome. The difference spectrum of the membranes at 77 K (Fig. 4, curve B) shows that the α -band of the cytochrome is not symmetrical. Possibly more than one haem component is present,

Fig. 3. Dithionite difference spectra of fractions of homogenates of cells before (left) and after (right) phagocytosis of latex separated on continuous sucrose gradients, showing clear separation of the absorption spectra of cytochrome *b* and myeloperoxidase

Absorbance scale marker = 0.0056 absorbance unit. The numbers indicate the density (g/ml) of the respective fraction.

or the absorption band of one component may tend to split at low temperatures. CO difference spectra of the two cytochrome *b*-containing fractions showed the presence of relatively low amounts of CO-binding pigments, compared with the unfractionated homogenate (Fig. 4, curves C and E).

The CO and dithionite difference spectra did not show any of the spectral characteristics of cytochrome oxidase or cytochrome *P*-450. The cytochrome *b* is distinguished by a low midpoint potential, in the range -120 to -140 mV in oxidative or reductive titrations. Titration points did not fit closely to an $n = 1$ line when E_h was plotted against $\log ([\text{oxidized cytochrome } b]/[\text{reduced cytochrome } b])$ and the value quoted is approximate. Difficulties in the titration may be caused by the relatively poor redox mediation afforded by 2-hydroxy-1,4-naphthoquinone, which is the mediator active over this low-

potential range (see Dutton, 1978). The cytochrome clearly is not cytochrome *b*₅, which has an oxidation-reduction midpoint potential around 0 mV and is reduced by NADH (see Lemberg & Barrett, 1973, p. 8), unlike the cytochrome described in the present communication.

Discussion

The work that resulted in the discovery of this novel cytochrome *b* in human neutrophils was performed independently and in ignorance of that of a number of Japanese workers who described almost identical spectral changes in horse (Hattori, 1961; Ohta *et al.*, 1966) and rabbit (Shinagawa *et al.*, 1966) neutrophils. They also interpreted the spectral changes as being due to a cytochrome *b*, which they too implicated in the oxidase process. The shift of the 560 nm-absorp-

Fig. 4. Dithionite (—) and CO (----) difference spectra of fractions separated from homogenates of human neutrophils by centrifugation on discontinuous sucrose gradients

Spectra of the fractions collected at the interfaces between sucrose of densities 1.10 and 1.17 g/ml (A, B and C) and between 1.17 and 1.21 g/ml (D) and of the whole cell homogenate (E) are shown. Spectrum A was measured in alkaline pyridine and B was at 77 K. Absorbance scale marker = 0.0056 absorbance unit.

tion band to 557 nm upon the addition of pyridine (Fig. 4, curve B) is characteristic of *b* cytochromes (Lemberg & Barrett, 1973, pp. 68–73). This important early Japanese work in animals was either not seen by workers on neutrophil O_2 metabolism, or its importance went unrecognized, as no further work has been done on it and it has never previously been identified in human cells. In studies on dithionite

difference spectra of human cells recently presented at an international meeting on phagocytes, the clearly defined peaks '... observed in the difference spectrum of granulocytes at 426 and 558 nm' were dismissed as being 'derived from either mitochondria or more likely the eosinophilic peroxidase' (Wever *et al.*, 1977).

It is likely that the *b* cytochrome is implicated in the oxidase process, since it is absent from two patients with chronic granulomatous disease and its spectrum is modified in the other two (Segal *et al.*, 1978). The observation that both obligate heterozygotes had abnormally low concentrations of cytochrome *b* further strengthens the case for the primary involvement of the *b* cytochrome in the disease, since these subjects are clinically unaffected. The myeloperoxidase difference spectra of all the patients were altered, although the activity of this enzyme was normal. This could be due to a change in its oxidation state, which markedly influences the spectral properties of myeloperoxidase (Odajima & Yamazaki, 1972), compound III absorbing at 452 nm, in the region occupied by peaks or shoulders in these patients.

The *b* cytochrome was reported previously to be localized to the neutrophil granules (Hattori, 1961) and then to the specific or A granules (Ohta *et al.*, 1966; Shinagawa *et al.*, 1966), which were purified by simple differential centrifugation. The present studies indicate that the *b* cytochromes have a dual localization. There is a peak of activity associated with the specific granules. However, there is another larger peak located with the light membranous organelles, almost free of myeloperoxidase, which is probably in the plasma membrane.

Phagocytosing neutrophils produce superoxide, a transient intermediate in the generation of hydroxyl radicals (Segal & Meshulam, 1979; Green *et al.*, 1979). This burst of superoxide generation does not occur in chronic granulomatous disease (Curnutte *et al.*, 1974), and it is therefore possible that the cytochrome *b*, which is defective in this condition, is normally responsible for the reduction of O_2 to superoxide. The phagosome becomes acidified soon after particle ingestion (Rous, 1925; Mandell, 1970), which has been attributed to the accumulation of lactate generated by glycolysis. Neutrophil-granule lysates are bactericidal, an effect that is most marked at acid pH (Hirsch, 1956a,b; Ginsburg & Sela, 1976). It thus seems feasible that the neutrophil electron transport could be involved in the acidification of the vacuole, possibly by a proton pump coupled to electron transport, as described in mitochondria (Mitchell, 1962, 1966; Lehninger, 1975).

Preliminary work has shown that the cytochrome *b* found in both of the purified fractions of human neutrophils (Fig. 4) that were separated on discontinuous sucrose gradients has a relatively low

oxidation-reduction midpoint potential (E_0) of between -120 and -140 mV. No clear difference in E_0 of the cytochrome *b* in the two fractions could be found.

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