Cytochrome b_{-245} of neutrophils is also present in human monocytes, macrophages and eosinophils

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A cytochrome b with a midpoint oxidation–reduction potential of -245 mV (cytochrome b_{-245}) that is a major component of the microbicidal oxidase system of human neutrophil leucocytes has been identified in human eosinophils, monocytes and macrophages at concentrations similar to that found in human neutrophils. It was absent from a variety of other cells. This cytochrome is present in phagocytic leucocytes and probably plays an important part in the specialized activities of these cells.

Neutrophil polymorphonuclear leucocytes (neutrophils) greatly increase their oxygen consumption as they phagocytose particles. This ‘extra respiration of phagocytosis’ (Baldridge & Gerard, 1933) is not mitochondrial, as it is not inhibited by inhibitors of cytochrome oxidase such as cyanide and azide (Sbarra & Karnovsky, 1959), and appears to facilitate the killing of ingested micro-organisms (Selvaraj & Sbarra, 1966). The products of the oxidase system are thought to include superoxide (Babior *et al.*, 1973), H_2O_2 (Iyer *et al.*, 1961) and hydroxyl radicals (Green *et al.*, 1979), and it has been proposed that these substances are directly toxic to the engulfed organism.

The oxidase enzyme has been the subject of considerable interest and controversy. We have shown that there is a very-low-potential ($E_{m,7.0} = -245$ mV) cytochrome b (Cross *et al.*, 1981) in the plasma membrane of these cells (Segal & Jones, 1979a, 1980a), which is incorporated into the phagocytic vacuoles (Segal & Jones, 1978) and has the characteristic features of an oxidase (Cross *et al.*, 1981). This cytochrome is reduced when the cells are stimulated under anaerobic conditions with phorbol myristate acetate, a potent activator of the oxidase system, and is reoxidized on re-exposure to air (Segal & Jones, 1979b). An important observation linking this cytochrome to the microbicidal oxidase system is that it appears to be missing or functionally abnormal in the rare syndrome of chronic granulomatous disease (Segal & Jones, 1980b). This is an inherited condition in which a predisposition to severe, often fatal, pyogenic infection is associated with a diminished killing of bacteria and fungi by the neutrophils and mono-

cytes (Quie *et al.*, 1967), which show no evidence of oxidase activity on stimulation (Holmes *et al.*, 1967). The microbicidal defect observed in neutrophils from these subjects has been attributed to their failure to generate toxic oxygen radicals and H_2O_2 , which is thought to provide the substrate for myeloperoxidase to chlorinate the microbe (Klebanoff, 1975). We have proposed that the oxidase system is part of an electron-transport chain (Segal & Jones, 1978, 1979a, 1980b), the function of which may be to regulate the pH in the lumen of the phagocytic vacuole (Segal *et al.*, 1981) and optimize conditions for killing by the contents of the cytoplasmic granules. Alternatively, it may produce toxic oxygen radicals that kill *per se*.

Other ‘professional’ phagocytes, such as monocytes (Reiss & Roos, 1978), macrophages (Johnston *et al.*, 1978) and eosinophils (Baehner & Johnston, 1971) also show evidence of this non-mitochondrial phagocytosis-associated respiration. They produce superoxide and H_2O_2 and chemiluminesce. We therefore studied these and other non-phagocytic cells to determine if the phagocytic cells also contain the cytochrome b_{-245} , as it seemed likely that they would share a similar mechanism for killing. We found that human eosinophils and mononuclear phagocytes, but none of the other cells we examined, contained a low-potential cytochrome b with the same redox properties as that of the neutrophil.

Materials and methods

Purification of cells

Leucocytes were isolated from normal human blood and from the blood of patients with (a)

monocytosis in response to an atypical mycobacterial infection, (b) idiopathic hypereosinophilia, (c) leukaemias of T-, B- or basophil cell lines, by sedimentation of erythrocytes in dextran and centrifugation of the supernatant on a discontinuous gradient of Ficoll/sodium metrizoate (Böyum, 1976). T-lymphocytes and monocytes from normal blood were further purified by 'rosetting' techniques (Kaplan & Clark, 1974), followed by a second centrifugation on Ficoll/sodium metrizoate. T-cells were obtained from the pellet after rosetting, and monocytes were obtained from the interface cells by adherence to fibronectin-coated plates (Ackerman & Douglas, 1978). Monocyte-enriched mononuclear-cell preparations were also obtained by using a discontinuous-Percoll-gradient separation (Ulmer & Flad, 1979) of the cells found at the interface after Ficoll/sodium metrizoate treatment of leucocyte-rich plasma (Böyum, 1976). Eosinophil- or basophil-enriched granulocyte preparations were obtained by subjecting the cells in the pellet after the Ficoll/sodium metrizoate step to centrifugation on a continuous gradient of Percoll (Segal *et al.*, 1980).

Platelets were obtained from concentrates (North London Blood Transfusion Centre) by low-speed centrifugation. Normal human fibroblasts (MRC 5 cell line) were grown in Eagle's minimal essential medium (Flow Laboratories, Irvine, Ayrshire, Scotland, U.K.) containing 10% (v/v) foetal-calf serum. Samples of pancreas were obtained from newborn piglets. Human erythrocyte 'ghosts', adipocyte plasma membranes and rat liver plasma membranes were gifts from Dr. M. J. A. Tanner and Dr. G. Belsham, Department of Biochemistry, University of Bristol, Bristol, U.K., and Professor J. Judah, University College, London, U.K., respectively.

Characterization of blood cells and macrophages

The cells were examined by optical microscopy after staining with Romanowsky stains. In addition, non-specific esterase staining (Yam *et al.*, 1971) was used to identify monocytes and macrophages and distinguish them from neutrophils. B-lymphocytes were identified by the presence of surface immunoglobulins and T-lymphocytes by rosetting characteristics (Kaplan & Clark, 1974).

Preparation of membranes

Crude membrane preparations were obtained by centrifuging cell homogenates or pancreas homogenates on discontinuous sucrose gradients (Segal & Jones, 1979a).

Spectroscopy and potentiometric titrations

Reduced-minus-oxidized difference spectra were measured after the addition of a few grains of dithionite to the sample cuvette, with a sensitive

split-beam spectrophotometer (see Jones & Saunders, 1972). Oxidative and reductive titrations of the cytochrome components were conducted under argon in a stirred cuvette fitted with platinum and calomel (Hg_2Cl_2) electrodes, as described by Dutton (1978). The following mediators were added to the suspension: phenazine methosulphate ($12.5\mu\text{M}$), phenazine ethosulphate ($12.5\mu\text{M}$), pyocyanine ($6\mu\text{M}$), 2-hydroxy-1,4-naphthoquinone ($12.5\mu\text{M}$), anthroquinone 2,6-disulphonate ($12.5\mu\text{M}$), anthroquinone ($10\mu\text{l}$ of a saturated solution in ethanol), 3,6-diaminodurene ($12.5\mu\text{M}$) and duroquinone ($12.5\mu\text{M}$). The extent of reduction of cytochrome *b* at different oxidation-reduction potentials (E_h) was calculated by measuring the height of the absorption band at 559 nm above the line joining the two troughs on each side of this band. The reference cuvette contained an identical sample of membranes without added mediators or reductant. The light-path was 10 mm. Addition of potassium ferricyanide to the reference cuvette caused no further oxidation of haem compounds.

Results and discussion

We were able to identify a cytochrome *b* in eosinophils and monocytes (Fig. 1) after the addition of dithionite to intact cells (Segal & Jones, 1980b). Dithionite penetrates membranes slowly, reducing molecules within the plasma membrane before intracellular organelles and their contents. The reduction of intact cells overcomes the problems of distortion of the spectrum by intracellular peroxidases and cytochromes that others have found so confusing when studying cell homogenates (Hamers *et al.*, 1980).

The leucocytes whose spectra are illustrated in Fig. 1 were prepared from one batch of human blood. Inevitably in such a preparation there was substantial contamination of the monocytes by lymphocytes and of eosinophils by neutrophils. However, the concentrations of the cytochrome *b* displayed in the difference spectra are such that they could not be due to contamination. For more precise determination of the concentration of low-potential cytochrome *b* in cells, it was necessary to use leucocytes prepared from patients with excessive production of particular types of white cell and to measure the amount of cytochrome *b* that was reduced only at potentials below -100mV . The concentration of this cytochrome *b* in monocytes was similar to that of neutrophils and was about half that in eosinophils (Table 1). To compare the cytochrome in these cells with that of neutrophils, the midpoint potential ($E_{m,7.0}$) was determined. Cytochrome *b*₋₂₄₅ was identified as the only obvious cytochrome in monocytes and comprised about half of the complement of cytochrome *b* in the eosino-

Fig. 1. Oxidized-minus-dithionite-reduced difference spectra of human peripheral blood leucocytes prepared from one sample of blood

Cells were suspended in aerated buffer before (—) and after (---) disruption with Triton X-100 (0.1%). (a) Neutrophils (1×10^8 /ml, 98% pure); (b) monocytes (64%) and lymphocytes (36%), 7.2×10^8 /ml; (c) eosinophils (51%) and neutrophils (49%), 2.4×10^7 /ml. Absorbance scale marker = 0.02, 0.04 and 0.01 absorbance unit before, and 0.04, 0.1 and 0.1 after, the addition of detergent to (a), (b), and (c) respectively.

Table 1. Concentrations of low-potential [$E_{m,7.0} < -100$ mV] cytochrome b in different cell types

Human cells were studied unless otherwise stated. The concentrations of cytochromes were calculated from the height of the absorption band at 559 nm, taking an approximate E_m value of 21 (Wikström, 1973). The potentiometric titrations of neutrophils, monocytes and eosinophils are shown in Fig. 2. The major cytochrome b in macrophages was of a low potential and the titrations were consistent with an $E_{m,7.0}$ of approx. -245 mV, although an accurate determination was prevented by the small numbers of cells in the sample (1 mg of protein/ml).

Cell type	[Low-potential cytochrome b] (pmol/mg of protein)
Neutrophils	80
Eosinophils	193, 284
Monocytes	50, 70
Macrophages	65, 152
Basophils	<10
B-lymphocytes	<10
T-lymphocytes	<10
Erythrocytes	0
Platelets	0
Fibroblasts	0
Pig pancreas	0
Rat adipocyte membranes	0
Rat liver membranes	0

phil membranes, the other half of which was a cytochrome b with an $E_{m,7.0}$ of -186 mV (Fig. 2). Since we have found that solubilized cytochrome b_{-245} of neutrophil membranes in detergent had an increased midpoint potential ($E_{m,7.0} = -188$ mV), it is possible that this second cytochrome b in eosinophils is cytochrome b_{-245} in an altered environment.

Tissue macrophages are thought to develop from circulating monocytes, and the change in habitat of these cells is accompanied by changes in composition, structure and function (Cohn & Benson, 1965). Cytochrome b_{-245} was identified in both preparations of human macrophages in concentrations similar to those observed in neutrophils. Whether this cytochrome is present in unstimulated resident macrophages or whether its presence in cells that have been elicited by active pathology indicates induced synthesis, or the recruitment of circulating monocytes that have not yet undergone complete involution, remains to be established.

Basophils are the other class of granulocytic cells in the peripheral circulation, where they are normally present in very small numbers. A preparation of such cells containing more than 90% basophils was prepared from a patient with leukaemia of this cell line, and membranes that were isolated from these basophils were found not to contain a low-potential cytochrome b , apart from the small

Fig. 2. Potentiometric titrations

Crude membranes from human neutrophils (a), eosinophils (c) and monocytes (e) were suspended in 50 mM-Mops (4-morpholinepropanesulphonic acid)/100 mM-KCl, pH 7.0, containing redox mediators (see the Materials and methods section). All the starting cell preparations were >95% pure. (●) Reductive titration with sodium dithionite solution; (○) oxidative titration with potassium ferricyanide. The lines through the points are for the theoretical curve for a one-electron-transfer reaction. The extents of reduction were calculated from reduced-minus-oxidized difference spectra (see the Materials and methods section), some of which are reproduced above: (b) neutrophil membranes (2.1 mg of protein/ml); (d) eosinophil membranes (1.1 mg of protein/ml); (f) monocyte membranes (2.9 mg of protein/ml). Abbreviation used: cyt, cytochrome.

amount attributable to the few contaminating neutrophils. This is noteworthy, because although basophils develop from the same progenitor cell as neutrophils and monocytes (Denegri *et al.*, 1978), their function is as a secretory rather than a phagocytic cell (Ishizaka *et al.*, 1969).

To confirm that the low-potential cytochrome b_{-245} is not necessarily implicated in all degranulation processes, we examined membranes prepared from pig pancreatic cells, which have an active exocrine function. There was no evidence of this cytochrome in the pancreatic cells, although it is

present in porcine neutrophils (F. Higson, personal communication).

Human erythrocyte 'ghosts' and fibroblast membranes, and rat adipocyte and liver cell membranes also appeared to lack this cytochrome. Human B- and T-lymphocytes, both leukaemic and normal circulating cells, were examined, and small quantities of cytochrome *b*₋₂₄₅ were identified in membrane preparations from both these cell types. However, the concentrations were less than 5% of those in similar membrane preparations from neutrophils and monocytes, making it impossible to distinguish confidently between minimal contamination of the lymphocyte preparations by these cells and the presence of small amounts of low-potential cytochrome in lymphocytes themselves.

Cytochrome *b*₋₂₄₅ has only been identified in high concentrations in the 'professional phagocytes', namely neutrophils, eosinophils, monocytes and macrophages. In neutrophils it is an important component of the microbicidal oxidase system, probably the terminal oxidase itself (Cross *et al.*, 1981). It is likely to serve the same function in the other cells in which it may control the pH within the phagocytic vacuole (Segal *et al.*, 1981), optimizing killing and subsequent processing of the target microbe or cell by granule contents as well as producing toxic oxygen products.

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