The development of cytochrome b_{-245} in maturing human macrophages*

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Cytochrome b_{-245} , the putative terminal component of the specialized cidal oxidase system of phagocytes, was measured in human monocytes in culture. There was a dramatic synthesis of the cytochrome, which increased by 27.3 ± 2.0 pmol/day per 10^7 cells. This represents an increase of about 40%/day in the early stages and an overall 7-fold increase after 16 days. The protein content increased 3-fold over the same period, resulting in a doubling of the specific content of the cytochrome b . The newly synthesized cytochrome b was identified as that specifically located in the microbicidal oxidase electron-transport chain, as titration demonstrated that, at day 16 of maturation, 70% of the total membrane cytochrome b had a very low midpoint potential (-260 to -220 mV), characteristic of that found in this oxidase system. This cytochrome distributed with the plasma membrane on analytical subcellular fractionation, and a close relationship was observed between the maturation-induced increase in the concentration of this molecule and the capacity of the cells to produce superoxide.

INTRODUCTION

Macrophages play an essential role in the defence of the body against a variety of pathogens and tumour cells. A very unusual oxidase system that generates superoxide and H_2O_2 has been shown to play a crucial role in this killing. Human monocytes develop many of the characteristics of macrophages when cultured *in vitro*. These include the secretion of lysozyme, the accumulation and release of lysosomal enzymes, an increase in cellular protein (Musson *et al.*, 1980), the uptake of particles through complement-subcomponent-C3b receptors (Newman *et al.*, 1980) and an increased cytotoxic activity (Musson, 1983). The respiratory burst results from the activation of an NADPH-oxidizing electron-transport chain located in the membrane of cells. Cytochrome b_{-245} is an integral part of this oxidase system and probably the terminal component itself, found in all professional phagocytes (Segal *et al.*, 1981). The present study was undertaken to examine the fate of cytochrome b_{-245} in human monocytes as they mature in culture, with particular reference to its relationship with the production of superoxide anion (O_2^-) by these cells.

EXPERIMENTAL

Preparation and culture of cells

Human monocytes were obtained from mixed, fresh, buffy-coat residues from between 12 and 16 normal donors at the South London Blood Transfusion Centre. The purification was conducted as described previously (Segal *et al.*, 1980) until after the Ficoll-Hypaque centrifugation step. The mononuclear cells at the

interface were washed four or five times with PBS by resuspension and centrifugation at $180g$ at room temperature, and suspended to a concentration of 10^7 /ml in RPMI 1640 medium, pH 7.4, containing Hepes (25 mM), glutamine (2 mM), $NaHCO_3$ (0.2%), penicillin (50 units/ml), streptomycin (50 μ g/ml) and amphotericin B (2.5 μ g/ml). This cell suspension was plated on to tissue culture dishes (Nunc; Gibco Ltd., Paisley, Renfrewshire, Scotland, U.K.) at a concentration of 5×10^5 /cm². The plates were incubated at 37 °C for 2–3 h under CO_2 /air (1:19), after which time the non-adherent cells were removed and discarded and the adherent cells were washed twice with PBS. RPMI medium, with the additions indicated plus 12% (v/v) fetal-calf serum (Gibco Ltd.), was then added to the plates. Cultures were maintained in a CO_2 /air (1:19) incubator, supplementing the cells every 4–5 days with fresh medium until they were harvested. The adherent cell population (approx. 2×10^5 /cm²) consisted of 81.1% monocytes ($n = 9$, S.E.M. 2.9) and 17.9% lymphocytes ($n = 9$, S.E.M. 2.5), as determined by both May-Grunwald-Giemsa and non-specific esterase staining (Yam *et al.*, 1971). These cells were harvested by gentle scraping with a latex stopper, after the addition of EDTA (2 mM) to the plates. They were washed with PBS containing EDTA (2 mM) and finally resuspended at a concentration of $(2-4) \times 10^7$ /ml in PBS containing EDTA (2 mM) and glucose (5 mM).

Subcellular fractionation

Cell pellets $[(2-7) \times 10^7]$ cells] were maintained in 6% (w/w) sucrose containing EDTA (1 mM) and heparin (5 units/ml) for 10 min, on ice, and then sonicated (amplitude 6 μ for 30 s, Soniprep 150; MSE Scientific

Abbreviations used: PBS, phosphate-buffered saline (137 mM-NaCl/2.7 mM-KCl/8.1 mM- Na_2HPO_4 /1.5 mM- KH_2PO_4 , pH 7.0); PMA, phorbol 12-myristate 13-acetate; O_2^- , superoxide anion.

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Instruments, Crawley, Sussex, U.K.). The sucrose concentration was immediately raised to 9% (w/w) by addition of the appropriate volume of a 30% (w/w) solution. This homogenate was loaded on an 8 ml continuous 34–60% (w/w)-sucrose gradient, which was centrifuged at 90000 *g* (r_{av} , 12 cm) for 15 h at 4 °C in a swing-out rotor (Sorvall AH-627). After centrifugation, four turbid bands could be distinguished. Seven fractions were collected from above with a Pasteur pipette, corresponding to: 1, cytosol [top of the gradient, density (ρ) 1.05–1.11 g/ml]; 2, the lightest band (membranes, ρ 1.11–1.14 g/ml); 3, the clear space between the membranes and mitochondrial band (ρ 1.14–1.16 g/ml); 4, 5 and 6, the three other bands (ρ 1.16–1.18, 1.18–1.20 and 1.20–1.24 g/ml), the lightest of which is enriched in mitochondria; 7, the clear solution at the bottom of the tube (ρ > 1.24 g/ml).

The further fractionation of membranes shown in Fig. 2 (below) was carried out on gradients of Ficoll 400 (Pharmacia AB, Uppsala, Sweden), as previously described (Taylor *et al.*, 1983). Briefly, 1.0×10^8 monocytes harvested at day 3 and containing 1540 pmol of cytochrome *b* were fractionated and fraction 2 collected and diluted fivefold with a solution of EDTA (1 mM) and heparin (5 units/ml). The membranes were pelleted at 100000 *g* (r_{max} , 8 cm) for 30 min in a Sorvall T865.1 rotor, resuspended with 2 ml of sucrose (70 mM) containing Tris/HCl, pH 7.7 (5 mM) and EGTA (1 mM), and subfractionated by centrifugation at 100000 *g* (r_{av} , 12 cm) on a continuous gradient of Ficoll 400 [5–18% (w/w)] (Taylor *et al.*, 1983), for 41 h at 4 °C in a Sorvall AH-627 swing-out rotor. The gradient was collected in 1.5 ml fractions as described above, by displacement with 20% (w/w) Ficoll 400.

For the assays described in Table 2 (below), the mitochondrial fraction was used as collected, whereas the membranes were pelleted and resuspended as described above to remove cytosolic proteins.

Enzyme assays

N-Acetyl- β -glucosaminidase (EC 3.2.1.30) and α -glucosidase (EC 3.2.1.20) were assayed as described by Rest *et al.* (1978), 5'-nucleotidase (EC 3.1.3.5) by the method of Douglas *et al.* (1972) as modified in our laboratory (Segal *et al.*, 1983), sulphatase C (EC 3.1.6.1)

by the method of Canonico *et al.* (1978), and cytochrome oxidase (EC 1.9.3.1) as described by Cooperstein & Lazarow (1951).

Superoxide formation

The rate of formation of superoxide was measured in a double-beam spectrophotometer at 37 °C. The incubation mixture contained PBS, cytochrome *c* (0.1 mM), glucose (1 mM) and portions of cell suspensions [$(3-7) \times 10^5$ cells], in a total volume of 1 ml. The reference cuvette contained superoxide dismutase (50 μ g) in addition. The reactions were started by addition of PMA (1 μ g; Sigma Chemical Co., Poole, Dorset, U.K.) in dimethyl sulphoxide (2 μ l), and the rate of reduction determined from the increased absorbance at 550 nm, by using an absorption coefficient of 21.1 mM⁻¹·cm⁻¹ (Van Gelder & Slater, 1962). Inhibition by NaN₃ (1 mM) was always less than 20%.

Determination and characterization of cytochrome *b*

Cytochrome *b* was measured in cell suspensions and subcellular fractions by reduced-minus-oxidized difference spectroscopy, on a Pye-Unicam SP8-200 double-beam spectrophotometer. A few crystals of sodium dithionite were added to one of the cuvettes to fully reduce the preparation, and spectra were recorded from 600 to 400 nm. The concentration of cytochrome *b* was calculated from the peak of absorption at 559 nm by using an absorption coefficient of 21.6 mM⁻¹·cm⁻¹ (Cross *et al.*, 1982).

Potentiometric titrations were performed as described previously (Segal *et al.*, 1981).

RESULTS

Monocyte maturation

The first objective of these studies was to determine the cytochrome *b* content of maturing monocytes. The maturation process was monitored by microscopic observation and by an increase of total cellular protein and lysosomal-enzyme activities (Table 1).

Cytochrome *b*

(a) **Quantification.** There was a dramatic increase in the cellular content of cytochrome *b*, which rose 7-fold

Table 1. Protein content and hydrolase activity as a function of maturation

Cells were harvested at the times indicated and assayed for protein content and hydrolase activity. The mean values (with the number of experiments indicated in parentheses) are expressed as a function of the average value on day 1. Absolute values per 10⁷ cells were: protein, 0.41 mg ($n = 7$, S.E.M. 0.03); *N*-acetyl- β -glucosaminidase, 450 ($n = 4$, S.E.M. 47); α -glucosidase, 14.4 ($n = 1$) and sulphatase C, 1.3 ($n = 2$, S.E.M. 0.3) nmol of 4-methylumbelliferone/h.

Time in culture (days) . . .	Value relative to that on day 1							
	2	3	6	7	8	9	13	16
Protein	1.2 (2)	1.1 (5)	1.8 (3)	2.0 (2)	3.2 (2)	2.0 (2)	2.7 (2)	2.9 (1)
<i>N</i> -Acetyl- β -glucosaminidase	1.2 (2)	1.8 (2)	3.7 (3)	3.0 (3)			26.8 (1)	
α -Glucosidase								15.5 (1)
Sulphatase C		1.2 (1)		2.3 (1)				8.0 (1)

Fig. 1. Cytochrome *b* content and superoxide-forming ability as a function of time in culture

Cytochrome *b* content (○) and O₂⁻-generating capacity (▲) of macrophages at different stages in development. The results are expressed as the percentage increase above the day-1 values, the absolute values of which were: cytochrome *b*, 65.1 pmol/10⁷ cells ($n = 6$, S.E.M. = 4.0), and rate of O₂⁻ generation, 21.0 nmol/min per 10⁷ cells ($n = 4$, S.E.M. = 1.6). The results of 15 separate experiments are shown.

in 16 days (Fig. 1). This increase was linear with time ($r = 0.93$, $P < 0.001$), and 2–3-fold greater than the increase in cellular protein.

(b) Characterization. (i) Subcellular localization. In order to implicate this cytochrome *b* in the NADPH oxidase system of monocyte-derived macrophages, it was necessary to identify it as the cytochrome *b* of the microbicidal oxidase. It was distinguished from mitochondrial cytochromes by determining its subcellular distribution. A large proportion of the cytochrome

(65%) was found to be present in the light region of sucrose gradients (ρ 1.11–1.13 g/ml) together with the plasma-membrane marker 5'-nucleotidase, well separated from the mitochondrial marker cytochrome *c* oxidase (Table 2). The specific content of cytochrome *b* present in this low-density fraction increased 2–3 times on maturation (results not shown), indicating a relative enrichment of the membranes in this component of the oxidase system. Further fractionation of membranes obtained from monocytes at day 3 of culture on a Ficoll gradient showed that the distribution of the cyto-

Table 2. Subcellular fractionation by isopycnic sucrose centrifugation

Cells were harvested at between 1 and 16 days in culture and fractionated on sucrose gradients as described in Experimental section. The distribution in the gradient and enrichments did not vary significantly with time in culture. Recoveries were 105.5% ($n = 3$, S.E.M. 12.9), 81.8% ($n = 3$, S.E.M. 13.3) and 54.9% ($n = 1$) for cytochrome *b*, 5'-nucleotidase and cytochrome *c* oxidase respectively.

	Membrane fraction		Mitochondrial fraction	
	%*	Enrichment†	%*	Enrichment†
Cytochrome <i>b</i>	64.7 ± 3.0 ($n = 7$)	7.4 ± 0.9 ($n = 5$)	8.9 ± 0.9 ($n = 7$)	1.5 ± 0.3 ($n = 7$)
5'-Nucleotidase	63.3 ± 9.0 ($n = 7$)	17.0 ± 4.7 ($n = 3$)	6.0 ± 2.5 ($n = 4$)	1.8 ± 1.0 ($n = 3$)
Cytochrome <i>c</i> oxidase	7.8 ± 2.5 ($n = 2$)	0.4 ± 0.2 ($n = 2$)	57.2 ± 8.9 ($n = 2$)	21.4 ± 0.9 ($n = 2$)

* Proportion of total in the gradient ($\pm$ S.E.M.).

† Enrichment = specific content or activity relative to that in the corresponding homogenate.

Fig. 2. Density distribution profile of the cytochrome *b* (c) in relationship to marker enzymes for plasma membrane (b) and endoplasmic reticulum (a) in a Ficoll gradient

Top and bottom of the gradient are on the left- and right-hand side of the Figures respectively. Recoveries were 88, 92 and 75% for cytochrome *b*, 5'-nucleotidase and sulphatase C respectively.

chrome *b* is similar to that of the plasma membrane and different from that of the endoplasmic reticulum (Fig. 2).

(ii) Midpoint potential. The cytochrome *b* was characterized by measuring its midpoint redox potential at pH 7 in subcellular fractions of $\rho = 1.11$ –1.13 and 1.17 g/ml from both immature and mature monocytes (days 2, 7 and 16 of culture). It was observed that 75.7% ($n = 3$, S.E.M. 7.2) of the cytochrome in the light membrane fraction had a low midpoint potential (–260 to –220 mV), in agreement with the value for the membrane cytochrome *b* of peripheral-blood monocytes (Segal *et al.*, 1981). In contrast, the cytochrome in the fractions of greater density was predominantly of a high midpoint potential (> -100 mV), consistent with a mitochondrial origin. No significant variation was observed in the proportion of cytochrome *b* with a low potential present in the membrane fractions from cells at different stages of maturation.

The relationship between maximal superoxide generation and cytochrome *b*_{–245} development

The relationship between the cytochrome *b* content of monocytes and their ability to generate O_2^- on stimulation was studied at different times during

maturation. The results shown in Fig. 1 indicate that the rate of increase of superoxide generation was slightly greater than that of the accumulation of cytochrome *b*, the slopes of the corresponding linear regression lines being 0.68 ($n = 14$, S.D. 0.08, $r = 0.92$, $P < 0.001$) and 0.42 ($n = 27$, S.D. 0.03, $r = 0.93$, $P < 0.001$) respectively.

DISCUSSION

The present study shows that there is a dramatic synthesis of cytochrome *b*_{–245} in association with maturation by human monocytes in culture. These cells clearly changed their characteristics in culture. They markedly increase their content of protein and lysosomal enzymes and their capacity to produce superoxide. This maturation was probably stimulated by lymphokines produced as a result of a mixed lymphocyte response. The extent of the increase in the capacity of the maturing cells to produce superoxide was surprising in view of the conflicting reports in the literature as to the ability of monocytes to generate oxygen metabolites upon culture. Some investigators described a marked decrease (Musson *et al.*, 1982; Nakagawara *et al.*, 1981), whereas others observed superoxide production to be maintained in the presence of bacterial products (Pabst *et al.*, 1982). This reported variability is almost certainly a reflection of differences in culture conditions, with particular reference to factors such as the substratum and cellular-derived mediators like lymphokines and interferons (Nakagawara *et al.*, 1982; Nathan *et al.*, 1983; Cassatella *et al.*, 1985), and in the assay conditions. For maximal superoxide generation the cells must be in suspension, probably because some of the membrane and an indeterminate proportion of the oxidase system and its products are separated from the superoxide detection system when the cells are spread on a surface. We also found that it is important to include glucose in the incubation mixture for optimal oxidase activity. In the absence of added glucose there is a decrease in apparent activity with increasing time in culture (results not shown).

A close relationship existed between the increase in the cellular content of cytochrome *b*_{–245} and the increase in the maximal superoxide-generating ability. The rate of superoxide generation was 5.4 and 8.0 mol/s per mol of cytochrome *b* in cells after 1 and 9 days in culture respectively, which is close to the steady-state rates of reduction of the cytochrome and superoxide generation in a partially purified solubilized oxidase preparation (Cross *et al.*, 1985). These results also suggest that the bulk of the newly synthesized cytochrome is functional. Our observations contrast with those of Berton *et al.* (1986), in whose study on mouse peritoneal macrophages no correlation was found between the cellular content of cytochrome *b*_{–245} and the rate of superoxide generation. In view of the kinetic data described above, the cytochrome would not be expected to be limiting at the low rates of superoxide generation they observed in resident macrophages. Other factors such as the reduced availability of substrate or the limitation of electron transport by other components of the chain could have restricted the rate of superoxide generation in that study. In our investigation these factors do not appear to restrict the oxidase, the rate of which seems to be dependent upon the cellular concentration of cytochrome *b*_{–245}.

This cytochrome provides a well-defined molecule that is clearly linked to the host defence capability of the macrophage. It might therefore provide a valuable indicator of the influence of mediators on macrophage maturation, which should be particularly useful with the advent of reagents that allow the assay of mRNA coding for its synthesis.

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