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# Rapid incorporation of the human neutrophil plasma membrane cytochrome b into phagocytic vacuoles

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Summary: Phagocytic vacuoles containing IgG coated latex particles were isolated from human neutrophils by floatation. The absorbance spectrum of the cytochrome b was associated with the vacuoles within 10 sec of particle uptake and the vacuolar concentration increased little thereafter. In contrast, the cytoplasmic granule proteins myeloperoxidase and vitamin $B_{12}$ binding protein associate with the vacuoles more slowly. The addition of dithionite to intact cells rapidly reduces most of the cytochrome b, whereas only a small proportion of the myeloperoxidase, which is located intracellularly, is reduced in the absence of detergent. Most of the cytochrome b appears to be localised in the neutrophil plasma membrane.

## INTRODUCTION

Neutrophil granulocytes demonstrate a short burst of respiration in association with phagocytosis (1). This respiration is non-mitochondrial (2) and is essential for the killing of certain bacteria (3). We have recently described a cytochrome b which is a central component in this oxidase system (4-6). Analytical subcellular fractionation by centrifugation on continuous sucrose gradients showed a dual localisation for this cytochrome, with most of the activity in the region of the plasma membrane and a small peak of activity associated with the specific granules (6). However, these studies were complicated by the lack of a definitive marker for the neutrophil plasma membrane. The rates of association of the cytochrome b with the phagocytic vacuole, and its reduction after the addition of dithionite to intact cells have been investigated, and compared with similar observations on proteins contained within the cytoplasmic granules, in a further attempt to demonstrate the situation of this cytochrome b within the plasma membrane.

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## MATERIALS AND METHODS

Isolation of neutrophils. Neutrophils were isolated from human buffy coat residues by dextran sedimentation of erythrocytes, centrifugation through a gradient of Ficoll/sodium metrizoate and hypotonic lysis of residual erythrocytes as described previously (7).

Isolation of phagocytic vacuoles. Phagocytic vacuoles were isolated at various times after the addition of the latex particles to the cell suspension as described previously (4). An aliquot of the neutrophil suspension (2.0 ml containing 2 x $10^8$ cells) in RPMI 1640 medium (Flow Labs. Irvine) containing 5 iu/ml heparin was rapidly stirred with a magnetic stirring rod in a thermostatically controlled (37°C) chamber, and the temperature allowed to equilibrate for 2 min. Latex particles (2 x $10^{10}$) coated with human IgG (8) were then added to the chamber. Phagocytosis was stopped at varying intervals by adding this suspension to 50 ml of cold (0°C) Hanks balanced solution (Flow Labs) containing tetrasodium ethylenediaminetetra-acetate (EDTA 1.0 mmol/l, pH 7.4, BDH Chemicals Ltd., Poole, England). The mixture was centrifuged at 75 g for 10 min at 4°C and the pellet was suspended in 50 ml of 11.2% (w/w) sucrose, and re-centrifuged at 75 g for 10 min at 4°C. The pellet was homogenised in 5.0 ml of 11.2% sucrose in a 7.0 ml Dounce homogeniser (Kontes Glass Co., Vineland, U.S.A.) with 100 strokes of a tight-fitting (B) pestle. The homogenate was mixed with 12.0 ml of 60% sucrose and divided into two aliquots. One aliquot (2.0 ml) was retained for determinations of marker enzyme activities. Phagosomes were isolated from the remaining 15.0 ml. The suspension was placed at the base of a 50 ml centrifuge tube (Sorvall), overlayed with 20.0 ml of 33% and then 5.0 ml of 11.2% sucrose and centrifuged at 14,500rpm for 20 min at 4°C in a Sorvall SS3 centrifuge with a 55-34 angle head rotor (g max = 25,359, g min = 13,426). Latex particles collected at the interface between the 11.2% and 33% sucrose and were harvested. The concentration of sucrose was determined with an Abbé refractometer and the sucrose was then diluted to a concentration of 11.2% with cold (4°C) $H_2O$ containing EDTA (1.0 mmol/l) and heparin (5 iu/ml). The suspension of latex particles was centrifuged at 14,500 rpm for 45 min at 4°C in Sorvall SS3 centrifuge and the pellet was suspended in 1.6 ml of 11.2% sucrose. All sucrose solutions contained EDTA (1.0 mmol/l, pH 7.4) and heparin (5 iu/ml).

Assays of Granule Contents. Myeloperoxidase was measured by a modification of the method of Bretz and Baggiolini (9). Just prior to spectrophotometry the assay mixture was mixed with 1 ml of methanol and centrifuged for 2 min at 8,000 g in an Eppendorf 3200 centrifuge to remove latex particles. Horseradish peroxidase (Sigma, type II 195 units/mg) was used as standard. Vitamin $B_{12}$ binding protein was measured as described by Kane & Peters (10). Latex particles were counted microscopically.

Spectroscopy. Dithionite difference spectroscopy was performed as described previously (6). Intact cells were washed and then suspended in Ringers Phosphate buffer (Gibco) immediately prior to examination.

## RESULTS AND DISCUSSION

The methods used in these studies for examining the time dependence of the association of neutrophil components with phagocytic vacuoles have been detailed previously (11). Biochemical and electron microscopic

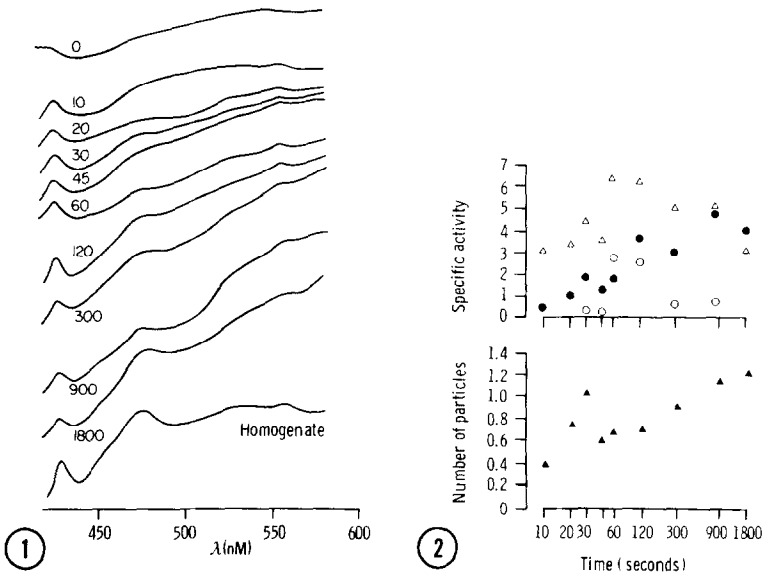


Figure 1. Dithionite reduced versus oxidised spectra of phagocytic vacuoles containing latex particles isolated from human neutrophils by floatation at different times after phagocytosis. The integrated areas under the curves have been normalised to a concentration of 1 x $10^9$ latex particles to correct for variation in the number of vacuoles. The figures associated with each spectrum indicate the time (seconds) after the addition of latex particles at which the cells were cooled and processed. Absorbance scale marker = 0.0028 of full scale.

Figure 2. The time dependence of the association of the cytochrome b Δ, vitamin $B_{12}$-binding protein O, and peroxidatic activity ● with the phagocytic vacuoles. These activities are expressed as a percentage of the activity in the whole homogenate and normalised to a latex concentration of 1 x $10^9$ particles. The numbers of particles isolated at each time interval are also shown▲.

morphological techniques revealed that the IgG opsonised latex particles are rapidly phagocytosed (half saturation time = 7 seconds) and that the specific and azurophil granule contents became associated with the phagosome later, with half saturation times of 69 and 85 seconds respectively.

Reduced minus oxidised spectra of the vacuole preparations showed a pigment with absorption band at 428, 528 and 559nm, characteristic of the cytochrome b, had become associated with the latex particles within ten seconds of the addition of the particles (Figs. 1 and 2). The differential rate of association of the cytochrome b and myeloperoxidase can be clearly seen in the spectra of the phagosomes (Fig.1) in which the 474 nm peak of myeloperoxidase showed a gradual increase in relation to the fairly constant

428 nm peak of the cytochrome b. Assay of peroxidatic activity in the vacuoles showed a similar rate of increase to that of myeloperoxidase assayed spectroscopically (Fig. 2). The activity of the 428 nm peak of the cytochrome b, described as a function of the number of latex particles, was fairly constant over the earlier time intervals and then increased at between 1 and 2 minutes. This increased activity coincided with the entry of the $B_{12}$ binding protein, which is contained in the specific granules (10), into the vacuoles. These data support the results of analytical fractionation studies (6) in which most of the cytochrome b was localised to the plasma membrane, with a second peak of activity that was distributed with the specific granules. This accentuation of the peak height by the specific granules could be due to the location within those of the same, or another, cytochrome b, or some other molecule with an absorbance in the region of 430 nm, resulting in an apparent increase in the height of the superimposed 428 nm cytochrome b peak. Unfortunately the $\alpha$ and $\beta$ peaks of the cytochrome b were not large enough for accurate quantitation and clarification of this point.

Further evidence for the plasma membrane location of the cytochrome b is furnished by the observation that it is rapidly reduced after the addition of dithionite to intact neutrophils (Fig.3). Reduction by sodium dithionite of cytochrome b within membranes may be time dependent (12), indicating that it penetrates membranes slowly. In our studies we observed the reduction of only a small proportion of the myeloperoxidase, which is known to be contained within a second membrane in the azurophil granule. The rapid reduction of myeloperoxidase by dithionite only after membrane permeability was increased by the addition of Triton X-100, indicated that it is relatively inaccessible to dithionite in the intact cell, and also that it is not naturally maintained in the fully reduced state. The addition of a small amount (0.03 mmol/l) of the detergent Triton X-100 influenced the spectra of both the cytochrome b and myeloperoxidase (Fig 3). There was a slight increase in the height of the Soret band of the cytochrome b, which also became sharper, possibly as a result of penetration

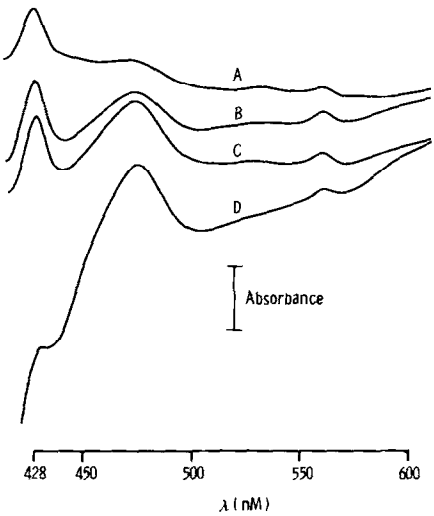


Figure 3. The effect of the addition of Triton X-100 on the development of dithionite difference spectra of intact neutrophils. A. - 2 x $10^7$ cells/ml in Krebs Ringers Phosphate medium ± dithionite. B. - as for A three minutes after the addition of 0.033 mmol/1 Triton X-100. C. B - 3 mins. later. D. 3 mins after the addition of 0.6 mmol/1 Triton X-100. Absorbance scale marker = 0.019 of full scale.

of dithionite into the specific granule. The 474 nm peak of myeloperoxidase showed a marked rise which was more obvious after six than after three minutes. At a higher concentration of Triton (0.60 mmol/1) there was a dramatic increase in the myeloperoxidase spectrum, and the γ peak of the cytochrome b was largely lost in the deep trough produced by myeloperoxidase at that wavelength. This differential reduction upon the addition of dithionite to intact cells greatly simplifies the spectroscopic demonstration of the cytochrome b because of the diminished interference by intracellular substances like myeloperoxidase and eosinophil peroxidase (13).

These studies strongly support the localisation of the cytochrome b in the plasma membrane. This is an apparently ideal situation for the oxidase system as the plasma membrane invaginates to comprise the wall of the phagocytic vacuole where it is interposed between a presumptive substrate in the cytosol and the lumen of the vacuole. The other components of this electron transport system and its precise function require identification.

Acknowledgements

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