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REDUCTION AND SUBSEQUENT OXIDATION OF A CYTOCHROME b OF HUMAN NEUTROPHILS AFTER STIMULATION WITH PHORBOL MYRISTATE ACETATE

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Summary: Difference spectroscopy of human neutrophils stimulated with phorbol myristate acetate as compared with unstimulated cells produced the characteristic spectral changes of the reduced b cytochrome that has recently been described in these cells. The reduction of this cytochrome b is enhanced under anaerobic conditions and is partially reversed upon the reintroduction of air. This reversible oxidation and reduction after stimulation with phorbol myristate provides additional evidence for the role of this cytochrome b as a component of the microbicidal oxidase system.

INTRODUCTION

Neutrophilic polymorphonuclear leukocytes (neutrophils) exhibit a burst of oxygen consumption in association with phagocytosis (1). This respiration does not result from the enhanced consumption of oxygen by mitochondrial respiration (2), and is important for the microbicidal activity of these cells (3). The burst of oxygen consumption is not only initiated by ingestion of a particle, but can also be stimulated by soluble agents. One of the most potent of these soluble stimuli is phorbol myristate acetate (4), an inflammatory component of croton seed oil.

The respiratory burst which is induced by phorbal myristate is thought to involve the same oxidase system as that activated by phagocytosis of a particle. Either stimulus causes the cell to generate superoxide and in Chronic Granulomatous Disease (5), a syndrome characterised by a profound predisposition to infection as a result of malfunction of this oxidase system (6), neither stimulus is effective.

We have recently identified a cytochrome b in human neutrophils (7). It appears to be located predominantly in the plasma membrane (8) and becomes

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incorporated into the phagocytic vacuoles (7). The characteristic spectral absorption of this cytochrome is missing or abnormal in cells from patients with Chronic Granulomatous Disease (9). The present study was conducted to examine human neutrophils for spectroscopic evidence of the involvement of this cytochrome b in the oxidase activity of stimulated cells.

MATERIALS AND METHODS

Separation of neutrophils. Neutrophils were separated from human blood and suspended at a concentration of 5 x 10^7 cells/ml in RPMI 1640 medium (Flow Labs, Irvine) containing heparin (5 iu/ml, Paines and Byrne, Greenford) as described previously (10).

Spectroscopy. Difference spectroscopy was performed in a split beam spectrophotometer as described previously (7). An aliquot of the cell suspension (2.5ml) was placed in each cuvette and the test cell was then stimulated by the addition of phorbol myristate (10 μg in 10 μl of DMSO, Sigma) or 100 μl of latex particles opsonised with human IgG (10). The cuvettes were rendered anaerobic by bubbling with oxygen free argon and the oxygen was reintroduced by bubbling the cuvettes with air, after which they were briskly shaken.

RESULTS

The addition of phorbol myristate to aerobic cells resulted within two minutes on the production of a small peak of absorbance at 428 nm (Fig.1 curve B). Under anaerobic conditions the spectral changes were much more dramatic with the rapid production of much larger peaks at 559, 530 and 428 nm (Fig.1 curve C). These spectral changes were very similar to those produced by the reduction with dithionite of a suspension of neutrophils (Fig 1. curve A) and the purest fraction prepared by analytical subcellular fractionation of a neutrophil homogenate on continuous sucrose gradients (Fig.1 curve D, (8)). The reoxygenation of the cuvette caused a decrease in the height of these peaks, and the appearance of a broad peak between 520 and 580 nm (Fig.1 curves E and F).

Phagocytosis of latex particles resulted in a very different spectrum (Fig.1 curve G). Under aerobic conditions the predominant features were a peak at 415 nm with another peak or shoulder at 444 nm and a broad peak at 560 nm. This pattern was little changed by anaerobiosis where the 444 nm shoulder became less obvious (not shown). The typical spectral changes of the reduced cytochrome b could not be distinguished.

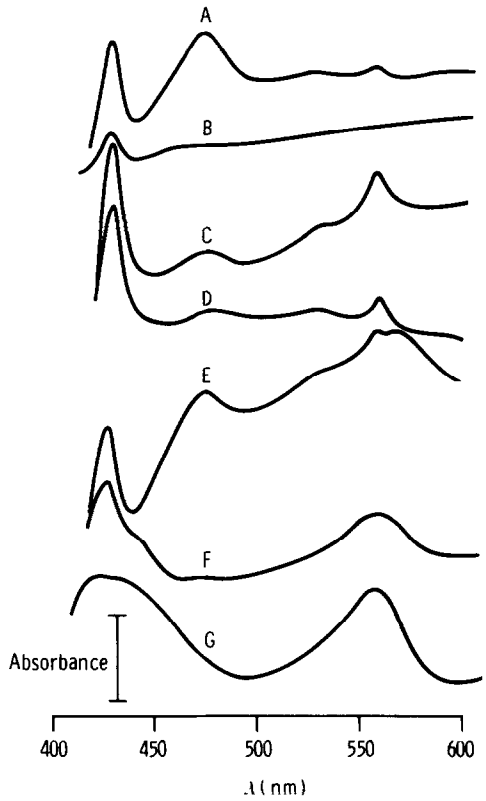

Fig. 1. Difference spectroscopy of purified human neutrophils.

A. Cells + dithionite
B. Aerobic cells ± phorbol myristate acetate (after ~2 min.)
C. Anaerobic cells ± phorbol myristate acetate (after ~2 min.)
D. Dithionite difference spectrum of purest fraction (density = 1.15g/ml) enriched in cytochrome b by analytical subcellular fractionation on continuous sucrose gradients (8).
E. Sample in C after reintroduction of air (~ 5 min after stimulation)
F. E ~ 15 min. after stimulation.
G. Cells ± latex particles (after ~ 2 min.)

Absorbance scale marker = 0.044 of full scale for A and G and 0.022 for B - F.

These spectral changes were not simply a result of the aerobic or anaerobic state per se, as shown by the lack of the production of a similar state in the reference cuvette, or in the test cuvette in the absence of phorbol myristate or latex.

DISCUSSION

Phorbol myristate acetate is a potent stimulus of the neutrophil oxidase system (4). The induction of the reduced state of the b cytochrome of these

cells by phorbol myristate, together with the enhancement of this reduction in anaerobic conditions and reversal upon reoxygenation is strong evidence for the role of this cytochrome as a component of this oxidase system. These data support the finding of absent or abnormal cytochrome b spectra in homogenates of neutrophils from patients with Chronic Granulomatous Disease, implicating defective function of this molecule as the causal lesion in this condition (9).

The spectral changes that are observed after stimulation of the neutrophils with opsonised latex particles are very different from those produced by phorbol myristate (See Fig.1). The main components being a broad peak at 415 nm with a shoulder or secondary peak at 444 nm and a broad peak in the region of 560 nm. It is not surprising that these two stimuli cause different spectral changes because they affect the cell very differently. The latex particle is phagocytosed into a vacuole into which the cytoplasmic granules then discharge their contents, included amongst which is myeloperoxidase. Phorbol myristate, on the otherhand, results in intracytoplasmic lysis of specific granules (11) and selective secretion of specific granule contents (12).

Oxygen radical production by neutrophils stimulated with these two agents is very different (13). Phorbol myristate causes the generation of the superoxide anion whereas cells which have been stimulated with latex particles produce almost pure hydroxyl radicals without any evidence of superoxide. Superoxide radicals are however produced if latex stimulation follows incubation of the cells with sodium azide (13). Under physiological conditions the oxidase system appears to involve the coordinated interaction of a number of components. We are uncertain as to the identity of the compounds responsible for the complex spectral changes observed after the addition of latex to the cells. The characteristic pattern of the cytochrome b was not seen, possibly because it was obscured by other, larger changes. Another possibility is that whereas phorbol myristate is a soluble stimulus which is likely to activate the cytochrome b uniformly throughout the plasma membrane, the latex particles probably only

activate that cytochrome located in the plasma membrane that invaginates to form the wall of the phagocytic vacuole, which is likely to represent only a small proportion of the total complement of the cell.

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REFERENCES

1. BALDRIDGE, C.W. and GERARD, R.W. (1933) AM. J. PHYSIOL. 103, 235-236.
2. SBARRA, A.J. and KARNOVSKY, M.L. (1959) J.BIOL. CHEM. 234, 1355-1362.
3. MANDELL, G.L. (1974) INFECT. IMMUN. 9, 337-341.
4. REPINE, J.E., WHITE, J.G., CLAWSON, C.C. and HOLMES, B.M. (1974) J.LAB. CLIN. MED. 83, 911-920.
5. BERENDES, H., BRIDGES, R.A. and GOOD, R.A. (1957) MINN.MED. 40, 309,312.
6. HOLMES, B., PAGE, A.R. and GOOD, R.A. (1967) J.CLIN. INVEST. 46, 1422-1432.
7. SEGAL, A.W. and JONES, O.T.G. (1978) NATURE 276, 515-517.
8. SEGAL, A.W. and JONES, O.T.G. (1979) BIOCHEM. J. (in press).
9. SEGAL, A.W., JONES, O.T.G., WEBSTER, D. and ALLISON, A.C. (1978) LANCET 2, 446-449.
10. SEGAL, A.W. and COADE, S. (1978) BIOCHEM. BIOPHYS. RES. COMM. 84, 611-617.
11. WHITE, J.G. and ESTENSEN, R.D. (1974) AMER. J. PATHOL. 75, 45-60.
12. ESTENSEN, R.D., WHITE, J.G. and HOLMES, B. (1974) NATURE 248, 347-348.
13. GREEN, M.R., HILL, H.A.O., OKOLOW-ZUBKOWSKA, M.J. and SEGAL, A.W. (1979) FEBS.LETTS. 100, 23-26.