ABSENCE OF CYTOCHROME *b* REDUCTION IN STIMULATED NEUTROPHILS FROM BOTH FEMALE AND MALE PATIENTS WITH CHRONIC GRANULOMATOUS DISEASE

Anthony W. SEGAL and Owen T. G. JONES

Department of Clinical Haematology, University College Hospital Medical School, University Street, London, WC1 and *Department of Biochemistry, University of Bristol, University Walk, Bristol, BS8 1TD, England*

Received 30 November 1979

1. Introduction

We have recently described a cytochrome *b* in human neutrophils [1]. It is located predominantly within the plasma membrane [2,3] and is rapidly incorporated into the phagocytic vacuole [1,3]. We believe that the microbicidal oxidase system of neutrophils contains an electron transport chain in which this cytochrome *b* is a central component. The evidence for the involvement of this cytochrome *b* in the oxidase system is 2-fold. Stimulation of neutrophils with phorbol myristate acetate (PMA) activates the oxidase system [4] and results in the reduction and subsequent oxidation of this cytochrome *b* [5]. This cytochrome *b* was not found in reduced–oxidised spectra of neutrophils from a group of patients with Chronic Granulomatous Disease (CGD) [6], a syndrome in which a predisposition to bacterial infection is associated with the absence of demonstrable oxidase activity in their neutrophils upon stimulation [7]. However, this cytochrome *b* was later found in some patients with CGD, particularly those with an autosomal mode of inheritance [8,9].

This study was conducted to investigate whether neutrophils from patients with CGD, both those with and those without spectroscopic evidence of the cytochrome *b*, demonstrate the normal pattern of reduction of this cytochrome after stimulation of the cells with PMA. It was found that the cytochrome *b* was not reduced in any of these patients. This strongly implicates this electron transport chain in the microbicidal oxidase system. It also confirms that the syndrome of CGD is the result of at least two different molecular aberrations; in some cases the cytochrome *b* is missing, in others it fails to become reduced upon stimulation of the cell, either because of the absence of a proximal component of the electron transport chain or because of the failure of some activation mechanism.

2. Patients, materials and methods

2.1. *Patients*

Four patients with CGD were studied. Patient 1 was a 30 year old female. There was no history of illnesses in her parents or 3 month old child. Patients 2–4, were a female and two males (respectively, patients U, Q and G in [6]). The diagnosis of CGD was confirmed in all these patients on the basis of a clinical history of pyogenic infection [7], defective killing of Staphylococci [10] impaired reduction of nitroblue-tetrazolium [11] by their neutrophils, and absence of the normal enhancement of oxygen consumption after stimulation of these cells with IgG opsonised latex particles [12] and PMA [4]. Informed consent was obtained from all the subjects.

2.2. *Isolation of neutrophils*

Venous blood (100 ml from patients 1–3 and 4 normal healthy controls, 50 ml from patient 4) was taken into heparin (5 IU/ml preservative free, Paynes and Byrne, Greenford, Middlesex). Neutrophils were isolated by dextran sedimentation, centrifugation through Ficoll/sodium metrizoate, and hypotonic lysis of residual erythrocytes [13]. The cells were resuspended in 20 ml Krebs-Ringers phosphate buffer containing 5 IU heparin/ml. The purity of the neutrophils was >95% in cells obtained from the normal controls. Those from the patients were contaminated with eosinophils [9] which constituted 9, 15, 11 and 5% of the cells in patients number 1–4, respectively.

2.3. *Spectroscopy*

A sensitive split-beam spectrophotometer [14] was used. Reduced–oxidised spectra were recorded after the addition of a few crumbs of dithionite to one of the cuvettes, each of which contained 2.5 ml cell suspension. The protein concentration was estimated in the unreduced samples by the method in [15].

The effect of stimulation of the cells with PMA was studied after the addition of 10 μg PMA in 10 μl dimethyl sulphoxide to 2.5 ml cell suspension after both the test and reference cells had been gassed with argon [5]. In some studies superoxide dismutase (SOD, 100 μg 2950 IU/mg, Sigma, from bovine erythrocytes) was added to both the reference and the test cuvette before the addition of PMA.

The effect of an $O_2^{\cdot-}$ generating system on the spectra of intact cells was examined by the addition of xanthine oxidase (10 μl 13.2 IU/ml Sigma, prepared from buttermilk) to aerobic cell suspensions in both cuvettes in the presence of hypoxanthine (10^{-3} M, Sigma) in the test cuvette. The generation of $O_2^{\cdot-}$ by this system was tested by the addition of cytochrome *c* (0.15×10^{-3} M, from horse heart, type III, Sigma) to the cell suspensions in both cuvettes, in the presence and absence of SOD (100 μg) and found to be 12.5 μmol/min taking $\epsilon = 21.1\ M^{-3}.cm^{-1}$ for cytochrome *c* at 550 nm [16].

3. Results

Reduced–oxidised spectra of intact cells from normal subjects (fig.1) clearly demonstrate the α, β and γ (or Soret) absorption bands of a cytochrome *b*, with minimal interference by myeloperoxidase or eosinophil peroxidase [9]. Similar spectra were observed with cells from the two female patients (1,2). The spectra obtained with cells from the male patients were markedly different. Both these patients had a broad peak of absorbance with maximal absorbance at 431 and 435 nm in patients 3 and 4, respectively, without evidence of the α and β peaks of a cytochrome *b*. No evidence of the cytochrome *b* was produced by oxidation of the reference cell with potassium ferricyanide.

Phorbol stimulation resulted in the reduction of the cytochrome *b* of the control cells as in fig.2 of [5]. However, in none of the patients with CGD was the spectrum of the reduced cytochrome *b* observed (fig.2). In these patients the major changes were the development of troughs at ~422 and 444 nm with broad peaks at ~433 and 463 nm.

The addition of SOD did not inhibit the PMA-induced reduction of the cytochrome *b* in normal cells, and the xanthine/xanthine oxidase $O_2^{\cdot-}$ generating system failed to reduce the cytochrome *b* in neutrophils from the normal controls.

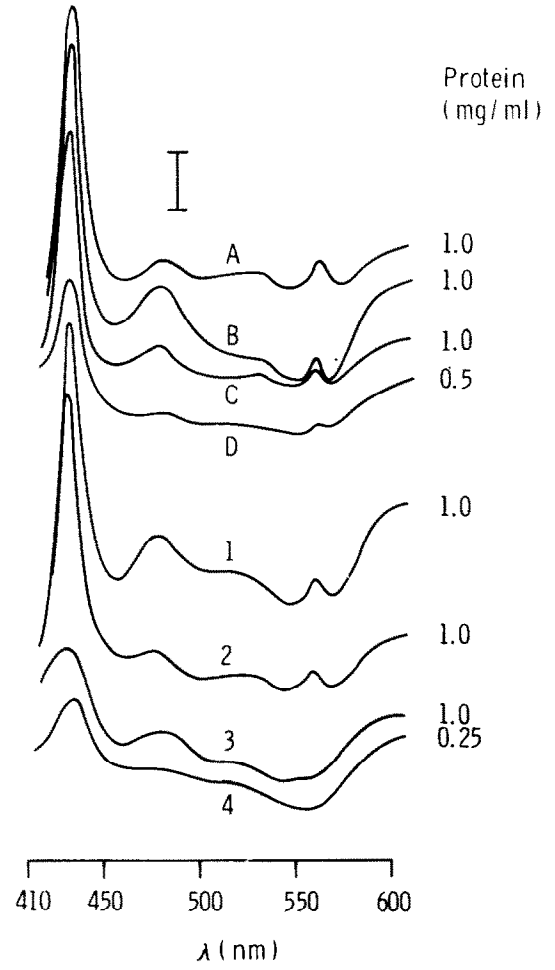

Fig.1. Dithionite difference spectra of intact neutrophils from 4 normal subjects (A–D), two female patients (1,2) and two male patients (3,4) with Chronic Granulomatous Disease. The peaks of the Soret bands were at 428–429 nm for the normal subjects and female patients, 431 nm for patient 3 and 435 nm for patient 4. The integrated areas under the peaks in the region of 430 nm have been normalised to 1 mg protein/ml, or proportions thereof. The original protein concentrations are shown in fig.2. Absorbance scale marker = 0.008 absorbance units.

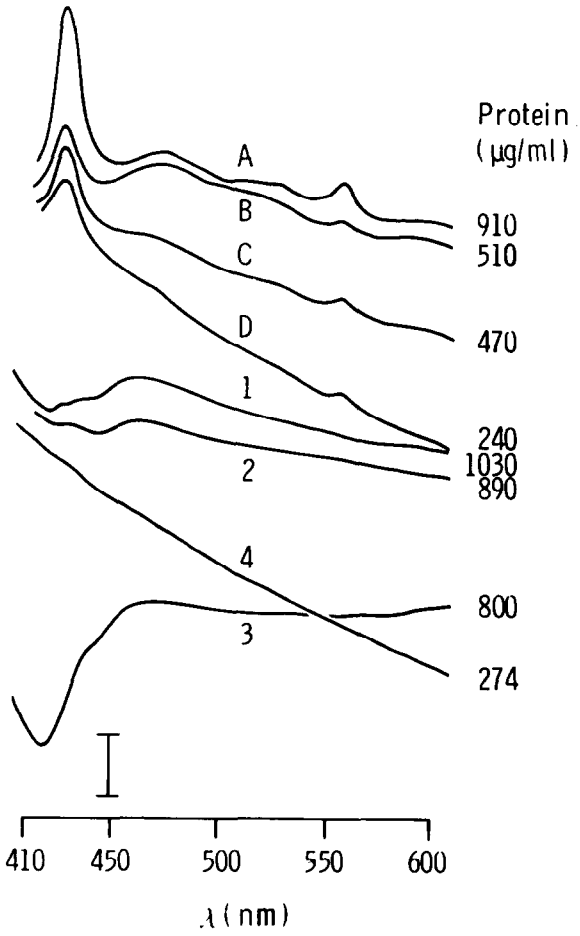

Fig.2. Difference spectra of neutrophils stimulated with phorbol myristate acetate compared with unstimulated cells. Spectra were run under anaerobic conditions 3 min after stimulation. Symbols refer to the same cell preparations as do those in fig.1. Absorbance scale marker = 0.008 absorbance units. The protein concentration of the cell suspensions has been measured as an index of the concentration of cells.

4. Discussion

These results indicate that neutrophils from patients with CGD, unlike controls, fail to reduce a plasma membrane-associated cytochrome *b* after stimulation of these cells with PMA. In the two male patients, failure of reduction of this cytochrome *b* appears to be attributable to the absence or primary abnormality of this molecule, whereas the cells from the female patients failed to reduce this cytochrome *b*, although a normal cytochrome *b* spectrum was found upon reduction with dithionite. We have proposed that the microbicidal oxidase system consists of a number of components which are integrated into a complex electron-transport chain [9]. In this case the failure of reduction of the cytochrome *b* in the cells of the female patients could be due to the absence of a proximal component of this electron transport chain, or to a defect in the mechanisms which activate or co-ordinate the system.

A possible explanation for the failure of the reduction of the cytochrome *b* in the patients with CGD was that this reduction is mediated by, rather than being responsible for, the production of superoxide. This does not appear to be the case because: phorbol stimulation was conducted under anaerobic conditions, although it is difficult to ensure complex anaerobiosis; the addition of superoxide dismutase did not inhibit cytochrome *b* reduction; and the addition of an $O_2^{\cdot-}$ generating system to intact cells failed to reduce this cytochrome.

The reduction of intact, rather than disrupted, cells with dithionite greatly improves the spectroscopic demonstration of the cytochrome *b* because this reducing agent penetrates membranes slowly [17], and thus it reduces the cytochrome *b* which is in the plasma membranes [2,3] more readily than components within the cytoplasmic granules. The major contribution of myeloperoxidase and eosinophil peroxidase to the spectral changes observed in reduced–oxidised spectra [9] can be avoided with this technique. Under these conditions we have observed broad absorption bands with peaks in the region of 430–436 nm in both male patients with CGD. This could be due to contaminating haemoglobin, which also absorbs in this region, but we have found that the spectral changes due to haemoglobin are different from those observed in these patients. The spectral changes in this region could indicate the presence of another redox compound. Similarly, failure of the normal reduction of the Soret band of the cytochrome *b* upon stimulation with PMA, permits a variety of spectral changes at 400–470 nm to be observed in cells from patients with CGD, which would be obscured in cells from controls. These spectral changes could result from redox changes in proximal components of an electron transport chain; e.g., the broad trough at ~444 nm would be consistent with reduction of a flavoprotein [18].

Failure of reduction of the cytochrome *b* in neutrophils from patients with CGD after stimulation with PMA, despite the demonstration of this mole-

cule in the cells from two of them, is further evidence that this cytochrome *b* is a component of the normal microbicidal oxidase system. Dysfunction of complex electron-transport systems as a whole due to different abnormalities of individual components is well known in bacterial mutants [19].

Acknowledgements

We thank the Wellcome Trust and Medical Research Council for financial support and Professors M. Greaves and J. Soothill and Dr D. Webster for allowing us to study patients under their care.

References

[1] Segal, A. W. and Jones, O. T. G. (1978) Nature 276, 515.
[2] Segal, A. W. and Jones, O. T. G. (1979) Biochem. J. 180, 33.
[3] Segal, A. W. and Jones, O. T. G. (1980) submitted.
[4] Repine, J. E., White, J. G., Clawson, C. C. and Holmes, B. M. (1974) J. Lab. Clin. Med. 83, 911.
[5] Segal, A. W. and Jones, O. T. G. (1979) Biochem. Biophys. Res. Commun. 88, 130.
[6] Segal, A. W., Jones, O. T. G., Webster, D. and Allison, A. C. (1978) Lancet ii, 446.
[7] Holmes, B., Page, A. R. and Good, R. A. (1967) J. Clin. Invest. 46, 1422.
[8] Borregaard, N., Staehr-Johansen, K., Taudorff, E. and Wandall, J. H. (1979) Lancet i, 949.
[9] Segal, A. W. and Jones, O. T. G. (1979) i, 1036.
[10] Quie, P. G., White, J. G., Holmes, B. and Good, R. A. (1967) J. Clin. Invest. 46, 668.
[11] Segal, A. W. and Peters, T. J. (1975) Clin. Sci. Mol. Med. 49, 591.
[12] Segal, A. W. and Coade, S. B. (1978) Biochem. Biophys. Res. Commun. 84, 611.
[13] Segal, A. W. and Peters, T. J. (1977) Clin. Sci. Mol. Med. 52, 429.
[14] Jones, O. T. G. and Saunders, V. A. (1972) Biochim. Biophys. Acta 275, 427.
[15] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265.
[16] Lemberg, R. and Cutler, M. E. (1970) Biochim. Biophys. Acta 197, 1.
[17] Bendall, D. S., Davenport, H. E. and Hill, R. (1971) Methods Enzymol. 23a, 327.
[18] Beinert, H. (1966) in: Biological Chemistry (Mahler, H. R. and Cordes, E. H. eds) p. 574, Harper Row, New York.
[19] Haddock, B. A. and Jones, C. W. (1977) Bacteriol. Rev. 41, 47.