Induction of synthesis of components of the hydrogen peroxide-generating oxidase during activation of the human monocytic cell line U937 by interferon- γ

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Increased amounts of cytochrome b_{-245} and a 45 kDa polypeptide component of the hydrogen peroxide-generating oxidase were detected in the human monocytic cell line U937 after incubation in interferon- γ . The time course of increase in cytochrome b_{-245} paralleled the induction of oxidase activity. Induction of the 45 kDa component had a different time course, having a greater lag before increased amounts were seen.

INTRODUCTION

Macrophages can be activated by interferon- γ to kill micro-organisms (Murray *et al.*, 1983; Nathan *et al.*, 1983; Byrne *et al.*, 1986) and tumour cells (Johnson *et al.*, 1983). Such activation of macrophages is an essential facet of immunity to intracellular parasites and tumours. Enhanced antimicrobial and antitumour activity has been shown to be dependent on an increased capacity of activated macrophages to produce toxic forms of oxygen, particularly H_2O_2 (Adams & Nathan, 1983; Lowrie, 1983; Murray, 1983).

The macrophage produces toxic forms of oxygen by the activity of an NAD(P)H oxidase similar to that of neutrophils. The oxidase contains FAD protein and a low-potential cytochrome b_{-245} (Berton *et al.*, 1985; Cross *et al.*, 1983, 1985). Recently, the polypeptide component of the flavoprotein has been identified by binding studies with the specific oxidase inhibitor diphenylene iodonium (DPI) (Cross & Jones, 1986; Hancock & Jones, 1987). In neutrophils and macrophages it is a protein of M_r 45000. DPI bound a 45 kDa polypeptide in macrophage homogenates and in solubilized oxidase from neutrophils. It specifically inhibited the respiratory burst in intact neutrophils and macrophages, with little effect on mitochondrial respiration. It was also a strong inhibitor of the solubilized oxidase, preventing the reduction of the flavin and cytochrome components (Cross & Jones, 1986).

An important question about macrophage activation is how the oxidase is modulated to enhance its capacity to reduce oxygen. We report here that, as a result of activation with interferon- γ , the mononuclear phagocytic cell line U937 has increased amounts of cytochrome b_{-245} and a DPI-binding polypeptide of M_r 45000. In addition, we compare the time course of changes in the levels of these proteins with the time course of induction of the oxidase after interferon- γ treatment.

MATERIALS AND METHODS

Chemicals

Recombinant-DNA-derived interferon- γ was a gift from Boehringer Ingelheim (Vienna, Austria). DPI was synthesized as described by Cross & Jones (1986).

Cell culture

The cell line U937 was maintained by twice-weekly passage in RPMI 1640 medium containing 2 mM-glutamine and 10% (v/v) heat-inactivated newborn-calf serum. For activation, cells were adjusted to a concentration of 1×10^6 /ml and treated with recombinant-DNA-derived human interferon- γ (100 units/ml) (produced by Genentech, San Francisco, CA, U.S.A.) as appropriate. Fresh interferon was given every 24 h.

Determination of oxidase activity

Oxidase activity was quantified by release of H_2O_2 after stimulation of cells with phorbol myristate acetate (1 μ g/ml), and the number of cells was determined by measurement of DNA as described previously (Jackett *et al.*, 1981).

Determination of oxidase components

At least 2×10^7 cells were collected into Hanks balanced salts solution containing (per ml) 5 μ g of phenylmethanesulphonyl fluoride, 1 μ g of chymostatin, 1 μ g of leupeptin, 1 μ g of antipain and 1 μ g of pepstatin, then disrupted by sonication (2 $\times$ 15 s at 50W).

Cytochrome b levels were measured spectrophotometrically as described previously (Cross *et al.*, 1984). The contribution of low-potential cytochrome b to the spectra obtained was distinguished, by potentiometry, from that of mitochondrial cytochromes as described elsewhere (Hancock & Jones, 1987). Potentiometric titrations were done as described previously (Cross *et al.*, 1981).

The 45 kDa polypeptide component was assayed by incubation of the homogenate with [125 I]DPI, followed by SDS/polyacrylamide-gel electrophoresis and subsequent autoradiography as described by Cross & Jones (1986).

RESULTS

The time course of changes in oxidase activity and in levels of oxidase components was studied in the cell line U937 treated with recombinant-DNA-derived human interferon- γ . Induction of oxidase activity occurred only after a lag of at least 8 h after the addition of interferon- γ , but subsequently increased over the following 40 h (Fig. 1a). Cells maintained in the absence of interferon showed no increase in oxidase activity.

Increased levels of low-potential cytochrome *b* within U937 closely paralleled increased oxidase activity (Fig. 1b). There was a lag of at least 8 h after interferon treatment before both increased to reach peak levels at 48 h. Amounts of cytochrome *b*₋₂₄₅ were only slightly changed in untreated cells over 72 h. The midpoint oxidation-reduction potential of the cytochrome *b* was determined as -245 mV at pH 7.0 by potentiometric titration.

Induction of the 45 kDa oxidase component showed different kinetics (Fig. 1c). A small increase in 45 kDa protein was seen in the first 24 h of interferon- γ treatment, which also was seen in untreated cells, followed by a period in which increased amounts of this protein were detected in homogenates of interferon-treated cells, which continued up to 72 h after commencement of the experiment.

DISCUSSION

Activation of macrophages is a complex process, but our data show that one effect of interferon- γ is to increase the amounts of two components of the NAD(P)H oxidase. Because cytochrome *b*₋₂₄₅ levels

parallel oxidase activity in these cells, we can speculate that levels of cytochrome *b*₋₂₄₅ are one of the initial factors limiting the rate of induction of the oxidase. The effect on the 45 kDa protein is more complex. Increase in the amount of this protein only becomes apparent after induction of the oxidase has begun. One explanation for this could be that U937 cells constitutively contain sufficient of the 45 kDa component to achieve a partially activated state by recruitment of these molecules to the site of the oxidase. However, to attain a fully activated oxidase under prolonged interferon- γ treatment, the cell must begin to produce additional 45 kDa protein.

Our results with cytochrome *b* differ from those of Berton *et al.* (1986) and Tsunawaki & Nathan (1984), who could not correlate oxidase activity with low-potential cytochrome *b* content. Our hypothesis of synthesis *de novo* and rearrangement of existing molecules could explain the difference between our results and those of Berton *et al.* (1986), who reported that mouse peritoneal macrophages activated *in vivo* with *Corynebacterium parvum* had no more cytochrome *b*₋₂₄₅ than did resident macrophages, but had more molecules of cytochrome *b*₋₂₄₅ at the oxidase site. Others have shown that macrophages can be activated to different degrees of H₂O₂ production and antimicrobial activity. *C. parvum*-activated macrophages represent an intermediate state (Murray & Cohn, 1980). *C. parvum* activation may only trigger a translocation of components of the oxidase without initiating synthesis *de novo*; this is sufficient to achieve some, but not complete, oxidase induction. In contrast, cells treated with interferon- γ are destined to be more activated and therefore synthesis of cytochrome *b*₋₂₄₅ begins.

It is more difficult to comment on the data of Tsunawaki & Nathan (1984). They reported only levels of total cytochrome *b* within mouse macrophages. They did not discriminate between high- and low-potential cytochrome *b*. Mononuclear phagocytes are rich in mitochondrial cytochrome *b*. Therefore only by poten-

Fig. 1. Induction of oxidase activity and synthesis of its components during macrophage activation by interferon- γ

(a) Oxidase activity, quantified by release of H₂O₂, (b) cytochrome *b* levels, and (c) levels of the 45 kDa polypeptide component were measured as described in the Materials and methods section. ●, Interferon- γ added; ○, cells maintained in the absence of interferon- γ .

tiometry, as done by ourselves and Berton's group (Berton *et al.*, 1985, 1986), is it possible to accurately determine changes in low-potential cytochrome *b*.

An alternative explanation for these differences is less complex. Maybe it is simply that the mechanism of activation of human and murine macrophages is different.

Whatever the exact mechanism of oxidase activation, the sequential synthesis of the two components of the oxidase after interferon- γ treatment provides an opportunity for studying, at the gene level, the immune regulation of macrophage antimicrobial function.

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