The subcellular localization of ubiquinone in human neutrophils

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Ubiquinone-10 has recently been proposed as a component of the microbicidal oxidase system of neutrophil leukocytes [Crawford & Schneider (1982) *J. Biol. Chem.* **257**, 6662–6668]. We have determined the subcellular localization of ubiquinone and could detect it only in the mitochondrial fractions. It was not enriched in phagolysosomes which were enriched in $O_2^{\cdot-}$ generating activity and cytochrome b_{-245} . It is proposed that ubiquinone is unlikely to play a direct role in the electron transport chain located in the plasma membrane which functions to produce microbicidal oxygen radicals.

Neutrophil polymorphonuclear phagocytes (neutrophils) exhibit an increased consumption of oxygen when phagocytosing microbes or following stimulation by a number of soluble agents. The products of this increased oxygen uptake, reduced oxygen radicals such as superoxide ($O_2^{\cdot-}$), peroxide (O_2^{2-}) and hydroxyl radicals ($OH^{\cdot}$), are not of mitochondrial origin, but rather of an electron transport chain located in the plasma membrane. The components of this electron transport chain have been suggested to be a flavin-containing dehydrogenase (Gabig & Babior, 1979; Light *et al.*, 1981; Gabig, 1983) and a low-potential b -type cytochrome (Segal & Jones, 1978; Cross *et al.*, 1981) which is associated with FAD in a ratio 1:1 in the plasma membrane and in a 110-fold purification of the cytochrome (Cross *et al.*, 1982*b*). This low-potential cytochrome b ($E_{m,7.0} = -245$ mV) has a dual location in the plasma membrane and in the specific granule membrane which fuse to form the phagolysosome (Segal & Jones, 1979).

In addition to these components it has been suggested that a quinone may be involved in this radical-generating system (Millard *et al.*, 1979; Crawford & Schneider, 1981; Sloan *et al.*, 1981), later identified as ubiquinone-10 (Crawford & Schneider, 1982; Cunningham *et al.*, 1982) which was suggested not to be mitochondrial in origin. These workers have also suggested that phagolysosomes isolated from neutrophils after ingestion of latex beads are enriched in ubiquinone content (Crawford & Schneider, 1983). To investigate the possible role of ubiquinone in this microbicidal oxidase system we have determined the subcellular localization of ubiquinone by linear sucrose density gradient fractionation of neutrophil homogenates

and the localization of ubiquinone after the phagocytosis of immunoglobulin-coated latex beads.

Methods

Preparation of human neutrophils and neutrophil homogenates

These methods have been fully described in Segal & Jones (1979). Neutrophils were 98% pure.

Analytical gradient fractionation and assay of marker enzymes

Methods were as described in Segal & Jones (1979) with the following modification. Density gradients were constructed by forming a discontinuous gradient consisting of 2 ml each of 55% and 60% sucrose; 4 ml of 50% sucrose; 5 ml each of 45%, 40% and 35% sucrose; 3 ml of 30% sucrose; 2 ml each of 25% and 20% sucrose (all w/w). The gradient was left to diffuse for 3 h at room temperature to give a gradient shape which was shallow and linear in the centre, but steeper at high and low densities to give maximum separation of plasma membrane and granule fractions. Each gradient was overlaid with 4.5 ml of homogenate, equivalent fractions from four gradients being pooled after centrifugation overnight in a 6 × 36 ml Sorvall swinging bucket rotor (AH 627) at 25 000 rev./min (83 100 *g*).

Cytochrome b_{-245} was assayed by reduced minus oxidized difference spectroscopy using $\Delta\epsilon_{559-540} = 21.6 \text{ cm}^{-1} \cdot \text{mM}^{-1}$ (Cross *et al.*, 1982*a*). Cytochrome oxidase was assayed both by the rate of oxidation of reduced cytochrome c (Cooperstein & Lazerow, 1951) and by reduced minus oxidized difference spectroscopy using $\Delta\epsilon_{605-630} = 24 \text{ cm}^{-1} \cdot \text{mM}^{-1}$ (Van Gelder, 1966).

Malate dehydrogenase was assayed by the method of Sottocasa *et al.* (1967).

Preparation of latex phagolysosomes

Neutrophils (7.6×10^6) were incubated with or without 7×10^{10} latex particles ($0.8 \mu\text{m}$) in 15 ml of RPMI medium containing 10 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] and 5 units of heparin/ml, at 37°C for 5 min. Ice-cold saline (15 ml) was added, the cells were pelleted by centrifugation (400g, 4 min), washed with 15 ml of 11.5% (w/w) sucrose containing 1 mM-EDTA and 5 units of heparin/ml (SVH medium) and resuspended in a volume of 5 ml. Cells were disrupted with 100 strokes of a tight-fitting pestle in a Dounce homogenizer, and 12 ml of 60% (w/w) SVH medium was then added [final sucrose concn. 46% (w/w)]. A discontinuous sucrose gradient was constructed consisting of 2 ml of 60% SVH, 2 ml of 50% SVH, 13 ml of homogenate, 6.5 ml of 35% SVH, 6.5 ml of 20% SVH, and 2 ml of 11.5% SVH (all w/w). The gradient was centrifuged at 55000g for 45 min and five fractions were collected. Under these conditions the latex phagolysosomes appear in fraction 2. The fractions were assayed for cytochrome *b* and ubiquinone-10 as described above, and for superoxide dismutase-sensitive NADPH-cytochrome *c* reductase activity as described by Cross *et al.* (1982a).

Extraction of quinone from subcellular preparations

Light petroleum extractions of quinone from fractions were performed as described by Redfearn (1967). Efficiency of extraction was checked by extraction of ubiquinone from rat liver mitochondria, which contain ubiquinones 9 and 10 (Olsen & Dialameh, 1960).

Determination of ubiquinone-10

Ubiquinone-10 was estimated in extracts of homogenates and in standards by oxidized minus reduced difference spectroscopy using $\Delta\epsilon_{275}$ of $12.25 \text{ cm}^{-1} \cdot \text{mm}^{-1}$ (Redfearn, 1967). Ubiquinone extracted from homogenates and subcellular fractions was estimated by u.v. absorption at 275 nm (or at 290 nm after borohydride reduction) after purification using reverse-phase performance liquid chromatography (h.p.l.c.) on a 200 mm Apex-ODS silica column ($5 \mu\text{m}$) eluted with acetonitrile/diethyl ether (4:1, v/v) using ubiquinone-10 as a standard (a gift from Dr. W. T. Griffiths, Biochemistry Department, University of Bristol).

Results and discussion

Isolation and identification of ubiquinone-10 from neutrophil homogenates

Light petroleum extraction of neutrophil homogenates yielded a quinone which had spectral and

chromatographic properties identical with those of authentic ubiquinone-10. The neutrophil homogenate contained 25.5 pmol of ubiquinone-10 per mg of protein.

Subcellular fractionation

The markers of subcellular organelles were well resolved in analytical subcellular fractionations (Fig. 1). There were two major peaks of cytochrome *b*₂₄₅ associated with the plasma membrane and specific granule fractions, as described previously (Segal & Jones, 1979), neither of which was associated with the mitochondrial markers cytochrome oxidase or malate dehydrogenase. Assay of the subcellular distribution of cytochrome oxidase by reduced cytochrome *c* oxidation activity or by absorbance in reduced minus oxidized difference spectra put the peak of activity in fraction 14. This was also the peak of malate dehydrogenase activity. The peak of distribution of ubiquinone exactly corresponded with the peak of distribution of cytochrome oxidase and malate dehydrogenase. The small peak of activity at the bottom of the gradient of the mitochondrial markers, cytochrome *b* and ubiquinone, was almost certainly due to a small quantity of aggregated material which had not been removed during the low speed centrifugation of the cell homogenate prior to loading on the gradient (Segal & Jones, 1979). No such peak was seen in another gradient (results not shown).

Neutrophils have relatively few mitochondria (20–30 per cell; Kirschner *et al.*, 1972) and the quantity of cytochrome oxidase we have found (2.14 pmol/mg of cell protein) is quite compatible with this amount, and with the concentration of mitochondrial cytochromes found in redox titrations (Cross *et al.*, 1981). Moreover the ratio of ubiquinone to cytochrome oxidase, 11.9:1, is in the range typically found in mitochondria (10.9–16.4; Szarkowska & Klingenberg, 1963) using the absorption coefficient for cytochrome oxidase of $\Delta\epsilon_{605-630} = 24 \text{ cm}^{-1} \cdot \text{mm}^{-1}$. This method is more reliable than calculations made from turnover numbers as used by Cunningham *et al.* (1982).

The results of the latex phagolysosome isolation are shown in Fig. 2. As expected, the bulk of the superoxide generation activity was recovered in fraction 2, which contained the phagolysosomes in the latex immunoglobulin G-coated treated cells. In this fraction there was a 5.6-fold enrichment in cytochrome *b*₂₄₅ but only a 1.08-fold enrichment of ubiquinone compared with the fraction obtained from cells which were not incubated with latex beads.

The quantity of ubiquinone found in neutrophils which we report here, 25.5 pmol/mg of cell protein, is less than that reported by previous authors [60–430 pmol/mg of cell protein (Millard *et al.*,

Fig. 1. Analytical subcellular fractionation of human neutrophil homogenates in sucrose gradients

The activities of the fractions were assayed as described in the Methods section and in Segal & Jones (1979). Mitochondrial markers were cytochrome oxidase (solid line) and malate dehydrogenase (broken line). The recovery of ubiquinone was 97.8%.

1979; Sloan *et al.*, 1981; Crawford & Schneider, 1981, 1982; Cunningham *et al.*, 1982)], whereas the quantity of cytochrome *b*₋₂₄₅ which we have found is greater (Cross *et al.*, 1981, 1982*a,b*) than that of the previous authors. This may be the result of differences of purity of neutrophil preparations; even minor contamination with other white blood cells which contain more mitochondria will significantly increase the quantity of ubiquinone. The purity of neutrophils used in these experiments was >98% as determined by microscopy.

We feel that the use of the same preparation of neutrophils to determine possible enrichment of ubiquinone in latex phagolysosomes compared with

the equivalent 'unfed' subcellular fraction is more reliable than using two separate neutrophil preparations which may differ in mitochondrial content due to purity or source. This may explain why we find no enrichment of ubiquinone in phagolysosomes, in contrast with Crawford & Schneider (1983) who compared the ubiquinone content of a latex phagolysosome preparation with the ubiquinone content of a separate preparation of neutrophils.

The subcellular localization of ubiquinone with the mitochondrial fraction and its non-incorporation into phagosomes suggest that it is unlikely that it is involved in the microbicidal oxidase system of

Fig. 2. Enrichment of superoxide-generating activity, cytochrome b_{-245} and ubiquinone in latex phagolysosomes. Phagolysosomes were prepared and activities measured as described in the Methods section. Under these conditions the phagolysosomes are isolated from fraction 2. Enrichment is expressed as activity in fractions from neutrophils 'fed' with latex beads over the corresponding fraction from 'unfed' neutrophils.

neutrophils, in keeping with thermodynamic considerations. The midpoint potential of ubiquinone ($E_{m,7.0} = +65$ mV; Urban & Klingenberg, 1969) even when in association with specific binding proteins (Rutherford & Evans, 1980), is much higher than that of superoxide ($E_0 = -330$ mV, $E_{m,7.0} = 160$ mV; Wood, 1974) or the other redox components of the neutrophil superoxide-generating system, a flavoprotein with $E_{m,7.0}$ (FAD/FADH $^+$) = -200 mV and $E_{m,7.0}$ (FADH $^+$ /FADH $_2$) = -280 mV (A. R. Cross, O. T. G. Jones, R. Garcia & A. W. Segal, unpublished work) and cytochrome b , $E_{m,7.0} = -245$ mV (Cross *et al.*, 1981).

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