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IDENTIFICATION AND QUANTITATION OF ELECTRON-TRANSPORT COMPONENTS IN HUMAN POLYMORPHONUCLEAR NEUTROPHILS

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Using dithionite difference spectra we have detected cytochrome *b* in highly purified human neutrophils at a concentration of 0.08 nmol/mg protein. The presence of quinone was identified in lipid extracts at a concentration of approx. 0.06 nmol/mg protein. It was identified as ubiquinone-10 by mass spectrographic analysis. Simultaneous measurements of cytochrome oxidase indicated that these compounds could not be attributed to mitochondrial contamination. These results are compatible with the hypothesis that initiation of the respiratory burst in human neutrophils involves a multicomponent electron-transport system.

Introduction

During the process of phagocytosis, human neutrophils undergo marked alterations in oxidative metabolism. These changes, which are collectively referred to as the respiratory burst [1], are characterized by increased oxygen consumption, increased glucose utilization via the hexose monophosphate shunt and the generation of superoxide anion and hydrogen peroxide. The mechanism of initiation of the respiratory burst is not clear, but attention has focused on activation of a reduced pyridine nucleotide oxidase which preferentially utilizes NADPH [1–3]. Such an enzyme has been solubilized [4] and shown to possess a requirement for FAD [5].

More recently, Segal et al. [6] have reported the presence of a unique cytochrome *b* in the neutrophil which appears to undergo reduction upon stimulation of the cell [7]. Furthermore, Sloan et al. [8] have described the presence of a quinone in the neutrophil and postulated a role for this substance in the initiation of the respiratory burst. These recent observations have led to the suggestion that the respiratory burst involves a multicomponent electron-transport chain rather than a single enzyme. The present experiments were devised to provide independent verification and quantitation of these electron-transport components in human neutrophils.

Methods

Extraction of ubiquinone from cells. Neutrophils were isolated from 1 unit of heparinized venous blood, purified to 98% purity by sequential plasma gel sedimentation and Ficoll-Hypaque centrifugation and pelleted by centrifugation at $280 \times g$. The pellet was resuspended in isotonic saline (0.85%) at a protein concentration of approx. 10 mg/ml. The average number of cells extracted was $1.8 \cdot 10^9$. The cell suspension was homogenized in a glass-glass homogenizer; a 0.1-ml sample removed for protein determination and the re-

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mainder transferred to a 250-ml Erlenmeyer flask equipped with a Teflon-lined screw cap. For each volume of cell suspension, 4 vol. of HPLC grade methanol were added. The flask was flushed with N_2, sealed and extracted for 1 h at room temperature in a controlled-environment incubator shaker (New Brunswick Scientific Co.) at an rpm setting of 300. After 1 h, 6 vol. of petroleum ether were added, the flask flushed with N_2, resealed and extracted in the shaker overnight at the same shaker speed. The extraction mixture was centrifuged in an International Model V centrifuge (2400 rpm, Model 259 rotor) for 15 min. The petroleum ether layer was removed and stored under an N_2 atmosphere in the dark. The aqueous layer was reextracted twice with 4 vol. of petroleum ether for one-half hour. The petroleum ether layers from all three extractions were combined, evaporated to dryness under N_2 and redissolved in 2.5 ml petroleum ether/ethanol/H_2O (90 : 9.5 : 0.5).

Spectrophotometric analysis. An oxidized-reduced spectrum was recorded using a Cary 219 spectrophotometer over the range 225–350 nm with a full scale of 0–0.2 absorbance units, and a slit width of 0.5 nm. Samples (1.0 ml) were placed in two matched cuvettes. One sample was reduced with a small amount of $NaBH_4$ and placed in the reference chamber. The other sample was placed in the sample chamber and the spectrum determined. To assure that the sample in the sample chamber was completely oxidized, 2 μl of 5% alcoholic KOH were added and the oxidized-reduced spectrum was measured again. The quinone demonstrated a peak at 275 nm. The concentration of ubiquinone was determined using an ϵ_{275} (oxidized − reduced) of 12 500 $M^{-1} \cdot cm^{-1}$, which is an extinction coefficient for ubiquinone-10.

Purification for mass spectrometry. The two 1-ml samples above were recombined with the remaining 0.5-ml sample and the total was washed by vortex mixing with an equal volume of deionized water to remove the $NaBH_4$ and KOH. The phases were separated by centrifugation in a desk-top IEC clinical centrifuge. The petroleum ether phase was removed and streaked on a silica gel H plate previously washed with $CHCl_3$. The ubiquinone was separated by thin-layer chromatography in a ligroine/diethyl ether/formic acid (90 : 60 : 6) system. The area of the plate corresponding to the ubiquinone standard was scraped and the ubiquinone eluted with two 5-ml aliquots of diethyl ether. The diethyl ether was evaporated under N_2 and the ubiquinone redissolved in 3.0 ml petroleum ether.

Mass spectrometry. Mass spectra were obtained on a Ribermag R10-10 GC/MS apparatus from R.D.S. Nermag. Electron impact spectra were obtained using a solid probe heated from 50 to 450°C at 60°C/min with filament current of 200 mA and a potential of 70 eV. Desorption/chemical ionization spectra were obtained using methane as the ionizing gas and a desorption filament current programmed from 200 to 350 mA at 15 mA/s.

Cytochrome oxidase determinations. Cell preparations in which cytochrome oxidase activity was measured were resuspended in 0.25 M Mann enzyme grade sucrose, pH 7.4, at about 10 mg/ml protein, homogenized in a glass-glass homogenizer and sonicated at the microtip limit using a Heat Systems sonicator. Sonication was done in two 15-s bursts with intermediate cooling in an ice-water slurry. The assays were performed immediately by the method of Sottocasa et al. [9]. Protein concentrations of cell suspensions were determined by the method of Lowry et al. [10].

Cytochrome b determinations. The procedure used was essentially that of Segal and Jones [6], employing whole homogenates of purified neutrophils. Samples contained 0.25 M sucrose, 75 mM Tris-HCl buffer, pH 7.4, and 1% Triton X-100/mg protein. The Triton X-100 was found useful for clarifying the suspension and minimizing light scatter. 2 ml of each sample were placed in the reference and sample cuvettes and a baseline spectrum recorded from 650 to 400 nm using a Cary 219 spectrometer. A small amount of dithionite was added to the sample cuvette and the difference spectrum recorded with the baseline spectrum automatically subtracted. Four peaks were observed in the difference spectra at 558, 530, 475 and 427 nm. The 558, 530 and 427 nm peaks were assigned to the α-, β-, and γ- (Soret) bands of leukocyte cytochrome *b* [7] with the α-band being characteristic of the *b* type of cytochrome. The 475 nm band was assigned to myeloperoxidase [6]. The concentration of cytochrome *b* was estimated using an extinction coefficient for the beef heart

mitochondrial cytochrome *b* (ΔA(reduced − oxidized) 558–571 nm) of 26 $mM^{-1} \cdot cm^{-1}$. In samples where the amplitude of the peak at 558 nm was insufficient to obtain an accurate measurement, the Soret band was used to calculate the cytochrome *b* concentration, assuming an extinction coefficient of 86 $mM^{-1} \cdot cm^{-1}$ for (ΔA(reduced − oxidized) 426–440 nm). This latter extinction coefficient was derived from spectra of samples with a relatively high concentration of cytochrome *b* and based on the mitochondrial cytochrome *b* extinction coefficient for the α-peak described above.

Materials

HPLC grade methanol, diethyl ether, formic acid and Tween 80 were purchased from Fisher Scientific Co. Nanograde petroleum ether was from Mallinckrodt. Ligroine (b.p. 60–80°C) was from Eastman Kodak Co., Rochester, NY. Silica gel H, cytochrome *c* and ubiquinone-10 were purchased from Sigma. Ethanol was 95% undenatured ethanol from Publicker Chemical Co., redistilled in glass. $CHCl_3$ from Fisher was also redistilled in glass.

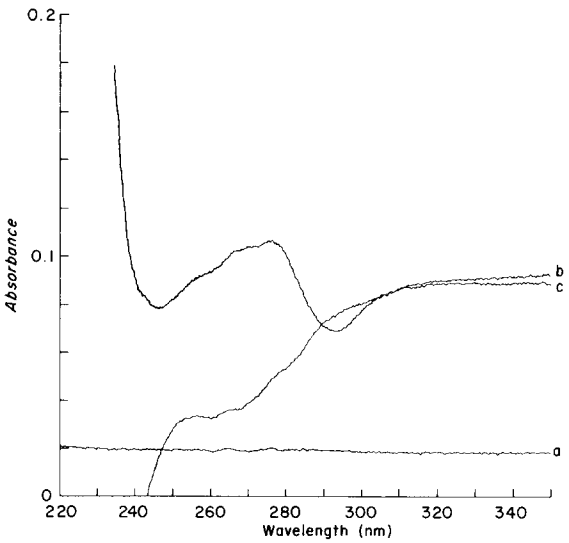

Fig. 1. Spectrum of ubiquinone isolated from neutrophils. Spectrum a is that of the solvent in which the samples were dissolved (baseline determination). Spectrum b is the air oxidized-$NaBH_4$ reduced spectrum of a solvent blank obtained by carrying volumes of those solvents used in the extraction of ubiquinone through the extraction procedure. Spectrum c is the air oxidized-$NaBH_4$ reduced spectrum of ubiquinone extracted from neutrophils.

Results

The oxidized − reduced spectrum of neutrophil extracts revealed a peak with an absorption maxima at 275 nm (Fig. 1c). A minimal estimate of ubiquinone present was calculated by drawing a line between the absorption minima at 293 and 260 nm. The point at which this line intersects at 275 nm was used to determine peak height, and ubiquinone present was calculated using the oxidized − reduced absorption coefficient of 12 500 $M^{-1} \cdot cm^{-1}$. Impurities in the large volumes of solvents used for extraction of the cells were assessed by carrying a volume of saline through the same extraction procedure and running its spectrum along with the ubiquinone sample (Fig. 1b). The decrease in absorbance observed below 310 nm was due to the $NaBH_4$ in the reference cuvette and the magnitude of the decrease was proportional to the $NaBH_4$ added. In no case was there a peak at 275 nm in solvent blank samples. An average value for ubiquinone extracted from six cell preparations is reported in Table I. This value is expressed in terms of ubiquinone/mg protein extracted as well as in terms of ubiquinone per cell.

Mass spectrometric analyses confirmed the presence of ubiquinone-10. Samples analyzed by electron impact using a heated-cup inlet yielded spectra having the predominant ions at 197 and 235 *m/e*. These spectra were similar to those obtained from authentic ubiquinone-10. Comparison of the sample extract 235 *m/e* ion intensity to that of a standard curve derived from a plot of 235 *m/e* ion intensity vs. ng ubiquinone indicated that 1.16 amol/cell were extracted. The difference between the latter value and that obtained by spectral analysis (Table I) was due to loss of ubiquinone in the purification steps preceding mass spectral analysis. Ubiquinone-10 standards carried through the TLC purification showed a 60% recovery in this step. Loss also occurred when the sample was washed to remove $NaBH_4$ and KOH. Thus, the two independent measurements of ubiquinone present in the neutrophil extract are in close agreement, when loss of sample between the two measurements is taken into account.

TABLE I

QUANTITATION OF ELECTRON-TRANSPORT COMPONENTS FROM HUMAN NEUTROPHILS

Values for both ubiquinone and cytochrome oxidase activity are averages of six cell preparations ±S.E. Values for cytochrome *b* are the average of determinations on two cell preparations.

Component	Concentration
Ubiquinone	
nmol/mg cell protein	0.058 ± 0.020
amol/cell	2.74 ± 0.91
Cytochrome *b*	
nmol/mg cell protein	0.08
amol/cell	3.78
Cytochrome oxidase	
nmol/cytochrome *c* oxidized/min per mg cell protein	0.80 ± 0.19
nmol/mg cell protein [a]	$2.2 \cdot 10^{-5}$
Ubiquinone/cytochrome oxidase	2600 (molar ratio)

[a] Estimated using the turnover number for the beef heart mitochondrial cytochrome oxidase (200 e^- transferred/s per molecule heme [21]) and the average value for the specific activity.

The desorption/chemical ionization mass spectrum of authentic ubiquinone displayed a molecular ion (U^+) at 862 *m/e*, and ions at 861 $(U^+ - 1)$, 863 $(U + 1)^+$, 864, 865 and 197 *m/e*. The latter three ions are consistent with ubihydroquinone (H^+) and a ubihydroquinone fragment $(H^+ - 681)$ having the empirical formula $C_{10}H_{13}O_4$. The peaks at 863 and 864 *m/e* are composed of both nominal mass ions and isotopic isomers of lower mass ions. Mass spectra of neutrophil extracts show high-mass ions of 862, 863, 864 and 865 *m/e* with the 8 : 1 ratio of 197 *m/e* to 863 *m/e* observed in authentic ubiquinone (Fig. 2). The extracts displayed a number of intense ions (e.g., 370 and 604 *m/e*) which were associated with impurities in solvents and the silicic acid used in purification of ubiquinone.

The cytochrome *b* concentration in the same preparations was remarkably close to a 1 : 1 stoichiometry with the ubiquinone (Table I). In contrast, the average cytochrome oxidase activity for six cell preparations yielded a far lower molar ratio (Table I). Calculations of the ubiquinone/cytochrome oxidase ratio yielded a ratio of 2600 : 1; a value far in excess of the 2.59–2.97 calculated for mitochondria [11–13]. Similar considerations hold for the cytochrome *b*/cytochrome oxidase ratio, indicating that mitochondrial contamination was far too low to account for the amounts of cytochrome *b* or ubiquinone measured in the cells.

Further evidence for low mitochondrial contamination was obtained from spectral analyses of purified neutrophil whole homogenates. In these preparations no absorbance was observed in the

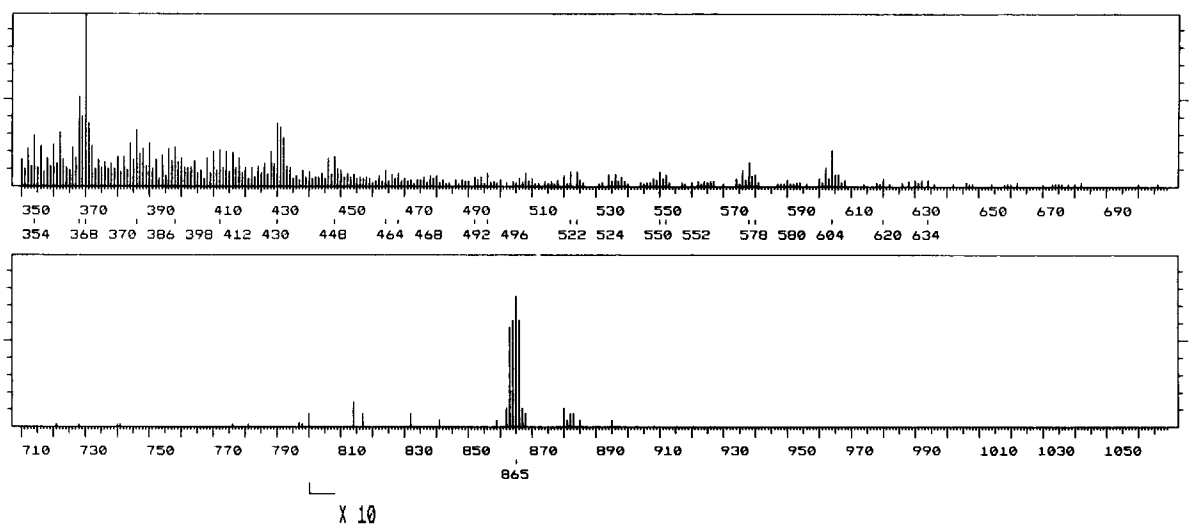

Fig. 2. Desorption/chemical ionization mass spectrum of ubiquinone extracted from neutrophils.

605 and 445 nm regions where reduced mammalian cytochrome oxidase normally absorbs. The same analyses demonstrated a pronounced cytochrome *b* spectrum. Furthermore, granule and membrane fractions prepared by sucrose density gradient separation of whole homogenate (unpublished procedure) demonstrated well defined cytochrome *b* spectra; there was no spectral evidence for cytochrome oxidase in any membrane containing fraction. Cytochrome oxidase should have been detected spectrally if ubiquinone in the neutrophil preparations was due to a mitochondrial contaminate, since the ubiquinone-cytochrome oxidase ratio in most mitochondria is close to 3 : 1. The low level of cytochrome oxidase activity and the lack of spectral evidence for cytochrome oxidase are therefore consistent observations, both indicating that the ubiquinone present cannot be attributed to mitochondrial contamination.

Discussion

Segal et al. [6] first described the existence of a unique cytochrome *b* in the human neutrophil. The involvement of this cytochrome in the respiratory burst was inferred from the following observations: (a) the cytochrome could be isolated from phagocytic vacuoles of neutrophils [6]; (b) activation of the respiratory burst in normal cells results in reduction of the cytochrome [6,14] and (c) cells obtained from patients with chronic granulomatous disease are unable to mount a normal respiratory burst upon stimulation; such cells either lack cytochrome *b* [15] or are incapable of reducing it upon appropriate stimulation [15]. The existence of cytochrome *b* in the human neutrophil has been confirmed by Light et al. [16] and by Sloan et al. [8]. Indeed, our value of 0.08 nmol cytochrome *b*/mg cell protein (Table I) is in close agreement with that of 0.06 nmol/mg reported by Sloan et al. [8] and that of 0.05–0.10 nmol/mg described by Segal et al. [17]. Because of this agreement, we have not performed the assay on a large number of different cell preparations.

The situation with the quinone is quite different, however. Millard et al. [18] first described a quinone in rat peritoneal leukocytes, and later the same laboratory described a quinone (based on spectral evidence) in human neutrophils [8]. Suggestive evidence for a role of the quinone in the respiratory burst was based upon the observations that exogenous quinones stimulated the respiratory burst in resting neutrophils while quinone analogs reduced the magnitude of the phagocytosis-induced respiratory burst [19]. In contrast, Cross et al. [20] reported no evidence of a quinone-type of material in human neutrophils. Our results are generally in agreement with those obtained in Schneider's laboratory. Difference spectra revealed the presence of a quinone in lipid extracts of highly purified human neutrophils (Fig. 1). This was confirmed by mass spectrometric analysis where the quinone demonstrated the spectral characteristics of ubiquinone-10 (Fig. 2).

One difference between our results and those of Sloan et al. [8] lies in the relative concentration of quinone measured. Sloan et al. [8] reported a concentration of approx. 0.43 nmol/mg protein in human cells; the same group measured concentrations of approx. 0.15 nmol/mg in rat leukocytes [18]. Our value of 0.06 nmol/mg (Table I) is appreciably lower although it is significant and was reproducible in six separate leukocyte preparations. The reason for the disparity in the reported concentrations is not apparent but it may relate to the efficiency of extraction. In order to isolate measurable amounts of quinone, we found it necessary to employ an overnight extraction with petroleum ether and vigorous shaking; short periods of extraction were completely inefficient. This might explain the inability of Cross et al. [20] to detect the presence of this compound.

Neither of the components can be attributed to mitochondrial contamination as demonstrated by the data on the concentration of cytochrome oxidase. Thus, the present results suggest that both a cytochrome *b* and ubiquinone are present in the human neutrophil. Although these data are consistent with the involvement of a respiratory chain in initiation of the respiratory burst, they in no way prove that this is the case. Further studies are clearly required to delineate the function of the respiratory components described.

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