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Partial purification of the superoxide-generating system of macrophages. Possible association of the NADPH oxidase activity with a low-potential (−247 mV) cytochrome *b*

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NADPH oxidase activity was solubilized by detergent treatment of subcellular particles obtained from guinea-pig peritoneal macrophages stimulated with phorbol myristate acetate. Gel filtration of the material containing the NADPH oxidase activity gave two peaks of proteins, one of which eluted with the void and the other with the included volume of an AcA 22 column. The material eluted in the void volume contained more than 50% of the NADPH oxidase activity and less than 10% of the NAD(P)H cytochrome *c* reductase activity. A *b*-type cytochrome with peaks of absorption at 558, 528 and 426 nm was also enriched in the fraction which contained the NADPH oxidase activity. The distribution of flavoproteins as revealed by the measurement of FAD was different from that of NADPH oxidase and cytochrome *b*, and followed the elution profile of NADH cytochrome *c* reductase. Studies in subcellular particles showed that the *b* cytochromes of mitochondria and endoplasmic reticulum reduced by selective biochemical means accounted for only a minor part of the total *b*-type cytochromes and that the new cytochrome *b* previously described in neutrophils is the major chromophore also in macrophages. Oxidation-reduction midpoint potential of the partially purified cytochrome *b* was shown to be −247 mV. Association of cytochrome *b* with the NADPH oxidase activity and its very low $E_{m7.0}$ makes it a suitable candidate to be part of the superoxide-generating system also in macrophages.

Introduction

Microbicidal and cytotoxic properties of neutrophils and macrophages are largely based on the production of reactive oxygen species like superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^\cdot$).

The molecular bases of this phenomenon have been elucidated only in part. Perturbation of the plasma membrane by suitable particulate or soluble stimulants activates an NADPH oxidase dormant in the resting cells, which reduces oxygen to O_2^- (see Ref. 1 for a review). Studies on intact cells or subcellular particles have provided strong evidence that in neutrophils a *b*-type cytochrome whose peculiar feature is a low midpoint potential ($E_{m\,7.0} = -245$ mV) participates in the transfer of reducing equivalents from NADPH to oxygen [2–6].

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Abbreviations: Cyt, cytochrome; PMSF, phenylmethylsulphonyl fluoride.

Many attempts have been made in order to isolate and characterize the NADPH oxidase from neutrophils [6–11]. Although instability of the oxidase activity has hampered complete purification, good evidence has been produced that cytochrome *b*-245 is a component of the NADPH oxidase [6,11,12]. Elements in favour of the participation of flavoproteins and the requirements for phospholipids have also been reported [8,9,12,13]. Although reactive oxygen species are known to play a central role in mediating the functions of macrophages in the defence against intracellular pathogens [14] and tumor cells [15] much less attention has been payed to the characterization of the NADPH oxidase of macrophages. Our previous studies performed on subcellular particles indicated that macrophages contain a membrane-bound NADPH oxidase with substrate specificity and kinetic properties similar to those of neutrophils [16,17]. Recently, the *b*-type cytochrome typical of neutrophils has been described in human monocytes [18] and mouse peritoneal macrophages [19]. In this paper, we report data on NADPH oxidase partially purified from guinea pig peritoneal macrophages. Particular attention has been given to the presence and the distribution during the purification of the putative components of this enzyme, i.e., the low-potential cytochrome *b* and flavoproteins. Some biochemical properties of the partially purified cytochrome *b* associated with NADPH oxidase have been also investigated.

Materials and Methods

Cells

Macrophages were obtained from the peritoneal cavity of guinea pigs injected five days before with sodium caseinate [17] and further purified by centrifugation on Ficoll-Hypaque. Contaminating lymphocytes of the final cell suspension were less than 20%. The macrophage preparations were totally freed of neutrophils. Macrophages (about $4–10^8$ in 10 ml) were suspended in Krebs–Ringer phosphate buffer [11] and stimulated for 5 min with 0.5 μg/ml phorbol myristate acetate as previously described [11].

Solubilization of NADPH oxidase and gel filtration chromatography

After stimulation with phorbol myristate acetate, macrophages were washed and resuspended in 0.34 M sucrose containing 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (PMSF) and 10 mM Tris (pH 7.4). After sonication for 3–5 s pulses at 100 Watt with a Branson sonifier, nuclei, intact cells and cell debris were removed by centrifugation at $400 \times g$ for 10 min and the post-nuclear supernatant spun for 30 min at $100\,000 \times g$. The pellet was resuspended in 50 mM sodium-phosphate buffer (pH 8.0) containing 2 mM sodium azide, 1 mM EDTA, 1 mM PMSF and 20% (v/v) glycerol (glycerol/phosphate buffer) at a protein concentration of about 6 mg/ml and then added with 0.4% Lubrol Px and 0.4% sodium deoxycholate.

After stirring for 10 min at 4°C the mixture was sonicated with 3–10 s pulses at 100 W and centrifuged at $100\,000 \times g$ for 1 h. The supernatant was applied to an Ultrogel AcA 22 column (3.8×16 cm) equilibrated with glycerolphosphate buffer (see above). Fractions of 4 ml were collected at a flow rate of 1 ml/min.

Biochemical assays

NADPH and NADH cytochrome *c* reductase activities and NADPH oxidase were assayed by measuring reduction of cytochrome *c* as previously described [11]. Cytochrome *b* was assayed by measuring dithionite-reduced-minus-oxidized difference spectra by using the absorption coefficient ($E_{559-540}$) of 21.6 $mM^{-1} \cdot cm^{-1}$ reported for the neutrophils cytochrome *b*-245 [4]. Since in the single fractions eluted from the AcA22 column (see above) the concentration of cytochrome *b* was too low to be quantified from absorption at 559 nm an $E_{426-410}$ of 151.0 $mM^{-1} \cdot cm^{-1}$ was used. Quantifications of total FAD content were done by the fluorimetric procedure previously reported [11]. Proteins were assayed by the method of Lowry.

Biochemical reduction of mitochondria and endoplasmic reticulum b-cytochromes

This was done in aerobic conditions as previously described by Thayer and Rubin [20] for mitochondrial *b*-cytochromes and by Sottocasa et

al. for cytochrome b_5 [21]. Spectroscopic analysis was done with a Beckman DU-8 spectrophotometer. Postnuclear supernatants prepared as described above, were centrifuged at $100\,000 \times g$ for 30 min and resuspended in 0.1 M phosphate buffer (pH 7.4) at a concentration of about 10 mg/ml. The absorbance of samples in the oxidized state was first measured and memorized. Then sequential additions of antimycin A (10 μg/ml), sodium succinate (10 mM) and NADH (0.1 mM) were done. Maximal measurable reduction of mitochondrial *b* cytochromes and cytochrome b_5 was accomplished within 5 min. The samples where the cytochromes have been biochemically reduced were then read as blanks and a few grains of sodium dithionite added to reduce all the remaining cromophores. This procedure was also used with guinea pig liver homogenates to define its reproducibility. Homogenates of liver from guinea pig were prepared by standard procedures in 0.25 M sucrose. After centrifugation of nuclei and cell debris at $400 \times g$ a crude fraction containing both mitochondria and endoplasmic reticulum was prepared by centrifugation at $100\,000 \times g$. The final pellet was suspended in 0.1 M phosphate buffer (pH 7.4) and analyzed as above.

Oxidation-reduction potential measurements

The midpoint oxidation-reduction potential of partially purified cytochrome *b* was determined with phenosafranin dye as potential indicator according to Light et al. [9] with some modifications. Spectra were recorded at 20°C with a 576 Perkin Elmer double-beam spectrophotometer. Reference and sample cuvette contained the NADPH oxidase suspensed in 2 ml of 50 mM sodium phosphate buffer (pH 7.0) containing 20% glycerol, 1 mM EDTA and 2 mM NaN_3. Reference sample was left air-equilibrated and without any addition in a opened cuvette. Sample solution was made anaerobic and was used for tritations as follows. Sample cuvette was stoppered with a ribbon plug bearing two 1.1×40 nm needles.

One needle was used for continuous flushing of oxygen-free nitrogen and the other one served for the outlet of nitrogen and for the various additions. Until anaerobiosis was obtained and after each addition, the solution was stirred by a micro-stirring bar driven by an external magnetic field placed under the sample cuvette. After ten minutes of equilibration with nitrogen, the background baseline was automatically corrected, than 15 μM phenosafranin was added to the sample cuvette and the difference spectrum was recorded. Reductive titration was then carried out by subsequent additions of small amount (0.2–2 nmol) of sodium dithionite and the scans were recorded. The reduction of cytochrome *b* was monitored as increase of its γ peak, measured as ΔA at 426–410 nm; the reduction of phenosafranin appeared as a progressive decrease of the peak at 525 nm. When the spectra showed maximum extent of cytochrome *b* reduction, the oxidative titration was done by adding small amounts of air to the sample cuvette and following the decrease of the cytochrome *b* peak and the increase of phenosafranin peak. When necessary, the reductive and oxidative tritations were repeated in order to acquire more experimental points. The system potential at each point in the titration was calculated from the Nernst equation using an $n = 2$ midpoint potential for phenosafranin of -252 mV. Cytochrome *b* potential was then calculated by plotting the log of the ratio of the oxidized to the reduced cytochrome versus the system potential.

Results

Solubilization of NADPH oxidase from phorbol myristate acetate-activated macrophages

We showed previously that NADPH-dependent O_2^- production by subcellular particles isolated from guinea pig peritoneal macrophages was enhanced about 20-fold if the cells had been stimulated with phorbol myristate acetate before disruption [16,17].

Table I shows that this activity can be successfully solubilized by treatment with 0.4% sodium deoxycholate plus 0.4% Lubrol Px. After ultracentrifugation of the detergent-treated subcellular particles, 85% of the NADPH oxidase was recovered in the supernatant. The O_2^- production by the solubilized oxidase was strictly dependent on NADPH as electron donor and the activity was less than 15% when NADH was substituted for NADPH (not shown). NADH and NADPH superoxide dismutase-insensitive cytochrome *c* reductase activities were also solubilized. Their activ-

TABLE I

SOLUBILIZATION OF NADPH OXIDASE AND OF NAD(P)H CYTOCHROME *c* REDUCTASES FROM PHORBOL MYRISTATE ACETATE ACTIVATED MACROPHAGES

Treatment with detergents is fully described in Materials and Methods. Values in parenthesis refer to percentage of recovery taking as 100% the activities in the 100000 × *g* pellet of the postnuclear supernatant. The results of duplicate assays of one typical experiment are reported.

	100000 × *g* pellet of the postnuclear supernatant	100000 × *g* super natant after detergent treatment (solubilized extract)	100000 × *g* pellet after detergent treatment
NADPH oxidase (nmol O_2^-/min per mg proteins)	19.8 (100)	22.6 (85)	14.7 (20.4)
NADPH Cyt *c* reductase (nmol Cyt *c* reduced/min per mg proteins)	15.2 (100)	21.2 (105)	8.2 (15.2)
NADH Cyt *c* reductase (nmol Cyt *c* reduced/min per mg proteins)	257 (100)	325 (95)	76 (8)
Proteins (mg)	38.9 (100)	29.3 (75.3)	10.7 (27.5)

ities were higher when NADH was used as substrate.

Partial purification of NADPH oxidase by gel filtration and its relationship with cytochrome c reductases, cytochrome b and flavoproteins

Fig. 1 reports the elution profile of NADPH oxidase and NADPH and NADH cytochrome *c* reductase activities of a detergent solubilized macrophage extract chromatographed through a column of Ultrogel AcA_{22}. Two main peaks emerged from the column. The first peak appeared in the void volume, thus reflecting the presence of materials with a molecular weight above 1200 kDa. About 70% of the proteins was eluted as a second broad peak in the included volume of the column. The first peak contained the bulk of the NADPH oxidase activity and only traces of the superoxide dismutase-insensitive NADPH and NADH cytochrome *c* reductase.

These latter activities were, on the contrary, associated with the second protein peak. Sodium dithionite-reduced-minus-oxidize difference spectra (see Fig. 2) showed that a *b*-type cytochrome

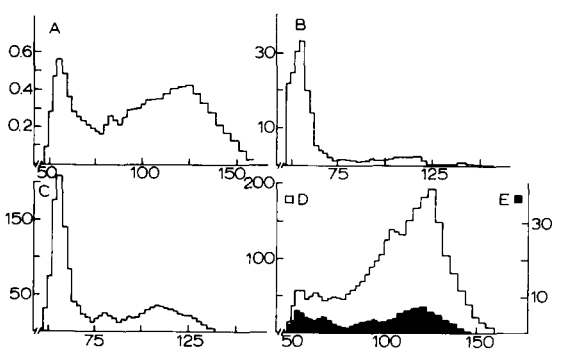

Fig. 1. Gel filtration of the solubilized extract of subcellular particles from phorbol myristate acetate-stimulated macrophages. A 6 ml portion of the solubilized extract was chromatographed on an Ultragel AcA22 column and fractions were collected and assayed as described in the text. The mean results of duplicate assays of one of two similar experiments are reported. Abscissa: elution volume (ml); ordinate: activities. (A) proteins (mg/ml); (B) NADPH oxidase (nmol O_2^-/min per ml); (C) cytochrome *b* (pmol/ml; (D) NADH cytochrome *c* reductase (nmol Cyt *c* reduced/min per ml); (E) NADPH cytochrome *c* reductase nmoles Cyt *c* reduced/min per ml.

with a Soret band at 426 nm and, when visible in the most concentrated fractions, an α peak at 558 nm, followed the same elution profile of NADPH oxidase. These results indicated that high-molecular weight complexes endowed with oxidase activity and a *b*-type cytochrome could be separated from other oxidoreductases. Since the small amount of materials did not allowed an accurate determination of cytochrome and flavin content in each single fraction of the column, we pooled the fractions constituting the two major peaks (from fraction 12 to 17 for the first peak and from 18 to 46 for the second one) and extended enzymatic and spectroscopic analysis.

Table II reports enzymatic activities and cytochrome *b* and FAD content of the two major peaks obtained by gel filtration. The specific activities of NADPH oxidase and cytochrome *b* increased by 3.4-times in the first peak eluted from the column which in total contained 50% of these two components. On the contrary, the second peak contained most of the NADH cytochrome *c* reductase and FAD. Data on the concentration of FMN were not obtained, since it was very low (less than 10% of FAD), and below the sensitivity limits of the assay. The recovery of NADPH oxidase was not optimal (61%) probably due to its inactivation during gel filtration as previously observed with neutrophils [10]. Also assuming that the aliquots which underwent inactivation was contained in the second peak, the distribution of enzymes, cytochrome *b* and FAD in the two peaks was clearly different. By comparing the relative amounts of enzymatic activities and of FAD and

TABLE II

ENZYMATIC ACTIVITIES, CYTOCHROME *b* AND FAD CONTENT OF THE TWO PEAKS OBTAINED BY GEL FILTRATION OF THE DETERGENT SOLUBILIZED MACROPHAGES EXTRACT.

The results of duplicate assays in pooled fractions of the experiment shown in Fig. 1 are reported. Values in parenthesis refer to percentage of recovery taking as 100% the activities in the detergent solubilized extract, which was applied to the column.

	Solubilized extract	First peak from gel filtration (fractions 12–17)	Second peak from gel filtration (fractions 18–46)
NADPH oxidase (nmol O_2^-/min per mg proteins)	22.6 (100)	71.8 (52.8)	2.6 (8.2)
NADH Cyt. *c* reductase (nmol Cyt *c* reduced/ min per mg proteins)	325 (100)	116 (6.7)	416 (91.1)
Cytochrome *b* (pmol per mg proteins)	138 (100)	470 (64)	79.6 (41)
FAD (pmol per mg proteins)	283 (100)	88.9 (5.9)	518 (130)
Proteins	29.3 (100)	5.5 (18.8)	21 (71.7)
NADPH oxidase-to-cytochrome *b* ratio	0.16	0.15	0.03
NADPH oxidase-to-FAD ratio	0.08	0.80	0.005
NADH Cyt *c* reductase-to-FAD ratio	1.10	1.30	0.80
Cytochrome *b*-to-FAD ratio	0.48	5.28	0.15

cytochrome *b* content of the partially purified enzyme (first peak from gel filtration) with those of the crude solubilized extract it can be seen that (1) the NADPH oxidase to cytochrome *b* ratio remained constant after purification, while the NADPH oxidase-to-FAD and the cytochrome *b*-to-FAD ratios changed by a factor of 10; and (2) a constant ratio between NADH cytochrome *c* reductase and FAD was maintained throughout the purification. Taken together these data suggest that copurification of NADPH oxidase and cytochrome *b* is not accompanyed by FAD which follows exclusively the NADH cytochrome *c* reductase.

Properties of the cytochrome b-558 of macrophages

Fig. 2 shows sodium dithionite-reduced-minus-oxidized difference spectra of the two major peaks eluted from the AcA22 column. The major chromophore which appeared upon reduction was a

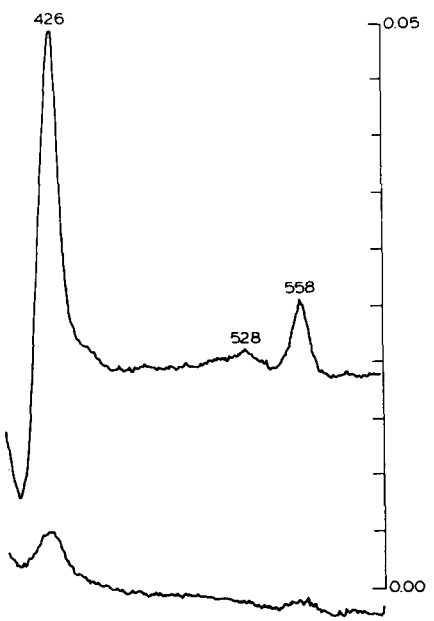

Fig. 2. Reduced-minus-oxidized difference spectra of the two protein peaks obtained by gel filtration of a solubilized extract of subcellular particles from phorbol myristate acetate stimulated macrophages. The fractions eluted from AcA22 column were pooled as specified in Table II. Reduction of cytochrome *b* was accomplished by addition of few grains of sodium dithionite. The concentrations of proteins were 0.3 mg/ml for peak no. 1 and 0.25 mg/ml for peak no. 2.

b-type cytochrome with peaks at 558, 528 and 426 nm. These spectroscopic characteristics are similar to those reported for the cytochrome *b*-245 of neutrophils.

Evidence that the cytochrome *b*-558 is the major chromophore also in macrophages were obtained by comparing biochemical reduction of mitochondrial and endoplasmic reticulum *b* cytochromes with chemical reduction with sodium dithionite. These studies were performed on subcellular particles ($100\,000 \times g$ pellet of a postnuclear supernatant) before solubilization to avoid possible damage of the electron-transport systems of mytochondria and endoplasmic reticulum. As shown in trace a of Fig. 3 the addition of succinate and antimycin A [20] to macrophage subcellular particles caused reduction of the mitochondrial *b* cytochromes. When NADH was added to the same preparation an increase of the total *b*-type cytochromes and a shift of the major γ peak towards shorter wavelengths was observed. This reflected the reduction of cytochrome b_5 present in the endoplasmic reticulum and external mitochondrial membrane [21] which was easily reduced by NADH in aerobic conditions also in microsomal preparations for guinea pig liver (not shown). Trace c of Fig. 3 shows a net-chemical-minus-biochemical difference spectrum of macrophage subcellular particles. When a baseline of absorbance was recorded by reading the sample of trace b as a blank, the subsequent addition of sodium dithionite caused the appearance of a new chromophore with major peaks at 558–559 and 426 nm and two shoulders at 474 and 442–444 nm probably indicative of the presence of myeloperoxidase, which is present in inflammatory peritoneal macrophages and cytochrome oxidase, respectively. When the same comparison between biochemical and sodium dithionite reduction of cellular chromophores was done with a $100\,000 \times g$ pellet of guinea pig liver homogenates, it was clear that after reduction of mitochondrial *b* and b_5 cytochromes the only chromophores reduced by sodium dithionite were Cyt $c + c_1$ and cytochrome oxidase (not shown). Although we had not definite evidence that mitochondrial and endoplasmic reticulum *b*-type cytochromes had been completely reduced, these data indicated that also in macrophages, the major chromophore was a *b*-type cyto-

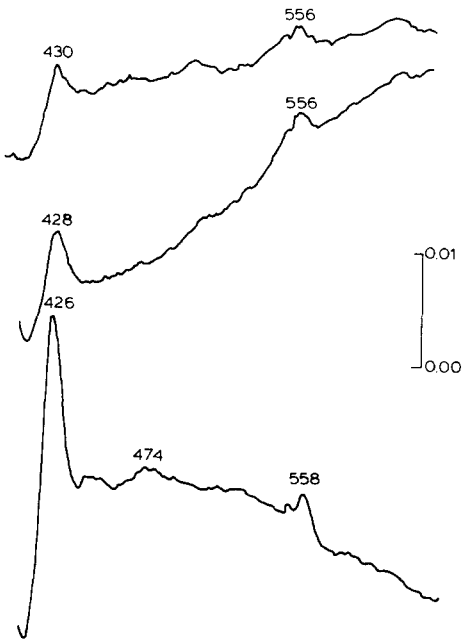

Fig. 3. Biochemical and chemical (sodium dithionite) reduction of *b*-cytochromes in macrophage subcellular particles. (a) Appearance of mitochondrial cytochromes *b* after their reduction with succinate in the presence of antimycin A. The absorbance of subcellular particles (3 mg in 0.3 ml) was first read and memorized. Antimycin A (10 μg/ml) was then added and after 2 min the *b*-cytochromes reduced by electron flow triggered by sodium succinate (10 mM final). (b) Absorption spectrum of the sample of trace a after further addition of NADH (0.1 mM final) and reduction of cytochrome b_5. (c) Chromophores reduced by sodium dithionite (net chemical reduction) after previous biochemical reduction of mitochondrial *b*-cytochromes and b_5. The sample of trace *b* was used to record background absorbance and then a few grains of sodium dithionite were added.

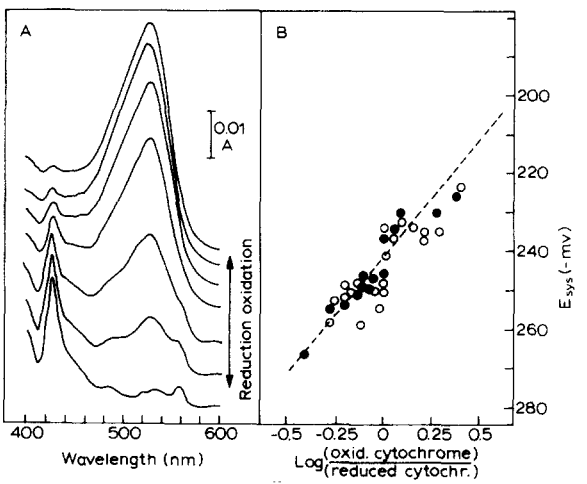

Fig. 4. Oxidation-reduction titration of partially purified cytochrome *b*. Panel (A) shows some reduced plus phenosafranin minus oxidized difference spectra obtained during titration. Panel (B) reports a Nernst plot of a complete potentiometric titration. ●———●, reductive titration with sodium dithionite; ○———○, oxidative titration with oxygen. The dotted line is the theoretical curve for a one-electron transfer reaction. Cytochrome *b* and phenosafranin concentrations were 0.12 μM and 15 μM, respectively. The potential (E) of the system was calculated as described in the text.

chrome with spectral characteristics similar to that of neutrophils.

During anaerobic titrations of cytochrome *b* obtained after AcA22 chromatography, difference spectra such as those reported in Fig. 4A were obtained. Cytochrome *b* reduction was clearly visible as increase of the γ peak at 426 nm, and this peak was completely separated from the peak of oxidized phenosafranin at 525 nm. The use of a partially purified preparation allowed optimal measurements of cytochrome *b* reduction without interference by other chromophores that could cause some difficulties in the calculations, as reported by Light et al. [9]. Fig. 4A also shows that phenosafranin is a particularly suitable indicator for these measurements, because the reduction of cytochrome *b* can be monitored in the same range of potentials where the dye is reduced and shows optical changes. Fig. 4A reports the calculation of the midpoint potential based on a number of different determinations. The $E_{m\ 7.0}$ of partially purified cytochrome *b* from guinea pig peritoneal macrophages resulted to be −247 mV, very close to the $E_{m\ 7.0}$ previously reported for the cytochrome *b* of both guinea pig [22] and human [2] neutrophils. It is evident, therefore, that the low-potential cytochrome *b* is a constant and crucial component of the oxidase system of different phagocytes.

Properties of the partially purified NADPH oxidase.

The NADPH oxidase activity present in the first protein peak eluted from the AcA22 column, displayed the same properties of the oxidase previously studies in macrophage subcellular particles [16,17], or partially purified from neutrophils [10]:

(i) The activity was insensible to the action of NaN_2, KCN, EGTA and EDTA at 1 mM concentrations, and was inhibited by 80 and 100% with 1 mM betaphenantrolin sulphonate and 0.1 mM *p*-chloromercuribenzoate, respectively.

(ii) Kinetic analysis by Lineweaver–Burk double reciprocal plots showed that Michaelis constant for NADPH was 0.066 ± 0.008 mM ($X \pm$ SD; $n = 2$);

(iii) When NADH was substituted for NADPH as substrate, the activity decreased to less than 15% and the Michaelis constant was above 0.4 mM.

Discussion

The molecular basis of the production of reactive oxygen intermediates by phagocytes has been object of intensitive investigation for the last twenty years. Most of this work has been done with neutrophils which can be obtained in high numbers from the circulation or sites of acute inflammation. There is now a general agreement that this phenomenon is due to activation of an oxido-reductase system which uses NADPH as substrate and transfers one electron to oxygen, thus forming superoxide anion. A great deal has been learnt about the biochemical properties of this NADPH oxidase as far as its kinetic properties, substrate specificities, sensitivity to different inhibitors and detergents, subcellular distribution and other parameters are concerned (see Ref. 1 for a review). Much less is known about the structure of the NADPH oxidase, i.e., its nature as a single enzyme or an electron-transfer system constituted by different components. Recent studies have produced strong evidence for an involvement of a *b*-type cytochrome in the NADPH oxidase activity. Spectral analysis of intact cells [2], membrane and granule fractions [23–24] and partially purified NADPH oxidase [6,9–12] have shown that neutrophils contain as their major chromophore a *b*-type cytochrome with peculiar spectroscopic properties and a low-midpoint potential ($E_{m\ 7.0} = -245$ mV).

The molecular bases of oxygen reduction in mononuclear phagocytes are less well known. Subcellular particles isolated from phagocytosing macrophages obtained from different sites and different species were shown to oxidize NADPH at a faster rate than subcellular particles from resting macrophages [25,26]. More recently, biochemical properties of NADPH oxidase have been reinvestigated in peritoneal guinea pig [16,17] and mouse [19,27,28] macrophages and rat pulmonary macrophages [29]. Also the presence of a *b*-cytochrome similar to that observed in neutrophils has been observed in human monocytes [18], in the promyelocitic human cell lines HL-60 upon differentiation in vitro [30] and in mouse macrophages [19]. A deeper knowledge of macrophage NADPH oxidase would certainly bring a great benefit to the understanding of complex phenomena like the enhanced respiratory burst activity of activated macrophages. Only recently, work along this line has been reported [19,27,28].

To out knowledge the data presented in this paper are the first which deal with the NADPH oxidase of peritoneal macrophages after solubilization and partial purification. As such, they demonstrate that the oxido-reductase system which is responsible for the respiratory burst in macrophages shows the same major properties that have been previously described in neutrophils.

Gel filtration of a detergent solubilized extract of subcellular particles isolated from phorbol myristate acetate stimulated macrophages enabled us to isolate a high-molecular-weight complex endowed with at least 50% of the NADPH oxidase activity. This complex contained only trace contaminants of other NADPH and NADH oxido-reductases which do not produce superoxide anion. NADPH oxidase activity of macrophages was copurified with a *b*-type cytochrome with the spectroscopic characteristic of the *b*-245 already observed in neutrophils.

Studies with subcellular particles showed that this *b*-type cytochrome is the major chromophore also in macrophages. In fact, when selectively reduced by biochemical means, mitochondrial and endoplasmic reticulum *b* cytochromes appeared to account for less than 20% of those chemically reduced by sodium ditionite. Potentiometric analysis showed that its midpoint potential was in effect −247 mV, and therefore suitable to be part of the O_2^--generating system.

Resolution by gel filtration in the same fractions of NADPH oxidase activity and cytochrome

b-247 was not accompanied by FAD. The cytochrome *b*-FAD ratio was in fact 5.28 : 1 in the first peak after gel filtration, i.e., 10- and 30-times higher than in the solubilized extract and the second peak, respectively. Also NADPH oxidase activity/FAD ratio was markedly higher in the first peak compared to the solubilized extract and the second peak. On the contrary, a relatively constant ratio with FAD in the three fractions analysed was found for NADH cytochrome *c* reductase. These data are in perfect accord with what was previously reported for neutrophils where a ratio of 19 cytochromes *b* for 1 molecule of FAD was found in the fractions enriched in NADPH oxidase activity [11]. The data strongly suggest a strict relation between the enzyme activity and the cytochrome *b*, but not between the enzyme and a flavin moiety. Most of FAD present in the solubilized extract is associated to SOD-insensitive oxidoreductase enzymes that can be chromatographically separated from NADPH oxidase. These results do not rule out the possibility that a little portion of FAD could be related with cytochrome *b* and oxidase activity, but indicate that the stoichiometry of the various candidate components of the oxidase system should be largely in favor of cytochrome *b*. This latter problem has been discussed in detail in a previous paper regarding the neutrophils enzyme [11].

In the recent years, a great deal has been learnt about the role played by reactive oxygen intermediates in the killing of microorganisms and tumor cells by macrophages. Progress in the procedures to isolate and purify the enzymatic system responsible for their production will significantly contribute to the understanding of the mechanisms of control and activation of macrophage effector functions. The results presented in this paper definitively establish that the enzyme responsible for the respiratory burst in macrophages is an NADPH oxidase, one of whose component is a cytochrome *b* with a midpoint potential of −247 mV.

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