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ANALYTICAL SUBCELLULAR FRACTIONATION OF RABBIT ALVEOLAR MACROPHAGES WITH PARTICULAR REFERENCE TO THE SUBCELLULAR LOCALISATION OF PYRIDINE NUCLEOTIDE-DEPENDENT SUPEROXIDE-GENERATING SYSTEMS AND SUPEROXIDE DISMUTASE

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Summary

Normal and *Bacillus Calmette-Guerin* (BCG) vaccine-induced rabbit alveolar macrophage homogenates were fractionated by isopycnic density gradient centrifugation. Superoxide dismutase-inhibitable NAD(P)H-dependent nitro-blue tetrazolium reductase was found localised to endoplasmic reticulum and mitochondria. The normal macrophages tended to contain more of this activity than the BCG-induced macrophages. Two superoxide dismutases were found: cyanide-sensitive superoxide dismutase was predominantly present in the cytosol, with a small proportion in mitochondria; cyanide-resistant superoxide dismutase was found confined to mitochondria. Neither differed in specific activity between the normal and BCG-induced macrophages.

Introduction

Superoxide anion ($^{\cdot}O_2^-$), an unstable free-radical of oxygen, plays a prominent role in the antimicrobial mechanisms of polymorphonuclear leukocytes, either

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Abbreviations: Hepes, *N*-2-hydroxyethyl piperazine-*N'*-2-ethane sulphonic acid; BCG, *Bacillus Calmette-Guérin*.

as a lethal agent itself or as an intermediate in the formation of hydrogen peroxide and further toxic free radicals [1—4]. The involvement of $^{\cdot}O_2^-$ in microbicidal mechanisms of macrophages is less certain [5].

Evolution of $^{\cdot}O_2^-$ in polymorphonuclear leukocytes is probably largely attributable to one-electron reduction of oxygen by reduced pyridine nucleotide-dependent oxidases. These enzymes have been demonstrated in subcellular granules [6—8] or on the plasma membrane [9—11]. Synthesis of $^{\cdot}O_2^-$ by enzymes in either location is appropriate to a microbicidal function; both plasma membrane and granule contents come into contact with micro-organisms during phagocytosis. Synthesis at sites remote from the target micro-organism would be inappropriate not only because $^{\cdot}O_2^-$ is inherently unstable and potentially toxic to the phagocyte but also because superoxide dismutase is present in the cytosol of the polymorphonuclear leukocyte [11,12] and so diffusion from a distance into phagocytic vacuoles would be limited.

The possibility that $^{\cdot}O_2^-$ might play a part in the microbicidal activities of alveolar macrophages was raised by demonstrations of $^{\cdot}O_2^-$ generation from these cells of mouse, guinea-pig [13], and rabbit [5,14]. However, the subcellular localisation of neither the $^{\cdot}O_2^-$-generating system nor superoxide dismutase in macrophages have previously been established. We report here the nature and subcellular distributions of these enzymes in normal and BCG-induced rabbit alveolar macrophages.

Materials and Methods

Homogenates of alveolar macrophages from normal and BCG-vaccinated rabbits were prepared as described previously [15]. Cells were homogenised in 0.25 M sucrose containing 1 mM disodium EDTA, (pH 7.4), 20 mM ethanol and 5 IU heparin/ml or in this buffer containing either 0.42 mM digitonin or 15 mM pyrophosphate. After centrifugation at $240 \times g$ for 15 min ($3600 \times g_{min}$) the supernatant was fractionated by isopycnic centrifugation on a sucrose density gradient, extending linearly with respect to volume and density from 1.05—1.28 $g \cdot cm^{-3}$, in a Beaufay small-volume automatic zonal rotor [16] as described by Peters [17]. After centrifugation at 35 000 rev./min at 4°C for 35 min (integrated angular velocity $3.3 \cdot 10^{10}$ $r^2 \cdot s^{-1}$) about 15 gradient fractions were collected and their density and weight recorded. Results from enzyme assays on these fractions were expressed as frequency-density histograms constructed as described by Peters [17].

NAD(P)H-dependent nitroblue tetrazolium reductase (EC 1.6.99.-)

This activity was estimated turbidometrically at 550 nm by following the reduction of nitroblue tetrazolium (Sigma) to an insoluble dark blue formazan [18]. The incubation mixture contained 150-μl sample plus 50 mM Hepes buffer, (pH 6.86), 0.1 mM disodium EDTA, 61 μM nitroblue tetrazolium and 25 μM NADH or 2.5 mM NADPH in 3 ml.

The system was observed to comply with Beer's law using nitroblue tetrazolium chemically reduced by the method of Segal and Peters [18].

The involvement of $^{\cdot}O_2^-$ in the enzymic reduction of nitroblue tetrazolium was investigated by assaying in the presence and absence of 150 μg (435 units)

bovine erythrocuprein (superoxide dismutase (EC 1.15.1.1), Sigma). Parallel assays were also done in the presence of 150 μg of superoxide dismutase that had been inactivated by autoclaving at 121°C for 15 min.

The rate of formazan production was not regarded as a measure of the rate of $^{\cdot}O_2^-$ generation. Not all nitroblue tetrazolium reduction was necessarily due to $^{\cdot}O_2^-$ and some $^{\cdot}O_2^-$ was undoubtedly lost in other reactions (e.g. spontaneous dismutation). Nevertheless the dismutase-inhibitable portion of the overall rate of formazan production was considered to reflect the net concentration of $^{\cdot}O_2^-$.

Superoxide dismutase

This enzyme was determined in polyacrylamide gels after electrophoresis. Samples were exposed to ultrasonication (60 W for 20 s, Biosonik microprobe, Bronwill Scientific Co., Rochester, NY) to disrupt subcellular organelles and 100-μl aliquots subjected to electrophoresis on 10% (w/v) polyacrylamide gels in 50 mM Tris-glycine buffer (pH 8.3), at room temperature [19]. Gels were negatively stained for superoxide dismutase activity with nitroblue tetrazolium in the presence or absence of 1 mM NaCN in 50 mM phosphate buffer, (pH 7.8), 1 mM disodium EDTA [20]. Activity was quantified against bovine erythrocuprein as a standard superoxide dismutase (3.5 units/μg, Sigma) [19] using a Joyce Loebl Chromoscan 200 scanning and integrating densitometer. Electrophoretic mobility was expressed relative to the mobility of the standard. Concentrations of up to 60% (w/v) sucrose in the sample did not affect the activity, resolution or mobility of superoxide dismutase.

Other assays

Protein and neutral α-glucosidase (EC 3.2.1.20) were assayed as described by Peters [17] except that neutral α-glucosidase was assayed at pH 7.5 rather than pH 8.0. Cytochrome *c* oxidase (EC 1.9.3.1) was assayed as described by Peters et al. [21], 5′-nucleotidase (EC 3.1.3.5) as described by Seymour and Peters [22], monoamine oxidase (EC 1.4.3.4) was assayed by the method of Wurtmann and Axelrod [23] using [2-^{14}C]tryptamine (New England Nuclear) as substrate and alkaline phosphodiesterase I (EC 3.1.4.1) was assayed as previously described [15].

Results

NAD(P)H-dependent nitroblue tetrazolium reductase

Nitroblue tetrazolium reductase activity was found in $3600 \times g_{min}$ supernatants from homogenised normal and BCG-induced macrophages. NADH showed a K_m of $1.3 \cdot 10^{-5}$ M, markedly lower than the K_m of $8.3 \cdot 10^{-3}$ M with respect to NADPH. Accordingly, assays were routinely carried out using (25 μM) NADH as substrate. Inclusion of 0.5 mM $MnCl_2$ or 0.1% (v/v) Triton X-100 in the assay medium resulted in 90.6 and 40.0% inhibition of activity, respectively, and the presence of 3 mM KCN produced a small (7.6%) increase in activity.

The total nitroblue tetrazolium reductase activity recovered in density gradient-fractionated $3600 \times g_{min}$ supernatants often substantially exceeded the activity detectable before fractionation, presumably through separation

from soluble inhibitors, therefore comparison of specific activity between normal and BCG-induced macrophages was made on the basis of activity recovered in gradients. The average specific activity of nitroblue tetrazolium reductase in three normal macrophage homogenates (ΔA_{550nm} 0.051 ± 0.029 (S.E.) $min^{-1} \cdot mg^{-1}$ protein) was greater than the average in twelve BCG-induced

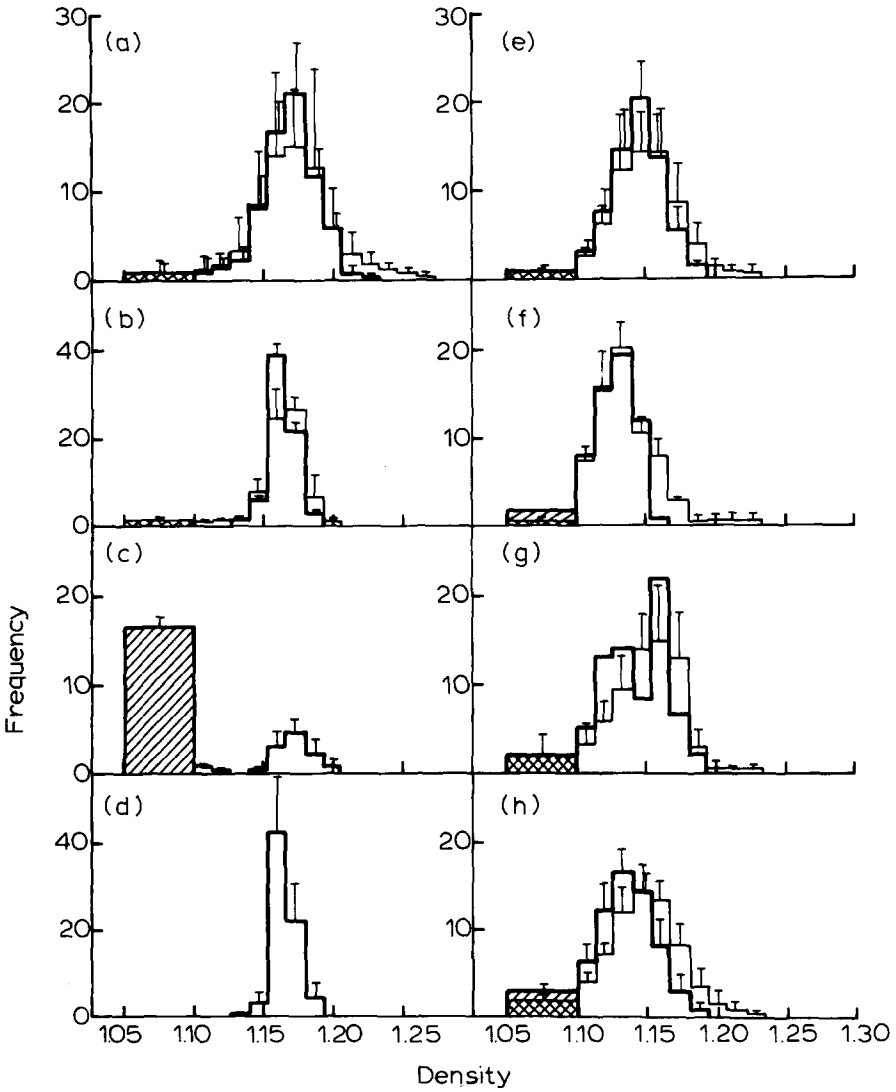

Fig. 1. Isopycnic fractionation of 3600 × g_{min} supernatant from homogenates of rabbit alveolar macrophages. Graphs show enzyme frequency-density distributions averaged from several experiments with normal (———) and BCG-induced (– – –) macrophages. The enzymes (number of experiments with normal and BCG-induced macrophages, respectively, being given in parentheses) are: (a) cytochrome *c* oxidase (8, 4); (b) monoamine oxidase (2, 4); (c) superoxide dismutase I (4, 0); (d) superoxide dismutase II (4, 0); (e) 5′-nucleotidase (11, 9); (f) alkaline phosphodiesterase I (1, 2); (g) nitroblue tetrazolium reductase (1, 6); (h) neutral α-glucosidase (6, 8). Frequency (mean ± S.D.) is defined as the fraction of total recovered activity in the subcellular fraction divided by the density span covered. The crosshatched area represents, over an arbitrary abscissa interval, the activity remaining in the sample layer.

macrophage homogenates (ΔA_{550nm} 0.0065 ± 0.0017 (S.E.) $min^{-1} \cdot mg^{-1}$ protein). Analysis of the log-transformed data showed that, despite substantial variation between animals, the difference just reached significance ($P \simeq 0.05$; Student's *t*-test).

Addition of superoxide dismutase to gradient fractions resulted in an inhibition of nitroblue tetrazolium reduction which, over nine gradients, averaged about 33%. The effect was highly significant ($P < 0.001$, $n = 9$). Proportionally similar inhibition was observed in all gradient fractions. The enzymic nature of inhibition was shown by lack of effect of heated superoxide dismutase ($P > 0.05$, $n = 3$). In one experiment using 2.5 mM NADPH as substrate, 17% inhibition of nitroblue tetrazolium reductase activity was observed with 50 μg dismutase/ml.

Superoxide dismutase

Two electrophoretically distinct superoxide dismutases were found in $3600 \times g_{min}$ supernatants from either normal or BCG-induced macrophages. One (superoxide dismutase I) was totally inhibited by 1 mM CN^- and had an electrophoretic mobility of 0.9 relative to erythrocuprein. The other (superoxide dismutase II) was unaffected by 1 mM CN^- and had an electrophoretic mobility of 0.7 relative to erythrocuprein. With normal macrophages the specific activity of superoxide dismutase I was 1.12 ± 0.21 (S.D., $n = 3$) units/mg protein and superoxide dismutase II 0.26 ± 0.04 (S.D., $n = 3$) units/mg protein. Similar specific activities ($P > 0.05$) were found with BCG-induced macrophages: 0.91 ± 0.32 (3) and 0.29 ± 0.05 (3) units/mg protein for superoxide dismutases I and II, respectively.

Subcellular localisation of superoxide dismutase and NAD(P)H-dependent reductase

Fig. 1 shows the frequency-density distributions of superoxide dismutase I, superoxide dismutase II and nitroblue tetrazolium reductase together with proposed marker enzymes of mitochondria (cytochrome *c* oxidase, monoamine oxidase), plasma membrane (5′-nucleotidase, alkaline phosphodiesterase I) and endoplasmic reticulum (neutral α-glucosidase) after isopycnic centrifugation of $3600 \times g_{min}$ supernatants. The specific activities of these enzymes was not significantly changed in BCG-induced cells with the exception of cytochrome *c* oxidase which was dramatically enhanced from 2.9 ± 0.8 (S.D., $n = 6$) munits/mg protein in BCG-induced cells. Recoveries of enzyme activity were usually in the range 68—90%. Negligible differences were found between normal and BCG-induced macrophages in the distributions of cytochrome *c* oxidase, 5′-nucleotidase and alkaline phosphodiesterase I (modal densities about 1.175, 1.15 and 1.13 $g \cdot cm^{-3}$, respectively).

The modal density of monoamine oxidase activity coincided with that of cytochrome *c* oxidase with BCG-induced cells but was slightly lighter with normal cells, at density 1.165 $g \cdot cm^{-3}$. Neutral α-glucosidase had a modal density of 1.15 $g \cdot cm^{-3}$ with induced cells but 1.135 $g \cdot cm^{-3}$ with normal cells. Lactate dehydrogenase and protein were found predominantly in the sample layer (cross-hatched area).

Over 80% of superoxide dismutase I was found in the sample layer with

normal cells and a small peak was found at 1.175 g · cm^{-3}, coincident with cytochrome *c* oxidase. Superoxide dismutase II sedimented as a single peak at 1.165 g · cm^{-3}, coincident with monoamine oxidase. Thus all of superoxide dismutase II and part of superoxide dismutase I appeared to be associated with mitochondria. The distributions of superoxide dismutase were not determined in experiments with BCG-induced macrophages.

The averaged distribution of nitroblue tetrazolium reductase from induced cells showed a peak of activity at 1.16 g · cm^{-3}, the distribution tailing towards the less dense region of the gradient. With normal cells two peaks of this activity were found, one at 1.169 g · cm^{-3} and one at 1.13 g · cm^{-3}. Nitroblue tetrazolium reductase activity assayed with 2.5 mM NADPH as electron donor had an identical distribution (not shown) to that found with the standard assay using 25 μM NADH. Activity was not detectable with 25 μM NADPH.

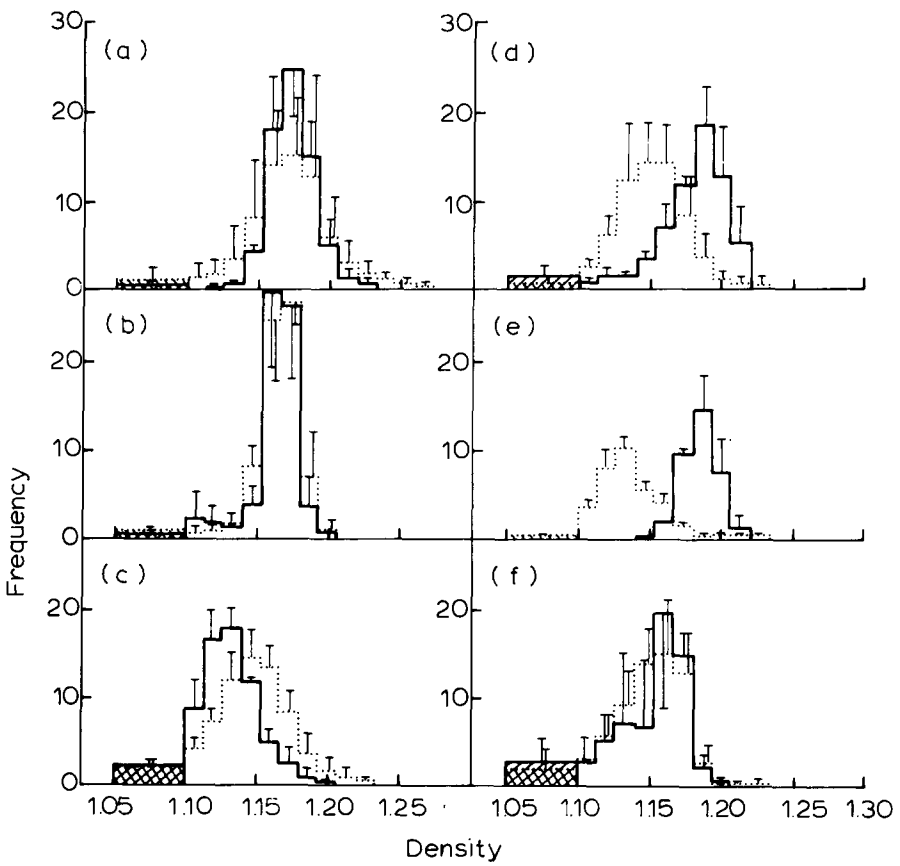

Fig. 2. Effect of homogenisation in the presence of 0.42 mM digitonin on enzyme distribution in isopycnic density-gradient fractions from BCG-induced macrophages. Frequency density distributions from experiments in the presence (———) or absence (· · · · · ·) of digitonin are shown. The enzymes are, with the number of experiments with and without digitonin, respectively, given in parentheses: (a) cytochrome *c* oxidase (3, 3); (b) monoamine oxidase (2, 4); (c) neutral α-glucosidase (3, 8); (d) 5′-nucleotidase (4, 9); (e) alkaline phosphodiesterase I (2, 2); (f) nitroblue tetrazolium reductase (2, 6). For further details see Fig. 1.

The tendency to a bimodal distribution of nitroblue tetrazolium reductase activity, particularly with normal cells, suggested association with more than one subcellular organelle. The peak at 1.16 $g \cdot cm^{-3}$ indicated a partial localisation to mitochondria but it was not clear whether activity in the lighter region of the gradient was associated with plasma membrane or endoplasmic reticulum; both subcellular organelles peaked in this region, as indicated by their marker enzymes 5′-nucleotidase, alkaline phosphodiesterase I and neutral α-glucosidase.

Effect of digitonin on enzyme distribution

Improved resolution of marker enzymes of plasma membrane, endoplasmic reticulum and mitochondria was achieved by homogenisation in the presence of 0.42 mM digitonin before fractionation. Results from experiments with BCG-induced macrophages are shown in Fig. 2. Essentially identical results were obtained with normal macrophages and for brevity are not shown. The most striking difference between treated and untreated cells was the large increase in the modal densities of 5′-nucleotidase and alkaline phosphodiesterase I, to 1.185 $g \cdot cm^{-3}$ from 1.15 and 1.13 $g \cdot cm^{-3}$ respectively. The peak of neutral α-glucosidase, at 1.13 $g \cdot cm^{-3}$, showed a lower equilibrium density compared to control (digitonin-free) BCG-induced cells. The distributions of cytochrome *c* oxidase and monomine oxidase were not affected by the digitonin treatment.

The distribution of nitroblue tetrazolium reductase activity was now bimodal, with peaks at 1.16 $g \cdot cm^{-3}$ (coincident with the peak of monoamine oxidase as before) and at 1.13 $g \cdot cm^{-3}$ coincident with neutral α-glucosidase at its new position. That the activity had not moved to a denser part of the gradient, in contrast to the plasma membrane markers, but had remained coincident with neutral α-glucosidase and monoamine oxidase appeared good evidence for its dual localisation in endoplasmic reticulum and mitochondria and absence from plasma membrane. Addition of digitonin to gradient fractions from macrophages homogenized without digitonin had no effect on the amount of detectable nitroblue tetrazolium reductase, showing that the agent had not selectively inhibited activity on plasma membrane.

Effect of pyrophosphate on enzyme distribution

Further evidence supporting this localisation of nitroblue tetrazolium reductase was obtained from experiments in which BCG-induced macrophages were homogenised in the presence of 15 mM pyrophosphate. The resultant enzyme frequency-density distributions are shown in Fig. 3. Substantial decreases in the modal densities of both neutral α-glucosidase and nitroblue tetrazolium reductase were observed. The neutral α-glucosidase peak shifted from a density of 1.15 to 1.12 $g \cdot cm^{-3}$ whilst most nitroblue tetrazolium reductase similarly shifted to peak at 1.115 $g \cdot cm^{-3}$. A second, small peak of nitroblue tetrazolium reductase activity was observed at a density of 1.175 $g \cdot cm^{-3}$, coincident with cytochrome *c* oxidase. Alkaline phosphodiesterase I was not detectable in the gradient; addition of pyrophosphate to gradient fractions prepared from previously untreated macrophages confirmed total inhibition of this enzyme by 15 mM pyrophosphate. The peak density of 5′-nucleotidase was unaffected after homogenisation with pyrophosphate, remaining at

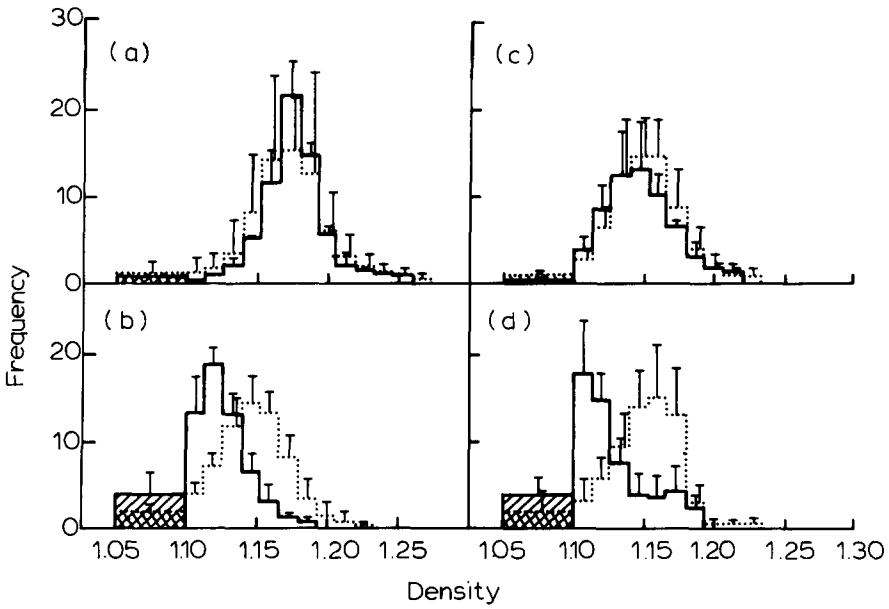

Fig. 3. Effect of homogenisation in the presence of 15 mM pyrophosphate on enzyme distribution in isopycnic density-gradient fractions from BCG-induced macrophages. Frequency-density distributions from experiments in the presence (———) or absence (· · · · · ·) of pyrophosphate are shown. The enzymes are, with the number of experiments with and without pyrophosphate, respectively, given in parentheses: (a) cytochrome *c* oxidase (2, 3); (b) neutral α-glucosidase (4, 8); (c) 5′-nucleotidase (3, 9); (d) nitroblue tetrazolium reductase (4, 6). For further details see Fig. 1.

1.15 $g \cdot cm^{-3}$, although the distribution was slightly skewed towards the less dense region of the gradient.

Discussion

Both a pyridine nucleotide-dependent $^{\cdot}O_2^-$-generating system and a $^{\cdot}O_2^-$-destroying system (superoxide dismutases) have been shown by this study to be present in rabbit alveolar macrophages and their subcellular localisations defined by reference to the fractionation of marker enzymes.

Marker enzymes

Neutral α-glucosidase was used here as the marker for endoplasmic reticulum, in recognition of its location in this material in other cells [24]. However, activity of this enzyme was not well separated in the gradient from that of 5′-nucleotidase, an enzyme that has become widely accepted as a suitable marker for plasma membrane in diverse cell types [25]. This was particularly so with induced cells where the particles carrying neutral α-glucosidase were of slightly greater density than with normal cells. This increased density perhaps reflected a greater abundance of ribosomes on rough endoplasmic reticulum. This would be consistent with an activated status of cells synthesising protein for storage in lysosomes or secretion [26]. Because absence of 5′-nucleotidase activity from rabbit alveolar macrophage plasma membrane has been claimed [27] the specificity and localisation of this

enzyme were studied further. Evidence is presented elsewhere [15] showing that the enzyme is a true 5′-nucleotidase and has a plasma membrane localisation.

Cytochrome *c* oxidase and monoamine oxidase are classical markers for mitochondria. Their distribution within the density gradient was here typical of mitochondrial enzymes of other fractionated cells [17]. Similar equilibrium densities of rabbit macrophage cytochrome *c* oxidase, distinct from values for other organelles, have been reported [28,29]. Most striking, however, was our observation of more than 10-fold greater cytochrome *c* oxidase specific activity in BCG-induced than in normal cells. The explanation of this phenomenon is not known but it was probably not simply a consequence of increased abundance of mitochondria because other mitochondria-associated enzymes, including monoamine oxidase, were not enhanced. However, activity of succinate dehydrogenase (EC 1.3.99.1), a mitochondrial enzyme not assayed in this study, has been reported enhanced in macrophages of BCG-elicited dermal granulomas in rabbits [30].

Lactate dehydrogenase (EC 1.1.1.27) has been widely used as a cytosol marker in studies with macrophages [31] as with other cells [17]. The present observation that most of this enzyme remained in the sample layer above the gradient confirmed the presence of this enzyme in cytosol.

Superoxide dismutase

The dual localisation of superoxide dismutase, cyanide-sensitive superoxide dismutase in the cytosol and mitochondria and cyanide-resistant superoxide dismutase exclusively in the mitochondria, is in agreement with reports from studies on diverse mammalian tissues [12,20,32—36].

NAD(P)H-dependent superoxide-generating systems

Although direct or $^{\cdot}O_2^-$-independent pathways may have contributed to the reduction of nitroblue tetrazolium, significant involvement of $^{\cdot}O_2^-$ was shown by the inhibitory effect of superoxide dismutase on nitroblue tetrazolium reduction. Since similar inhibition was observed in all gradient fractions the amount and distribution pattern of nitroblue tetrazolium reductase activity was considered to reflect that of the NAD(P)H-dependent $^{\cdot}O_2^-$-generating systems.

The absence of pyridine nucleotide-dependent $^{\cdot}O_2^-$-generating activity from the plasma membrane of the rabbit alveolar macrophage is in striking contrast to the situation in human polymorphonuclear leukocytes [9—11]. The distribution and characteristics of the hydrolase-containing granules in the density gradient have been previously reported [37] and were readily distinguished from those of nitroblue tetrazolium reductase as described here. Thus, in further contrast to findings with polymorphonuclear leukocytes [6—8], pyridine nucleotide-dependent oxygen-reducing systems are not present in hydrolase granules in the rabbit alveolar macrophage.

Endoplasmic reticulum nitroblue tetrazolium reductase. The use of neutral α-glucosidase as a marker for endoplasmic reticulum is well established [17,38]. The finding that a peak of nitroblue tetrazolium reductase activity coincided in the density gradient with that of neutral α-glucosidase even after cell disruption in the presence of digitonin or pyrophosphate constituted strong evidence that

the enzyme was present in the endoplasmic reticulum. This activity could be a manifestation of a mixed function oxidase system. Roerig et al. [39] have demonstrated nitroblue tetrazolium reductase activity in a rat liver NADPH-dependent cytochrome *c* reductase system whilst the generation of superoxide by cytochrome *P*-450 has also been shown [40,41].

The selective increase in equilibrium density of plasma membrane by digitonin treatment was in accord with observations with other cells [42] and effectively resolved plasma membrane from nitroblue tetrazolium reductase and neutral α-glucosidase. Decrease in equilibrium density by pyrophosphate treatment, as observed here with nitroblue tetrazolium reductase and neutral α-glucosidase, is a characteristic of rough endoplasmic reticulum. Amar-Costesec et al. [43] showed that the effect is a consequence of the removal of membrane-bound ribosomes by pyrophosphate. The large effect observed here was in agreement with electron-microscopical evidence of abundant ribosomes on endoplasmic reticulum in rabbit alveolar macrophages [26].

Mitochondrial nitroblue tetrazolium reductase. Monoamine oxidase and cytochrome *c* oxidase are markers of the outer and inner mitochondrial membrane, respectively [44,45]. The observation that monoamine oxidase consistently had an equilibrium density slightly lower than that of cytochrome *c* oxidase perhaps reflected the presence of a biochemically heterogeneous population of mitochondria within these cells. Wilson and Cascarano [46] have reported evidence of heterogeneity among rat liver mitochondria. The presence of a nitroblue tetrazolium reductase within mitochondria is not unexpected; evolution of $^{\cdot}O_2^-$ from mitochondrial elements has been reported by others [47—49].

In two respects the present evidence does not support the hypothesis that pyridine nucleotide-dependent generation of $^{\cdot}O_2^-$ plays a major role in the antimicrobial mechanisms of the macrophage: (1) the localisation of the $^{\cdot}O_2^-$-generating system in the endoplasmic reticulum and mitochondria of these cells is not appropriate for delivery of $^{\cdot}O_2^-$ into the microbial environment because the abundant superoxide dismutase in the cytosol could destroy much of the $^{\cdot}O_2^-$ before it reached the phagocytic vacuole; (2) although BCG-induced rabbit alveolar macrophages have greater microbicidal power than normal rabbit alveolar macrophages [50], the specific activity of the NAD(P)H-dependent $^{\cdot}O_2^-$-generating systems of the BCG-induced cells is not greater than that of the normal cells. However it should be emphasised that in the present studies only non-phagocytosing cells were fractionated. Phagocytic stimuli have been found to enhance $^{\cdot}O_2^-$ production from intact macrophages, particularly with macrophages primed by an appropriate treatment of the cell donor such as BCG vaccination [14,51]. It is possible that the subcellular distribution of pyridine nucleotide-dependent $^{\cdot}O_2^-$-generating systems will be found to be different in phagocytosing cells.

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