# Biochemical properties of macrophage fractions and their relation to the mechanism of superoxide production

Michael S. Diamond and Andrij Holian*

*Cardiovascular-Pulmonary Division of the Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA*

Received 21 October 1985; revised version received 22 December 1985

Guinea pig alveolar macrophages are separable by density gradient centrifugation into three subpopulations whose capacity for biological activity (e.g. $O_2^-$ production and chemotaxis) varies directly with buoyant density [(1983) J. Reticuloendothel. Soc. 33, 157–164]. This study demonstrates that the activity per cell of various other enzymes remains constant among the subpopulations. When normalized for cell volume, enzyme activity diminishes with decreasing buoyant density. Intracellular calcium mobilization, linked to formyl peptide and concanavalin A-stimulated $O_2^-$ production, similarly diminishes. Formyl peptide receptor distribution and affinity remain constant. Decreased responsiveness of lower density cells is probably due to lower concentration of enzyme(s) involved in the transduction of signal distal to ligand recognition (or binding).

*Macrophage* *Percoll* *Formyl peptide* *Peptide receptor* $Ca^{2+}$

## 1. INTRODUCTION

In a previous study, a method was described for separating guinea pig alveolar macrophages by continuous Percoll density gradient centrifugation into 3 subpopulations [2]. These subpopulations, referred to as fractions 3, 4, and 5 in order of increasing density, exhibited average cell volumes which varied inversely with buoyant density. In a companion paper, it was observed that the level of functional activities of these cells (stimulated $O_2^-$ production, stimulated cell migration, and pinocytosis) varied directly with buoyant density [1]. Fraction 3 macrophages, the large-volume low-density cells, were least active, and fraction 5 macrophages, the low-volume high-density cells, were most active. In addition, absolute protein content per cell was conserved among the cell fractions. Therefore, protein concentration per unit of cell volume varied directly with buoyant density.

Here it is hypothesized, on the basis of the observations above, that the diminished responsiveness of the lower density cells could be explained in terms of the lower concentration of enzyme(s) involved in the transduction of the signal responsible for macrophage activation. To test this hypothesis, two lines of investigation were pursued. First, experiments were performed to demonstrate a generalized decrease in the specific activities of various enzymes in proportion to buoyant density (mitochondrial oxygen consumption, 5′-nucleotidase, $(Na^+ + K^+)$-ATPase, lactate dehydrogenase, $\beta$-glucuronidase, and lysozyme). Secondly, events linked to the transduction sequence were examined. Formyl peptide receptor number and affinity were measured as well as stimulated calcium mobilization for each subpopulation of macrophages. The results suggest that the decreased responsiveness of the lower density cells is due to the lower concentration of enzyme(s) involved in the transduction of signal

* To whom correspondence should be addressed at: Department of Internal Medicine, 6431 Fannin, Suite 1.274, University of Texas Medical School, Houston, TX 77030, USA

*Published by Elsevier Science Publishers B.V. (Biomedical Division)*
00145793/86/$3.50 

distal to formyl peptide receptor-ligand interaction.

## 2. MATERIALS AND METHODS

Bronchoalveolar cells were recovered by lavage from normal guinea pigs [3] and subpopulations were isolated on discontinuous gradients of Percoll at 4°C. An iso-osmotic working solution of Percoll (Pharmacia, Uppsala, specific gravity 1.123) was prepared as in [2]. From the working solution 4 Percoll solutions of specific gravities 1.035, 1.048, 1.058 and 1.078 were prepared in order to isolate subpopulations referred to as 3, 4 and 5 (located between the 3 pairs of interfaces, respectively) which correspond to those obtained using continuous Percoll gradients [2]. The 4 specific gravities were gently layered in 3-ml portions in a 15 ml glass centrifuge tube (Corning Glass, Corning, NY). Approx. 1 ml of 20–50 $\times$ $10^6$ cells in $Ca^{2+}$- and $Mg^{2+}$-free Hank's buffered salt solution was gently layered on top then centrifuged at 400 $\times$ $g$ for 30 min. Cells at the interfaces were removed by gentle aspiration. Total cell counts, viability by trypan blue exclusion and Diff-Quik (Harleco, Philadelphia, PA) differential staining were assayed as described [2].

Cell volume was estimated from measurements of the $^3H_2O$-permeable and [$^{14}$C]polyethylene glycol-impermeable spaces according to [4]. Assuming that the cells maintained a spherical shape in suspension, the mean cell radius was calculated from the formula, $r = \sqrt[3]{3V/4\pi}$. The [$K^+$] of the cells was measured using a Varian 275 atomic absorption spectrophotometer of nitric acid-solubilized cell pellets as described [4].

The $K_d$ and number of binding sites for [$^3$H]-FNLLP (New England Nuclear, Boston, MA) were determined by Scatchard analysis as previously described [5]. However, obtaining enough cells for complete Scatchard analysis was not always possible. Therefore, an alternative procedure for determining the number of binding sites made use of the value for the $K_d$ ($1.5 \times 10^{-7}$) after it was determined that the $K_d$ was invariant. In this procedure, the number of receptors were determined using quadruplicate samples of cells incubated at one concentration of [$^3$H]FNLLP at or near the $K_d$ and calculated from: [unbound receptors] = $K_d$ $\times$ [bound receptors]/[[$^3$H]FNLLP]$_{free}$. The [bound receptors] are the number of receptors bound at [[$^3$H]FNLLP]$_{free}$. Subsequently, the total number of receptors can be calculated as the sum of unbound receptors and bound receptors.

Total cellular content of 5'-nucleotidase, lactate dehydrogenase, lysozyme and $\beta$-glucuronidase was measured on cells (5–10 $\times$ $10^6$/ml) lysed (89% effective) at 4°C after 10 min in hypotonic medium (0.25 ml cells in MHBSS and 0.75 ml $H_2O$) and passed 10$\times$ through a 30 gauge needle with a 1 ml syringe. Aliquots (50–200 $\mu$l) of this lysate were then used for enzyme assays. Units of enzyme activity were then calculated/$10^6$ cells. Lysozyme activity was measured by the rate of lysis of *Micrococcus lysodeikticus* cells (Sigma) as determined by the decrease in absorbance at 450 nm, with crystalline egg white lysozyme (Sigma) as the standard. $\beta$-Glucuronidase was assayed at pH 4.5 by measuring the formation of *p*-nitrophenol at 405 nm, with $\beta$-glucuronidase (Sigma) as the standard. Lactate dehydrogenase was measured with Sigma kit 340-LD. 5'-Nucleotidase activity was determined using Sigma kit 265-UV.

Efflux of $^{45}Ca^{2+}$ was monitored similarly to [6]. The uptake of $^{86}Rb^+$ was monitored as in [7]. Oxygen consumption was measured as described [8].

[$^3$H]$H_2O$, [$^{14}$C]polyethylene glycol, $^{86}Rb^+$ and $^{45}Ca^{2+}$ were obtained from Amersham (Arlington Heights, IL). Ouabain was obtained from Sigma (St. Louis, MO).

## 3. RESULTS

### 3.1. *Oxygen consumption and content of specific enzyme markers*

Oxygen consumption was measured as a marker for mitochondrial activity. 5'-Nucleotidase activity and ($Na^+$ + $K^+$)-ATPase (estimated by the rate of $^{86}Rb^+$ uptake) were measured as plasma membrane protein markers. Lysozyme and $\beta$-glucuronidase activities were measured as indicators of granular enzyme activity. Lactate dehydrogenase activity was measured as an enzyme marker of the cytosolic space. The results of these measurements are given in table 1 and fig.1. For $O_2$ consumption, 5'-nucleotidase, ($Na^+$ + $K^+$)-ATPase and the granular enzymes there was no significant variation in activity among the different fractions. Therefore, when corrected for cell volume or surface area, the relative enzyme activities diminished

Table 1
Oxygen consumption and enzyme content of guinea pig alveolar macrophage fractions

| | Unfractionated cells | Fraction 3 | Fraction 4 | Fraction 5 |
|---|---|---|---|---|
| $O_2$ consumption (nmol/5 min per $10^6$ cells) | 2.27 ± 0.20 | 2.01 ± 0.43 | 2.08 ± 0.20 | 2.39 ± 0.24 |
| 5'-Nucleotidase activity (units × $10^{-3}$/ $10^6$ cells) | 2.50 ± 0.22 | 2.66 ± 0.28 | 2.80 ± 0.42 | 2.73 ± 0.23 |
| Lysozyme activity (units/$10^6$ cells) | 21.5 ± 6.2 | 26.2 ± 4.9 | 25.3 ± 6.6 | 27.4 ± 7.6 |
| $\beta$-blucuronidase activity (units/$10^6$ cells) | 2.86 ± 0.27 | 3.27 ± 0.55 | 3.86 ± 0.99 | 3.76 ± 0.30 |
| Lactate dehydrogenase activity (units/ $10^6$ cells) | 1116 ± 138 | 1879 ± 129 | 1590 ± 243 | 1159 ± 75 |
| Lactate dehydrogenase activity (units/ $\mu$l volume) | 1670 ± 367 | 1791 ± 253 | 1797 ± 334 | 1834 ± 249 |

Each of the individual enzyme activities and oxygen consumption measurements were carried out as described in section 2 for unfractionated cells and for cells from each of the fractions. The units of lactate dehydrogenase activity/$\mu$l cell volume were calculated from parallel measurements of cell volume as described in section 2. Data represent means (± SE) of 4 experiments

with decreasing buoyant density, as did protein concentration. However, for the cytosolic enzyme, lactate dehydrogenase, the total enzyme activity increased with cell size. When the lactate dehydrogenase activity was corrected for cell volume (also shown in table 1) the concentration of activity was similar for all the fractions.

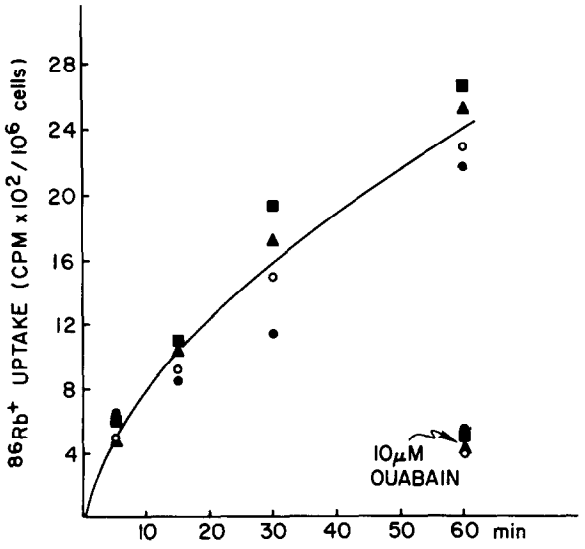


Fig.1. $^{86}Rb^+$ uptake by alveolar macrophage fractions. Uptake measurements were conducted as described in section 2 at 21–23°C for cells from fractions 3 (●), 4 (■), 5 (▲) and unfractionated cells (○). To record ouabain-insensitive $^{86}Rb^+$ uptake the cells were pretreated for 5 min with 10 $\mu$M ouabain prior to the addition of $^{86}Rb^+$. Each point represents the mean of 3 experiments (SE was ± 15% of the mean).

As can also be seen in fig.1, the passive permeability of $^{86}Rb^+$ (measured in the presence of 10 $\mu$M ouabain) is very low in all fractions, indicating no significant variation in membrane permeability to $^{86}Rb^+$ despite the differences in cell size. These results suggested that there might be a variation in the cytosolic potassium concentration among the 3 fractions. This was confirmed by atomic absorption measurements of intracellular [$K^+$] which gave values of 47.7 ± 6.9, 57 ± 12.7, 144 ± 12, and 98.7 ± 6.6 mM (mean ± SE, $n$ = 3) for fractions 3, 4, 5, and the whole population, respectively.

### 3.2. *Number and affinity of formyl peptide-binding sites*

To determine whether the differential activity of the macrophage fractions was explicable at the receptor level or at an intracellular level, the affinity and number (surface density) of FNLLP receptors were measured for each of the macrophage fractions (table 2). There did not appear to be any significant variation in the binding affinity, as determined from Scatchard plots, with the $K_d$ being approx. $1.5 \times 10^{-7}$ M for each cell population. However, the larger cells (fractions 3 and 4) had significantly more available binding sites than frac-

Table 2
Affinity and number of receptor-binding sites for [$^3$H]FNLLP on guinea pig alveolar macrophage fractions

| | Unfractionated cells | Fraction 3 | Fraction 4 | Fraction 5 |
|---|---|---|---|---|
| $K_d$ ($10^{-7}$ M)[a] | 1.47 ± 0.12 | 1.36 ± 0.07 | 1.56 ± 0.19 | 1.61 ± 0.05 |
| Binding sites/cell ($\times 10^4$)[b] | 5.47 ± 0.55 | 8.78 ± 0.82 | 9.19 ± 1.18 | 6.64 ± 0.56 |
| $H_2O$ volume/cell (pl)[c] | 0.668 ± 0.067 | 1.049 ± 0.076 | 0.885 ± 0.029 | 0.632 ± 0.045 |
| Approximate surface area/cell ($\mu m^2$)[c] | 367 ± 24 | 498 ± 25 | 445 ± 10 | 355 ± 17 |
| Binding sites/$\mu m^2$ surface area [a] | 151 ± 15 | 178 ± 17 | 216 ± 28 | 191 ± 23 |

[a] No significant differences were obtained with any comparisons
[b] $P \leq 0.05$ comparing fractions 3 or 4 with unfractionated cells or fraction 5
[c] $P \leq 0.02$ comparing fractions 3 or 4 with unfractionated cells or fraction 5

The binding affinity and number of sites for [$^3$H]FNLLP were measured as described in section 2. The approximate surface area of the cells was calculated from parallel measurements of cell volume. From the cell volume the radius was calculated using the formula $r = \sqrt[3]{3V/4\pi}$, then the surface area was calculated using the formula $A = 4\pi r^2$ assuming a spherical shape of the cells in solution. Data represent the means (± SE) of 4 experiments. Student's $t$-test values are given for the data as described

tion 5 or unfractionated cells. The surface density of the formyl peptide receptors was calculated assuming a spherical shape for cells in solution [2]. This results in a minimum calculated surface area and does not take into account possible irregularities in membrane architecture. When the number of sites were corrected for the different surface areas there was no significant difference in the number of sites/$\mu m^2$ (178–216) among the fractions.

### 3.3. $^{45}Ca^{2+}$ efflux

It has been demonstrated that for formyl peptides and concanavalin A (Con A) calcium mobilization (as can be monitored by an increase in cytosolic calcium or $^{45}Ca^{2+}$ efflux) and $O_2^-$ production are linked [6]. No difference in magnitude of $^{45}Ca^{2+}$ loading per cell was noted among the fractions (not shown). Unstimulated, FNLLP- and Con A-stimulated efflux of $^{45}Ca^{2+}$ from the 3 fractions is shown in fig.2A–C. As can be seen, the extent of either FNLLP- or Con A-stimulated efflux increases in going from fraction 3 to fraction 5. Comparison between fraction 3 and 5 of the extent of stimulated efflux at 5 min by FNLLP or Con A indicates that the differences were significant ($P \leq 0.05$ and $P \leq 0.10$, respectively). The plots of unstimulated efflux of $^{45}Ca^{2+}$ from the 3 fractions are shown together in fig.2D. The difference in efflux between fractions 3 and 5 was significant at all time points ($P \leq 0.05$). There was no clear evidence of any variation in the time of onset of the FNLLP-stimulated efflux. However, for Con A stimulation the magnitude of $^{45}Ca^{2+}$ efflux varied directly and time of onset appeared to vary inversely with buoyant density in parallel to the

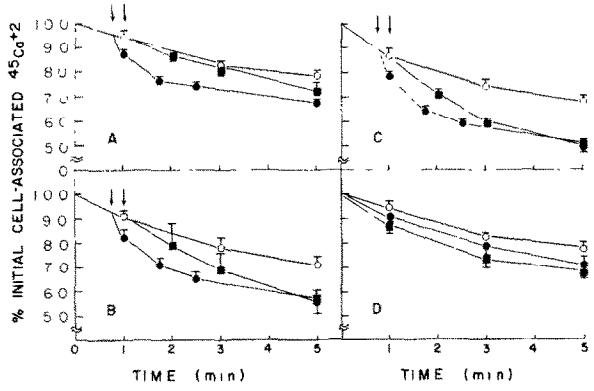


Fig.2. $^{45}Ca^{2+}$ efflux from alveolar macrophage fractions. Efflux measurements were conducted as described in section 2 at 21–23°C for cells from fractions 3 (A), 4 (B) and 5 (C). 10 $\mu$M FNLLP was added at 0.75 min (first arrow) or 250 $\mu$g/ml Con A at 1.0 min (second arrow); unstimulated efflux (○), FNLLP-stimulated efflux (●) and Con A-stimulated efflux (■). Unstimulated efflux from each of the fractions is shown in D; fractions 3 (○), 4 (●) and 5 (■). Data are expressed as the percent of cell-associated radioactivity with the first control point normalized to 100%. Data represent the means ± SE of 4 experiments.

magnitude and onset of initial rates of $O_2^-$ production reported earlier [1].

## 4. DISCUSSION

This study confirms the hypothesis that the previously observed decrease in protein concentration in proportion to buoyant density of macrophage subpopulations [1] is reflected in a similar decrease in activities of several specific cellular enzymes. This observation holds for two plasma membrane markers (5'-nucleotidase and ($Na^+$ + $K^+$)-ATPase), two granular enzymes (lysozyme and $\beta$-glucuronidase), mitochondrial $O_2$ consumption, and one intermediate signal in stimulated $O_2^-$ production (intracellular calcium mobilization). Two exceptions to this trend were the cytosolic marker lactate dehydrogenase and formyl peptide receptor density.

These findings contain a number of implications as to the nature of macrophage subpopulations and of the respiratory burst. The observed decrease in cell [$K^+$] in the lower density cells, in the absence of any significant change in passive permeability to $K^+$, would predict an altered resting membrane potential. Numerous studies have observed early changes in the membrane potential during phagocytic cell stimulation, but other studies have suggested that changing the resting electrical potential is without consequence for stimulated $O_2^-$ production in the macrophage [7]. It may be hypothesized that the lower density subpopulations represent older, damaged cells that have lost the capacity for $K^+$-dependent cell volume regulation that we have observed for fraction 5 cells (unpublished). In general, it has been observed that aerobic respiration, synthesis of ATP and $Na^+$ pump function are among the cellular metabolic functions most vulnerable to cell injury, with cellular swelling being one of the first manifestations of injury [9,10]. Thus it would be appropriate for LDH to be conserved in the face of a decrease in mitochondrial $O_2$ consumption.

There are two possible explanations for the compensatory increase in formyl peptide receptor number in the larger cells, thus maintaining a relatively constant receptor density among the subpopulations. There may be either synthesis of new receptors or exposure of latent receptors as the cells increase in size. There is some evidence to support the latter hypothesis [11–13]. In addition, in our laboratory we have observed increased numbers of receptors on fraction 5 cells that were induced to swell by hypotonic treatment (not shown). Finally, there has been no description of de novo synthesis of formyl peptide receptors. However, this does not rule out the possibility of new receptor synthesis in these studies.

The similar pattern of responsiveness of Con A and formyl peptide stimulation in the various fractions [1] also supports the hypothesis that in the low-density cells there is a lower concentration of an enzyme(s) involved in the transduction of signal following formyl peptide receptor binding. An early event preceding formyl peptide and Con A-stimulated $O_2^-$ production has been demonstrated to be a rapid increase in cytosolic calcium followed by an efflux of cell-associated calcium [6]. Recent evidence suggests that this calcium mobilization may be coincident with or follow polyphosphatidylinositol breakdown yielding inositol phosphates (the source of calcium mobilization) and diacylglycerol (activator of protein kinase C) [14]. Therefore, the rate and extent of $Ca^{2+}$ mobilization may be a direct reflection of the cell's potential to produce $O_2^-$. However, this study does not ditinguish between a decrease in signal to mobilize calcium versus a decrease in a specific pool of releasable calcium. Additional evidence that multiple enzymes are involved in this pattern of decreased responsiveness may be adduced from preliminary results which show that phorbol dibutyrate, a functional analog to diacylglycerol [15], follows the same pattern of diminished activation of the lower density macrophages (not shown). This implies diminished activity of protein kinase C or of a subsequent enzyme in the pathway, in addition to the previously noted decrease in the earlier signal for calcium mobilization.

These results strongly support the hypothesis that the observed generalized decrease in biological activity with decreasing buoyant density of macrophage subpopulations [1] is due to the lower concentration of enzyme activity involved in the transduction of signal distal to formyl peptide receptor-ligand interaction. In addition, these subpopulations may provide a tool for examining the nature of the regulation of the phagocyte

respiratory burst as well as for examining the nature of physiological aging at the cellular level.

## ACKNOWLEDGEMENTS

This work was supported by grants HL-26266 and HL-28758 from the National Heart, Lung and Blood Institute.

## REFERENCES

[1] Holian, A., Dauber, J.H., Diamond, M.S. and Daniele, R.P. (1983) J. Reticuloendothel. Soc. 33, 157–164.
[2] Dauber, J.H., Holian, A., Rosemiller, M.E. and Daniele, R.P. (1983) J. Reticuloendothel. Soc. 33, 119–126.
[3] Dauber, J.H. and Daniele, R.P. (1978) Am. Rev. Respir. Dis. 117, 673–684.
[4] Holian, A., Deutsch, C.J., Holian, S.K., Daniele, R.P. and Wilson, D.F. (1979) J. Cell. Physiol. 98, 137–144.
[5] Daniele, R.P., Diamond, M.S. and Holian, A. (1982) Am. Rev. Respir. Dis. 126, 274–280.
[6] Holian, A. and Daniele, R.P. (1982) J. Cell. Physiol. 113, 87–93.
[7] Holian, A. and Daniele, R.P. (1982) J. Cell. Physiol. 113, 413–419.
[8] Holian, A. and Daniele, R.P. (1979) FEBS Lett. 108, 47–50.
[9] Ginn, F.L., Shelburne, J.D. and Trump, B.F. (1968) Am. J. Cell Biol. 53, 1041–71.
[10] Saladino, A.J. and Trump, B.F. (1968) Am. J. Pathol. 52, 737–776.
[11] Liao, C.S. and Freer, R.J. (1980) Biochem. Biophys. Res. Commun. 93, 566–576.
[12] Zigmond, S.H., Sullivan, S.J. and Lauffenburger, D.A. (1982) J. Cell Biol. 92, 34–43.
[13] Fletcher, M.P. and Gallin, J.I. (1980) J. Immunol. 124, 1585–1588.
[14] Holian, A. and Stickle, D.F. (1985) J. Cell. Physiol. 123, 39–45.
[15] Castagna, M. Tasai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y. (1982) J. Biol. Chem. 257, 7847–7851.