

Production of oxidants in alveolar macrophages and blood leukocytes

T.S. Haugen*, O.H. Skjøsberg**, H. Kähler*, T. Lyberg*

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ABSTRACT: Increased production of oxidants subsequent to phagocyte stimulation has been associated with tissue damage in lung inflammatory disorders. The overall oxidative burden of the lung may vary with inflammatory cell composition.

Flow cytometry using three different dyes, dihydroethidium (DHE), dichlorofluorescein diacetate (DCFH-DA) and dihydrorhodamine 123 (DHR), all compounds that by interaction with oxidants are transformed to fluorescent products, was used to examine the production of intracellular oxidants in alveolar macrophages (AMs), including size-defined subpopulations, monocytes (Ms) and polymorphonuclear neutrophils (PMNs) during *in vitro* incubation in the presence or absence of phorbol myristate acetate (PMA).

PMA stimulation led to slightly increased (two-fold) ($p < 0.05$) DHE-induced fluorescence in AMs, whereas it was greatly increased in Ms and PMNs (13-fold and 113-fold, respectively). The levels of DCFH-DA- and DHR-induced fluorescence were significantly ($p < 0.05$) increased (four-fold and 110-fold, respectively) by PMA stimulation of PMNs, but not of AMs and Ms. Significant differences ($p < 0.05$) in the levels of DHE- and DCFH-DA-induced fluorescence in small and large AMs were also demonstrated.

The results show that the potential to increase the generation of various oxidants upon stimulation was: PMNs > Ms > AMs, suggesting that the total oxidative burden of the lungs is dependent on the type of inflammatory cells present, as well as on their state of activation.

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*Research Forum and **Dept of Pulmonary Medicine, Ullevaal University Hospital, Oslo, Norway.

Correspondence: T.S. Haugen, Research Forum (FUS), Ullevaal University Hospital, N-0407 Oslo, Norway. Fax: 47 22026811

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Oxidants, counterbalanced by the antioxidant system, are a prerequisite in the antimicrobial and antitumour defence mechanisms of the lung [1, 2]. However, excessive production of oxidants may lead to oxidative damage to the tissue [3, 4], and involvement of oxidants has been associated with a number of pulmonary diseases, such as adult respiratory distress syndrome (ARDS), idiopathic pulmonary fibrosis (IPF), sarcoidosis and asthma [5–8].

Upon activation, a burst of oxygen consumption (respiratory burst) occurs in inflammatory cells as a result of the catalytic action of the enzyme reduced nicotinamide adenine dinucleotide phosphate oxidase. In this process, the superoxide anion (O_2^-) is produced [3, 4]. The O_2^- formed can be rapidly converted to hydrogen peroxide, hypochlorous acid (formed from H_2O_2 and chloride ions by myeloperoxidase released from neutrophil granules), hydroxyl radical ($\cdot OH$) and other toxic species, which collectively are termed reactive oxygen species (ROS) [5]. These are regarded as the most important biologically active oxidants and are responsible for injury to microorganisms and the surrounding tissue. Closely related to O_2^- is the powerful oxidant peroxynitrite ($ONOO^-$), generated through the interaction of O_2^- with nitric oxide ($NO\cdot$) [9]. The major result of the reaction of $ONOO^-$ with pro-

teins is the addition of a nitro group in the *ortho* position of tyrosine residues to form nitrotyrosine [10]. The presence of nitrotyrosine has frequently been suggested as a marker of peroxynitrite-dependent damage *in vivo*, and has been detected immunohistochemically in human acute lung injury [11, 12], as well as in ischaemic rat lung injury [13].

Alveolar macrophages (AMs) are believed to be the main source of oxidants in the healthy lung, but, in inflammatory disorders, invading blood phagocytes like monocytes (Ms) and polymorphonuclear neutrophils (PMNs) contribute significantly to the oxidative burden [2, 3]. In addition to increased influx of blood leukocytes, changes in AM maturity may occur [14, 15], associated with changes in cell size, density and functional properties [16–18]. Thus, the predominant inflammatory cell population of the lung has been reported to vary according to the character of the disease [14, 15, 19–21]. Consequently, the overall oxidative burden of the lung may be dependent not only on the cell activation state but also on the inflammatory cell composition and the ability of the involved cells to produce oxidants. Therefore, the capacity for intracellular production of oxidants in AMs, including size-defined subpopulations, Ms and PMNs from healthy volunteers during *in vitro* incubation and phorbol

myristate acetate (PMA) stimulation was examined using three different oxidant-sensitive fluorescent probes.

Materials and methods

Study population

Bronchoalveolar lavage (BAL) was performed in six healthy nonsmokers, four males and two females; mean age 27 yrs (range 24–36). Citrated blood samples for the analyses of Ms and PMNs were taken simultaneously from the same individuals. The participants all had normal lung function (spirometry) and used no medication. Informed consent was given and the study approved by the Regional Ethics Committee.

Bronchoalveolar lavage procedure

After premedication (0.6 mg intramuscular atropine and 10 mg hydrokon bitartrate perorally) and local anaesthesia *via* inhalation of atomized lignocaine, a fiberoptic bronchoscope (Olympus 30; Olympus Optical Co. Ltd., Tokyo, Japan) was wedged in a segmental bronchus of the middle lobe. BAL was performed using three consecutive 50-mL aliquots of sterile isotonic saline solution (37°C), which was recovered by gentle suction and thereafter immediately cooled on crushed ice.

Lung alveolar macrophage preparations

After filtering the lavage fluid through one-layered sterile gauze, cytocentrifuge preparations (Cytospin; Shandon, Runcorn, UK) were stained using a modified May-Grünwald Giemsa method (Diff Quick; Dade, Dade Diagnostika, Munich, Germany) for differential cell counting. Total cell counts (Bürker haemocytometer Assistent, Germany) and viability (trypan blue exclusion test) were assessed after centrifugation (822 × g, 7 min, 4°C) and resuspension of bronchoalveolar cells in phosphate-buffered saline (PBS; 0.01 M phosphate buffer, 0.0027 M KCl, 0.137 M NaCl, pH 7.4). The cells were washed twice (300 × g for 5 min) in PBS and finally resuspended in basic cell culture medium supplemented with 20% heat-inactivated (56°C, 30 min) foetal calf serum (BioWittaker, Walkersville, ML, USA). The basic tissue culture medium used was endotoxin-free (<50 pg·mL⁻¹) RPMI 1640 from Gibco-Biocult (Paisley, UK).

Cell culture conditions and preparation for flow cytometry

AM suspensions ($1.6 \pm 0.6 \times 10^6$ cells·mL⁻¹) and citrated whole blood were incubated in polystyrene tubes equipped with ventilation caps in the presence or absence of PMA (100 ng·mL⁻¹) (Sigma, St Louis, MO, USA) for up to 24 h at 37°C (5% CO₂ in humidified air). Samples of AMs and whole blood were withdrawn for flow cytometric analysis of oxidants after 0, 0.5, 1, 2, 4 and 24 h.

After each incubation time 100 µL of AM suspension ($1.6 \pm 0.6 \times 10^5$ cells) and 50 µL of whole blood were incubated with 10 µL of dihydroethidium (DHE) (Sigma), 2,7-dichlorofluorescein (DCFH) diacetate (DCFH-DA)

(Sigma) and dihydrorhodamine 123 (DHR) (Molecular Probes, Leiden, the Netherlands) (final concentrations 5 µM) for 15 min at 37°C in the dark. Cell suspensions or blood with 10 µL PBS served as controls of autofluorescence.

Thereafter, AM suspensions were washed twice with PBS (2 mL) before cell fixation in 1% paraformaldehyde (PFA), whereas whole blood was incubated with 1.5 mL of an erythrocyte-lysing solution (NH₄Cl 155.5 mM, NaHCO₃ 1 mM, ethylenediamine tetraacetic acid (EDTA)-Na₃ 0.109 mM) for 15 min at 18°C. After centrifugation (300 × g, 5 min) and washing with 2 mL PBS, blood leukocytes were fixed in 1% PFA and like the AMs kept cold (4°C) and in the dark until flow cytometry was performed, within 24 h.

Flow cytometry

Nonfluorescent DHE, DCFH (intracellularly transformed intermediate of DCFH-DA) and DHR have been reported to be oxidized within cells to fluorescent products by oxidants [22–24], which enables monitoring of intracellular oxidant generation by measuring cell fluorescence intensity. This was done using a FACSort flow cytometer equipped with a FACStation™ and Cell Quest™ software (Becton Dickinson, Rutherford, NJ, USA). Ten-thousand and 5,000 events were registered from each blood and AM sample, respectively. The blood leukocyte subpopulations, *i.e.* lymphocytes, Ms and PMNs were separated *via* electronic gating, using the PC-Lysys 1.0 software program (Becton Dickinson). Comparisons between AM subpopulations of different cell size were carried out by gating into three separate regions, whereby the majority of the cells were within region 2 (intermediate size) (fig. 1). The intensity of the specific fluorescence was calculated and expressed as relative linear median fluorescence intensity (RLMFI), *i.e.* as the ratio of specific to nonspecific (autofluorescence) linear fluorescence [25, 26].

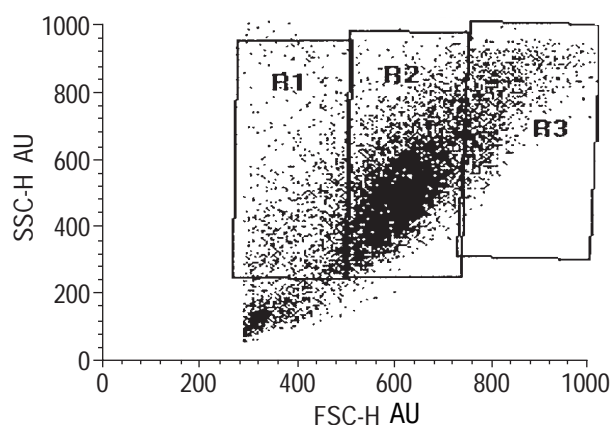


Fig. 1. – Gating of alveolar macrophages (AMs) into subpopulations of different size in a forward (FSC-H)/side scatter (SSC-H) dot plot, in which the abscissa (FSC-H) represents cell size and the ordinate (SSC-H) cell granularity. The gates R1, R2 and R3 represent small, intermediate and large AMs, respectively. AU: arbitrary units.

Statistics

All data are presented as mean \pm SEM. Statistical analyses were performed using the Wilcoxon signed rank test of paired data and Wilcoxon two-sample rank sum test. Differences were considered significant when $p < 0.05$.

Results

Recovery and cell distributions

The recovery of the BAL fluid was $73 \pm 1.5\%$ (109 ± 2.2 mL) of instilled fluid. The total cell count in the recovered lavage fluid was $8.2 \pm 2.9 \times 10^6$ cells. Differential cell counts showed $90 \pm 1\%$ macrophages, and the viability was $89 \pm 3\%$. The regional distribution of AM was: region 1 (small cells), $13 \pm 2\%$; region 2 (intermediate cells), $70 \pm 4\%$ and region 3 (large cells), $15 \pm 1\%$. The total was not 100% because of difficulties in performing accurate gating.

Production of oxidants in alveolar macrophages, monocytes and polymorphonuclear neutrophils

PMA stimulation induced a two-fold increase in RLMFI in DHE-incubated AMs (fig. 2), a 13-fold increase in Ms and a 113-fold increase in PMNs, the differences between the cell populations being statistically significant ($p < 0.01$). The basal RLMFIs were the same in the total populations of AMs, Ms and PMNs throughout 24 h of unstimulated *in vitro* incubation.

In DCFH-DA-incubated PMNs (fig. 2), a modestly (four-fold), but significantly ($p < 0.05$) increased RLMFI was observed following PMA stimulation, whereas no significant changes were seen during 24 h incubation in the presence or absence of PMA in AMs and Ms, and the

basal RLMFI levels were the same in all three cell populations.

PMA stimulation resulted in a 110-fold increase in RLMFI in DHR-incubated PMNs, but no significant increases were observed during PMA stimulation in AMs and Ms (fig. 2). The basal RLMFI in Ms was four-fold and six-fold higher than that of AMs and PMNs ($p < 0.01$), respectively.

Production of oxidants in subpopulations of alveolar macrophages

Following incubation with DHE, small AMs (fig. 1, region 1) showed significantly ($p < 0.05$) higher RLMFIs than the larger AMs (regions 2 and 3) after 1 h and 2 h of PMA stimulation (fig. 3).

As regards DCFH-DA-induced fluorescence, however, an opposite trend was observed, RLMFI being greatest in large AMs (fig. 1, regions 2 and 3) in unstimulated as well as in PMA-stimulated cells ($p < 0.05$).

After incubation with DHR, RLMFIs were similar in all three subpopulations of AM and neither incubation alone nor PMA stimulation had significant modulating effect on the formation of oxidants.

Discussion

In the present study, marked differences were demonstrated between blood leukocytes and AMs as regards capacity to increase intracellular oxidant formation upon PMA stimulation, *i.e.* PMNs > Ms > AMs. Further, significant differences were also demonstrated between small and large AMs with respect to oxidant formation.

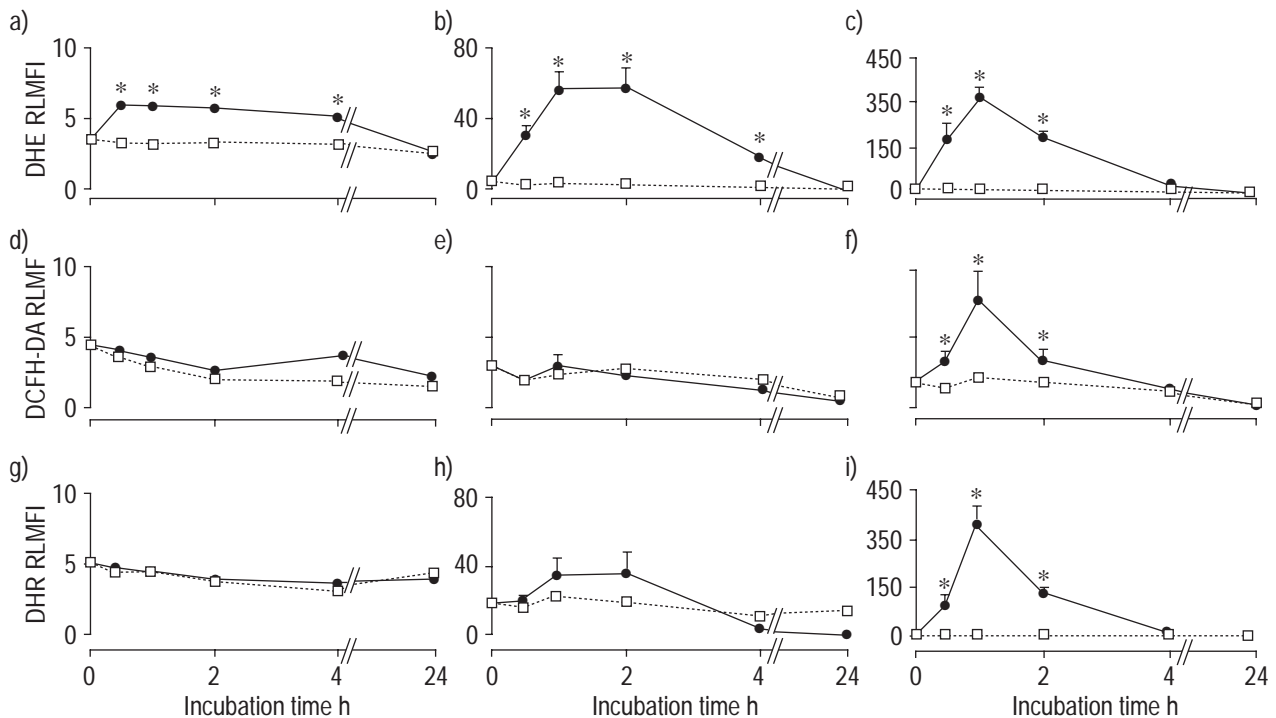


Fig. 2. – Relative linear median fluorescence intensity (RLMFI) after incubation with: a–c) dihydroethidium (DHE); d–f) 2, 7-dichlorofluorescein diacetate (DCFH-DA); and g–i) dihydrorhodamine 123 (DHR) in a, d, g) alveolar macrophages; b, e, h) monocytes; and c, f, i) polymorphonuclear neutrophils ($n=6$) in the presence (●) or absence (□) of phorbol myristate acetate (PMA) ($100 \text{ ng}\cdot\text{mL}^{-1}$). Values are given as mean \pm SEM. A value of 1 indicates no detectable specific fluorescence. *: $p < 0.05$, PMA-stimulated *versus* unstimulated cells.

It has previously been claimed that DHE, DCFH-DA and DHR indicate the production of mainly O_2^- , H_2O_2 and $ONOO^-$, respectively [22–24]. Based on this assumption, the present demonstration of probe-related differences in fluorescence intensity indicates that AMs, Ms and PMNs possess significant differences in their production capacities for O_2^- , H_2O_2 and $ONOO^-$. However, in the authors' opinion, the specificity of DHE, DCFH-DA and DHR in monitoring individual oxidants should be considered doubtful, at least until further evidence to this end has been gathered. It should also be emphasised that comparisons of oxidant levels in different cell types are valid only if DHE, DCFH-DA and DHR penetrate the cell membranes to a similar extent and if RLMFI is linearly related to the concentrations of the oxidants. These methodological problems were not the objective of the presented study, which, however, clearly illustrates the necessity of using different fluorescent probes when intracellular oxidant levels are examined by means of flow cytometry.

Conflicting results have been published regarding the oxidant production capacity of Ms and AMs. Some authors have reported similar ROS production capacity in Ms and AMs upon activation [27, 28], whereas others have demonstrated greater oxidation activity in AMs [29]. However, a weaker respiratory burst in resident macrophages compared to Ms has also been reported, as well as decreasing secretion of ROS when Ms mature to macrophages *in vitro* [30]. These latter data are in accordance with the present results, which are also supported by a series of reports in which O_2^- production was shown to be greater in high-density, *i.e.* small monocyte-like AMs compared to low-density AMs [14, 31–34]. In apparent contrast to this is the present finding that large cells, probably representing low-density AMs [16], had higher intracellular oxidant levels than small AMs, when monitored using fluorescence of oxidised DCFH. Dif-

ferent methodology for estimating oxidant production, *e.g.* measuring either secreted or intracellular (as in this report) oxidants, may explain the divergent results.

It has been claimed that HOCl is the major oxidant generated by PMNs [35], but recently it was reported that activated PMNs and Ms predominantly generate O_2^- [36]. The present demonstration of equal oxidant production in activated PMNs when using two different probes (DHR and DHE) suggests that not only one oxidant predominates in activated PMNs, provided that DHR and DHE demonstrate mainly different oxidants. In accordance with these results are the observations of STRAUZ *et al.* [37], the ROS release per cell being higher in PMNs compared to AMs in BAL from patients with idiopathic pulmonary fibrosis (IPF). In that study, it was demonstrated that ROS production in AMs from IPF patients was markedly increased compared to that in control subjects. In contrast, the present study on AMs from healthy individuals showed only a very modest PMA-induced increase in oxidant production. This discrepancy between AMs from IPF patients and healthy persons may be explained by the effects of priming compounds, *e.g.* cytokines, in inflammatory diseases [3, 29, 38].

The present study demonstrates a significantly greater increase in production of oxidants in PMNs than in Ms upon PMA stimulation, particularly for those oxidants detected by DHE and DHR fluorescence. These results support the view that an association exists between oxidant injury mechanisms and the crucial role of PMNs in ARDS [19].

The relative contributions of alveolar macrophages, monocytes and polymorphonuclear neutrophils to the overall respiratory burst in lung inflammatory disorders probably varies for each specific disease [4], as indicated in studies in patients with adult respiratory distress syndrome, sarcoidosis and idiopathic pulmonary fibrosis

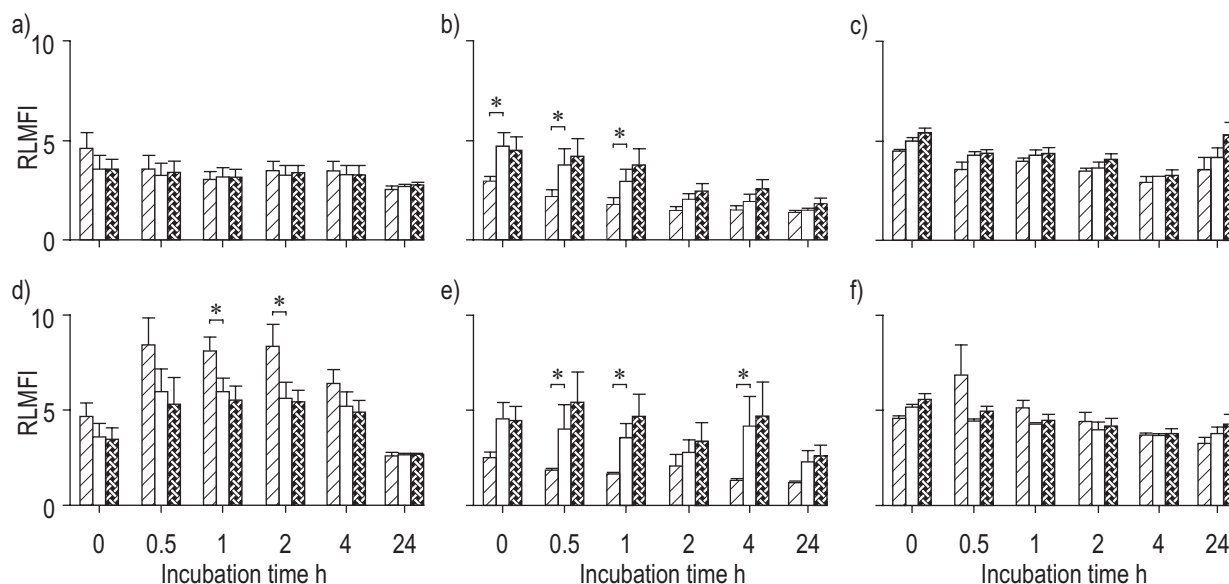


Fig. 3. – Relative linear median fluorescence intensity (RLMFI) after incubation with: a, d) dihydroethidium; b, e) 2, 7-dichlorofluorescein diacetate; and c, f) dihydrorhodamine 123 in a size-defined: a–c) unstimulated; and d–f) phorbol myristate acetate-stimulated ($100 \text{ ng}\cdot\text{mL}^{-1}$) alveolar macrophages (AMs); ▨: small AMs; □: intermediate AMs; ▩: large AMs. Values are given as mean \pm SEM. A value of 1 indicates no detectable specific fluorescence. *: $p < 0.05$.

[5, 6, 32, 37, 39] as well as in animal studies, using experimental models of lung injury [40, 41]. In conclusion, the present study clearly demonstrates that alveolar macrophages, monocytes and polymorphonuclear neutrophils possess significant differences in potential to increase their intracellular levels of oxidants upon stimulation. The results also suggest that the overall oxidative burden of the lung is critically dependent on the predominant inflammatory cell population, as well as on the activation state of the inflammatory cells.

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