Differentiated U937 cells and human monocytes exhibit a differential production of extracellular oxygen species: \(O_{2}^{\cdot-}\) excretion versus \(H_{2}O_{2}\) diffusion

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Abstract

The nature and the localization of the oxidative response triggered by different stimuli in either differentiated U937 cells and peripheral blood-derived human monocytes was investigated using luminometric and cytofluorometric techniques. Differentiated U937 cells essentially produced extracellular superoxide anion \(O_{2}^{\cdot-}\), whatever the stimulus used. Monocytes, however, responded to \textit{Salmonella typhimurium}, phorbol esters, and opsonized zymosan by an intracellular, an extracellular, and both an intra- and extracellular production of oxygen species, respectively. Furthermore, \(H_{2}O_{2}\) but not \(O_{2}^{\cdot-}\) was detected in the extracellular oxidative response of monocytes. Using differentiated U937 cells, luminol was found to be as efficient as lucigenin in the detection of extracellular \(O_{2}^{\cdot-}\), providing sufficient concentrations of extracellular horseradish peroxidase were present. However, both azide and histidine inhibited the lucigenin-enhanced chemiluminescence, suggesting an initial and transient production of singlet oxygen by differentiated U937 cells. Taken together these results strongly suggest that, when stimulated, differentiated U937 cells directly excrete \(O_{2}^{\cdot-}\) in the extracellular medium while, within monocytes, \(O_{2}^{\cdot-}\) is rapidly dismutated in \(H_{2}O_{2}\) which can eventually diffuse outside the cell. Such differences in the oxidative response between the two cell types could be explained by the lack of total closure of the phagosome, only observed in differentiated U937 cells.

Keywords: U937 cells; Monocytes; Oxidative burst; \textit{Salmonella typhimurium}

1. Introduction

The human histiocytic lymphoma cell line U937 can be differentiated along the monocyte/macrophage pathway by various inducers, such as phorbol esters (PMA), retinoic acid (RA), and 1,25-dihydroxyvitamin D3 (VD) [1–5]. The combination of RA and VD has been shown to exert synergistic effects on the terminal differentiation of U937 cells [6,7]. This differentiation was assessed both by the inhibition of proliferation and the acquisition of functional properties such as adherence, phagocytosis, and generation of an oxidative burst. Recently we reported that RA/VD-differentiated U937
cells were capable of developing an oxidative metabolic burst in response to *Salmonella typhimurium* infection [8]. This burst was found to affect neither the viability nor the multiplication of *S. typhimurium*, suggesting that it plays only a minor role in the host defence. Nevertheless, such a release of highly toxic oxygen species during phagocytosis could have a physiological significance by causing tissue damage. Despite the key role played in the host defence against infectious agents, the extracellular production of such oxygen metabolites by leukocytes also contributes to the emergence of various inflammatory diseases [9]. During phagocytosis, the increased uptake of molecular oxygen leads to the production of $\mathrm{O}_2^-$ by activation of the membrane fraction of the NADPH oxidase [10]. In order to limit tissue damage, the generated $\mathrm{O}_2^-$ is theoretically secreted into the phagocytic vacuole where it gives rise to other toxic oxygen derivatives [11,12]. Within the cell, scavenging enzymes, such as superoxide dismutase (SOD) and catalase, detoxify $\mathrm{O}_2^-$ and $\mathrm{H}_2\mathrm{O}_2$ respectively. However, these enzymes are essentially intracellular, and are found at only very low levels in the extracellular fluids [13,14]. Furthermore, the co-existence of $\mathrm{O}_2^-$ and $\mathrm{H}_2\mathrm{O}_2$ may generate the highly reactive hydroxyl radical 'OH, for which no physiological detoxification system has been reported. It has been argued that the relative lack of extracellular scavenging enzymes could allow the extracellularly released reactive oxygen species to function as bioeffector molecules [14].

Although *S. typhimurium* has been reported to induce an oxidative burst in different cell types [15–17], the localization of the oxidative response was poorly studied, probably because *S. typhimurium* was generally found resistant to this oxygen-dependent microbicidal mechanism. The aim of the present study was to investigate both the nature and the localization of the oxidative response triggered by *S. typhimurium* in RA/VD-differentiated cells and to compare this with peripheral blood-derived human monocytes. Surprisingly, the oxidative burst developed by the two cell types in response to *S. typhimurium* infection was at different cellular locations. Moreover, when other soluble or particulate stimuli were tested, the nature of the oxidative burst observed in the two types of cells was different: RA/VD-differentiated U937 cells excreted $\mathrm{O}_2^-$, whatever the stimulus used, whereas human monocytes showed different responses and, in certain cases, produced extracellular $\mathrm{H}_2\mathrm{O}_2$. Our results, completed by microscopic observations, strongly suggest that, in RA/VD-differentiated cells, the excretion of $\mathrm{O}_2^-$ was allowed by a delayed closure of the phagocytic vacuole.

2. Materials and methods

2.1. Reagents

All-trans-retinoic acid (RA), phorbol 12-myristate 13-acetate (PMA), superoxide dismutase (SOD), catalase, horseradish peroxidase (HRP), sodium azide, histidine, zymosan A, lucigenin (bis-N-methylacridinum nitrate) and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemie Sari (France). Luminol (5-amino-2,3-dihydro-1,4-ptalazinedione) was from Boehringer Mannheim (France). The 1,25-dihydroxyvitamin D3 (VD) was a generous gift from Dr. C. Damais (INSERM U313, Paris, France). Stock solutions of RA, VD (0.1 mM in ethanol) and PMA (1.6 mM in DMSO) were stored at $-70^\circ C$. The dihydrorhodamine 123 was from Interchim (France) and the stock solution (10 mg/ml in dimethylformamide) was stored at 4°C. Stock solutions of luminol (10 mg/ml in DMSO) and lucigenin (30 mg/ml in DMSO) were stored at room temperature. Zymosan (10 mg) was suspended in 1 ml phosphate-buffered saline (PBS), boiled for 30 min and washed twice in PBS. Opsonization was achieved by incubation in fresh pooled normal human serum (NHS) for 30 min at 37°C. After washing twice, opsonized zymosan was resuspended in PBS at 10 mg/ml and stored at $-70^\circ C$.

2.2. Bacteria

A wild-type virulent strain of *S. typhimurium* LT2 from ATCC (Rockville, MD) was stored at $-70^\circ C$ in trypticase soya broth supplemented with 10% (v/v) glycerol. Samples from the frozen stock were cultured overnight at 37°C with aeration in trypticase soya broth. Subcultures were grown for 3 h in the same conditions and harvested when in logarithmic phase. The bacteria were collected by
centrifugation (5 min, 6000 X g), washed twice with PBS and suspended in PBS at a density of 5 X 10^9/ml by adjustment of the turbidity at 600 nm. Bacteria were quantified by spreading a suitable dilution in PBS on trypticase soya agar.

2.3. Cells

U937 cells from ATCC were cultured in RPMI 1640 (Gibco BRL Sarl, France) supplemented with 10% (v/v) heat-inactivated (56°C, 30 min) foetal calf serum (Sigma Chimie Sarl, France) and 2 mM glutamine, at 37°C in a 95% air, 5% CO₂ atmosphere. The absence of contamination by mycoplasma was tested by 4,6-diamidino-2-phenylindole (DAPI) fluorescence. Cell viability was checked by Trypan blue exclusion. To induce differentiation, cells were cultured during 72 h at an initial density of 2 X 10^5 cells/ml in the presence of 100 nM RA and VD. The final concentration of the stock solution solvent (ethanol) had no effect on cell growth and differentiation. Before use, the U937 cells differentiated by RA/VD were harvested by vigorous pipetting and washed twice in cold PBS by centrifugation (10 min, 200 X g).

Monocytes were obtained from buffy-coats prepared from blood of healthy human volunteers, collected on citrate phosphate dextrose. The buffy-coat was diluted (1/1) in PBS and monocytes were directly separated from other blood cells by gradient of centrifugation using the Nycoprep d = 1.068 solution (Nycomed, Norway). The monocyte suspension was then washed twice in cold PBS by centrifugation (10 min, 200 X g) and cell viability was checked by Trypan blue exclusion. The contamination by polymorphonuclear cells was less than 5%. The presence of lymphocytes (15–20%) did not constitute a drawback for the chemiluminescence assay. Moreover, the population of monocytes could be separated using a gate in the cytofluorometric analysis.

2.4. Chemiluminescence assay

Differentiated U937 cells and human monocytes were adjusted in PBS at 5 X 10^5 and 1 X 10^5 cells/ml respectively. Cell suspensions (0.5 ml) were placed in 75 X 11 mm plastic tubes (Sarstedt, France) and 0.5 ml of luminol or lucigenin (stock solutions diluted 1/1000 in PBS) was added. When bacteria were used as a stimulus, 5% (v/v) NHS were also added. In some experiments, 3 μl of DMSO, or 200 U of catalase and/or SOD, or 4 U of HRP and/or 1 mM sodium azide, or 10 mM histidine were added. The tubes were shaken gently, placed in the luminometer (Lumicon, Hamilton, Switzerland) chambers (at 37°C) and allowed to equilibrate. The different stimuli: PMA (1.6 μM), opsonized zymosan (0.5 mg) or bacteria (10/cell) were then added as 50 μl of an appropriate dilution in PBS. The tubes were shaken gently and light emission was recorded with automatic background subtraction. Resting cells luminescence was measured in parallel runs. The viability of the cells was checked by Trypan blue exclusion at the beginning and the end of each experiment. Chemiluminescence readings were made at 30 s intervals over a 30 min period (for bacteria and opsonized zymosan) or at 5 s intervals over a 15 min period (for PMA), using a repeat mode. The amplitude of the response was computed and the results were expressed as the integral of counts per 1 X 10^6 monocytes or 5 X 10^5 differentiated U937 cells. At the end of some experiments, cells were washed twice in cold PBS to eliminate most of the extracellular bacteria, then resuspended at the initial volume in complete RPMI medium and cytocentrifuged. The slides were fixed in methanol, stained with Color Rapid (Scientec, France) and examined under immersion oil (×1000).

2.5. Cytofluorometric assay of the oxidative burst

Differentiated U937 cells and human monocytes were adjusted in PBS at 5 X 10^5 and 1 X 10^5 cells/ml, respectively. Cell suspensions (1 ml) were placed in 5-ml polypropylene tubes and preincubated in a shaking water bath at 37°C in the presence or absence of 5% fresh NHS. The different stimuli: PMA (1.6 μM), opsonized zymosan (0.5 mg), bacteria (10/cell) were then added at time zero, as 50 μl of an appropriate dilution in PBS. Controls were performed in the absence of stimulus. After appropriate dilution of the stock solution in PBS, the dihydrorhodamine 123 was added at the final concentration of 30 ng/ml, at the time t = 10 min. Samples were then tested at t = 15 and t = 20 min. The dihydrorhodamine 123 has been selected as the ade-
quate fluorochrome after elimination of dichlorofluorescein-diacetate (totally inhibited in the presence of NHS) and hydroethidine (which gave too much background with monocytes). The dihydrorhodamine 123, rapidly trapped within the cells, can be oxidized to a highly fluorescent compound in the presence of superoxide anion and hydrogen peroxide and was found to be not significantly affected by the presence of NHS. Since the addition of dihydrorhodamine 123 prior to, or simultaneously with, the phagocytic stimulus abrogates or diminishes the formation of measurable oxygen species, dihydrorhodamine 123 has to be added 10–20 min after initiation of the respiratory burst [18]. An optimal time point of 10 min was determined, in our system, by preliminary data. The cell population was analyzed in a Coulter Epics Profile (Coulter Cytometry, Hialeah, FL) flow cytometer equipped with a 25 mW argon laser (488 nm emission filter, photomultiplier voltage 1000 volts). Cells were distinguished by the combination of low angle forward scattered and right angle side scattered laser light. Debris and aggregates were excluded from the analysis as well as remaining peripheral blood cells different from monocytes. At least 10,000 cells were examined and results were expressed as the mean fluorescence of the cell population, using a logarithmic amplifier. The viability of the cells was checked by Trypan blue exclusion at the beginning and the end of each experiment.

3. Results

3.1. The infection of RA/VD-differentiated U937 cells by S. typhimurium triggers a release of extracellular O$_2^-$

The oxidative burst triggered by S. typhimurium in RA/VD-differentiated U937 cells, in the presence of NHS, was investigated. Fig. 1 shows that more than 80% of the luminol-enhanced chemiluminescence was inhibited in the presence of SOD, a large molecular scavenger of O$_2^-$ that has no access to intracellular compartment. On the other hand, the oxidative response remained unchanged in the presence of catalase (a scavenger of H$_2$O$_2$) or DMSO (a scavenger of hydroxyl radical). Lucigenin-enhanced chemiluminescence, which specifically detects extracellular O$_2^-$, was also measured. These results indicate that the oxidative response triggered by S. typhimurium in RA/VD-differentiated U937 cells is essentially constituted by an extracellular release of O$_2^-$.

3.2. The oxidative response of human monocytes differs according to the stimulus used, but extracellular O$_2^-$ cannot be detected

The oxidative response of monocytes was assessed by the luminol-enhanced system developed by Dahlgren and co-workers [19–21]. This system discriminates between the intra- and extracellular parts of the response. The intracellular response was measured in the presence of SOD and catalase, large molecular scavengers that do not enter the cells. The measurement of the extracellular response was based on the fact that the luminol-enhanced chemiluminescence is myeloperoxidase (MPO)-dependent [22–24] and therefore completely inhibited in the presence of azide, a potent inhibitor of MPO. The addition of exogenous HRP, a large molecule insensitive to azide, restores only the extracellular response. A lucigenin-enhanced system was simultaneously used.
to detect the specific extracellular production of $O_2^{•−}$ [25]. Our results show that the localization of the response varies according to the stimulus used (Fig. 2). No significative inhibition occurred in the presence of SOD and catalase nor was the azide-inhibited response restored by the addition of HRP. Thus, the oxidative burst elicited by \textit{S. typhimurium} in the presence of NHS was essentially intracellular. On the contrary, when PMA was used as a stimulus, the oxidative response was essentially extracellular since it was inhibited by both SOD and catalase and, HRP restored the azide-inhibited response. The intensity of the response triggered by PMA was approximately 4-fold lower than those induced by the bacteria but was increased in the presence of HRP. As luminol requires MPO for the enhancement of chemiluminescence, such a result seems to indicate that, during PMA stimulation, MPO was inadequately released from the cells and was therefore a limiting factor for the detection of oxygen species in the extracellular compartment. Finally, the response induced by opsonized zymosan was both extra and intracellular with a global intensity similar to that triggered by \textit{S. typhimurium}. Whatever the stimulus used, no extracellular $O_2^{•−}$ production could be detected with the lucigenin-enhanced system. These results indicate that the extracellular fraction of the oxidative burst developed by monocytes and inhibited by the SOD/catalase mixture was essentially $H_2O_2$. We confirmed this by showing that, in the luminol-enhanced system, catalase alone was responsible for the inhibition of the extracellular responses (data not shown).

### 3.3. RA/VD-differentiated U937 cells respond to different stimuli by excretion of $O_2^{•−}$

The oxidative burst developed by RA/VD-differentiated U937 cells was investigated using the double-system described above. Whatever the stimulus used, RA/VD-treated U937 cells produced a response detected by lucigenin and inhibited by the

### Table 1
Localization of the oxidative response triggered by different stimuli in RA/VD-differentiated U937 cells (cf. legend Fig. 2). Some controls were performed in the presence of HRP.

<table>
<thead>
<tr>
<th></th>
<th>PMA</th>
<th>OZ</th>
<th>Stm</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Luminol</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Assay</td>
<td>11.54(± 29.5)</td>
<td>14996(± 802)</td>
<td>1179(± 174)</td>
</tr>
<tr>
<td>+ HRP</td>
<td>4365(± 772)</td>
<td>58239(± 6184)</td>
<td>9901(± 1434)</td>
</tr>
<tr>
<td>+ azide</td>
<td>41(± 30)</td>
<td>77(± 25)</td>
<td>38(± 15)</td>
</tr>
<tr>
<td>+ azide/HRP</td>
<td>375(± 114)</td>
<td>1774(± 210)</td>
<td>159(± 28)</td>
</tr>
<tr>
<td>+ catalase/SOD</td>
<td>99(± 42)</td>
<td>104(± 49)</td>
<td>135(± 45)</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>52(± 15)</td>
<td>104(± 13)</td>
<td>8376(± 179)</td>
</tr>
<tr>
<td>+ HRP</td>
<td>406(± 191)</td>
<td>8376(± 179)</td>
<td>8376(± 179)</td>
</tr>
<tr>
<td><strong>Lucigenin</strong></td>
<td>514(± 119)</td>
<td>763(± 771)</td>
<td>1358(± 172)</td>
</tr>
<tr>
<td>Control</td>
<td>53(± 21)</td>
<td>763(± 771)</td>
<td>1238(± 65)</td>
</tr>
</tbody>
</table>

Fig. 2. Localization of the oxidative response triggered, in human monocytes, by different stimuli: PMA, opsonized zymosan (OZ) and \textit{S. typhimurium} (Stm). The addition of azide/HRP and SOD/catalase to the luminol-enhanced system allowed the separation between the extra- and intracellular parts of the oxidative response, respectively. The lucigenin-enhanced chemiluminescence, which specifically detects extracellular $O_2^{•−}$ was also measured. When bacteria were used as a stimulus, NHS (5%, v/v) was added. Controls were performed in the absence of stimuli. Results (mean ± S.D. of three separate experiments) were expressed as the integral of counts per 10⁷ cells during 15 or 30 min depending on the stimulus used.
SOD/catalase mixture and by SOD alone (not shown) in the luminol-enhanced system, showing that it was extracellular $O_2^-$ (Table 1). When lucigenin was used to enhance the chemiluminescence, quantitative variations were seen depending on the stimulus used, opsonized zymosan being the most potent inducer. Such variations were not detected in the presence of luminol unless exogenous peroxidase was added. Indeed, the quantities detected in the presence of luminol plus HRP were similar to those obtained with lucigenin. These results indicate that a low extracellular concentration of MPO can greatly limit the possibilities of the luminol-enhanced system. However, the addition of HRP can restore the sensitivity of this system. The HRP-amplified response could be inhibited by SOD alone (data not shown), therefore, in the presence of exogenous peroxidase, luminol can be as efficient as lucigenin for detecting extracellular $O_2^-$. Moreover, these results imply that, in the absence of $H_2O_2$, MPO may use $O_2^-$ as a substrate.

Despite arguments in favour of the extracellular nature of the oxidative burst developed by RA/VD-differentiated U937 cells, the addition of HRP could not restore the response inhibited by azide. This result can be explained by the fact that azide is also a potent scavenger of singlet oxygen [26]. $O_2^-$ formation results from the interaction of molecular oxygen with the NADPH-oxidase. Spontaneously reversible formation of singlet oxygen may occur at the time of an increase in energy, for example during phagocytosis. It is thus possible that azide prevents $O_2^-$ formation by scavenging the singlet oxygen. In order to verify this hypothesis, the action of azide on the lucigenin-enhanced system was tested. Using polymorphonuclear cells or monocytes, this system has been described as MPO-independent and thus insensitive to azide [25]. The lucigenin enhanced chemiluminescence intensity remained unchanged in the presence of HRP when RA/VD-differentiated cells were used (data not shown). Consequently, if azide inhibited such a reaction, it would not be by inhibiting MPO but rather by scavenging singlet oxygen. Our results show that both azide and histidine, an other scavenger of singlet oxygen [27], inhibited the lucigenin-enhanced chemiluminescence (Table 2). Since such a system specifically detects extracellular $O_2^-$, the observed inhibition strongly suggests that the consumption of singlet oxygen by scavengers such as azide or histidine prevents the formation of $O_2^-$. 

### Table 2

<table>
<thead>
<tr>
<th></th>
<th>PMA</th>
<th>OZ</th>
<th>Stm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azide</td>
<td>39.0 (+0.7)</td>
<td>73.8 (+0.9)</td>
<td>72.6 (+1.3)</td>
</tr>
<tr>
<td>Histidine</td>
<td>51.4 (+0.23)</td>
<td>95.1 (+1.1)</td>
<td>91.3 (+0.6)</td>
</tr>
</tbody>
</table>

Fig. 3. Cytofluorometric assay of the oxidative burst developed by either monocytes (solid line) or differentiated-U937 cells (dashed line) in response to different stimuli. Cells were pre-incubated at 37°C and each stimulus (PMA, O; opsonized zymosan, O; S. typhimurium, O) was added at $t = 0$. Dihydrorhodamine 123 was added at $t = 10$ min. Assays were performed at $t = 15$ and $t = 20$ min. Controls, with (■) or without NHS and in the absence of stimulus, were run in parallel. Results were expressed as the mean ($\pm$ S.D.) fluorescence of the cell population from three separate experiments.

3.4. Cytofluorometric assay of the oxidative burst developed by human monocytes and RA/VD-differentiated U937 cells

To confirm our results, the oxidative burst was investigated by a cytofluorometric technique. Fig. 3 shows that, whatever the stimulus used, RA/VD-differentiated U937 cells failed to develop an intracellular oxidative burst. On the contrary, the intensity of the intracellular oxidative response of monocytes depended on the stimulus used: a weak re-
response to PMA, a strong response to bacteria and an intermediate response to opsonized zymosan. These results were in agreement with those obtained in luminometry.

3.5. RA/VD-differentiated U937 cells show a delayed closure of the phagosomal compartment

Cytocentrifuged preparations of RA/VD-differentiated U937 cells infected by *S. typhimurium* were carefully examined. A lack of total closure of the phagocytic vacuole was observable, at least in some infected cells. Fig. 4 shows that the phagosomes remained linked to the extracellular compartment by a channel which could allow a direct excretion of \( \text{O}_2^- \). Such a phenomenon was impossible to detect, in the same conditions, with human monocytes.

4. Discussion

Our results show that the oxidative burst developed either by human monocytes or RA/VD-differentiated cells differed in its nature and/or location. In human monocytes, the oxidative response varied in its location depending on the stimulus used. *S. typhimurium* produced an intracellular burst, while PMA induced extracellular \( \text{H}_2\text{O}_2 \) and opsonized zymosan triggered both an intra- and extracellular oxidative response. In RA/VD-treated U937 cells, the burst was characterized by extracellular \( \text{O}_2^- \), whatever the stimulus used, and a delayed closure of the phagosome of infected cells was observed. On the contrary, within monocytes, *S. typhimurium* seemed to be in a completely closed vacuole in which the reactive oxygen species remain localized. This mechanism focuses the microbicidal arsenal on the phagocytosed bacterium and protects neighbouring tissues from oxidative injury. Oxygen species may diffuse across the biological membranes and could also affect intracellular components [28]. The cell, however, has evolved a potent system of self-protection; outside the phagosome, the dismutation of \( \text{O}_3^- \) is considerably increased by the cytosolic SOD and the resulting less toxic \( \text{H}_2\text{O}_2 \) is then hydrolysed by catalase or glutathion peroxidase. Therefore, an absence of detectable extracellular oxygen species may occur during the interaction between monocytes and bacteria. In the case of opsonized zymosan, the number and the size of the phagocytosed particles as well as a greater oxidative response could lead to a
fraction of the H₂O₂ produced escaping the enzymatic neutralization and being released outside the cell. More intriguing is the difference in the production of extracellular oxygen species in response to a soluble stimulus such as PMA, by the two types of cell. The absence of detectable extracellular O₂⁻ in the case of monocytes, strongly suggests that the O₂⁻ produced is dismutated inside the cell and that most of the resulting H₂O₂ escapes the enzymatic hydrolysis to be released outside the cell. The hypothesis that extracellular release of SOD can happen only in the case of monocytes seems unlikely. Despite the fact that cancer cells generally contain less SOD than normal cells, the process of differentiation allows them to recover an optimal level of SOD [29]. Moreover, SOD is an intracellular, high molecular weight enzyme and, even during inflammatory states, the levels of extracellular SOD are extremely low [13,14]. The quasi-absence of extracellular SOD is corroborated by the fact that the administration of low doses of SOD gave encouraging results in the treatment of the oxidative injury following myocardial ischemia reperfusion [30]. Consequently, the release by monocytes of most of the H₂O₂ produced by intracellular dismutation of O₂⁻ strongly suggests that PMA needs to enter the cell before triggering the oxidative burst. This internalization of PMA was confirmed by the weak intracellular response observed in flow cytometry within PMA-elicited monocytes.

Taken together, our results lead to the definition of two different mechanisms of production of extracellular oxygen species. First, H₂O₂, which results from the spontaneous intracytosolic dismutation of the O₂⁻ (released from a closed phagosome) passively diffuses across the cell membrane. Second, O₂⁻ is directly excreted from a still open phagosome. The excretion of O₂⁻ certainly requires a highly energetic mechanism to prevent the immediate closure of the vacuole by fusion of the membrane. Whereas the mechanism of membrane fusion remains still largely unknown it has been suggested that the conformation of proteins involved in fusion may change, showing an unfolded conformation during the step of fusion and being folded again, certainly by a highly energetic mechanism [31].

Our results suggest that, during the oxidative burst developed by the RA/VD-differentiated U937 cells, the oxygen was spontaneously and transitionally excited to form singlet oxygen. It is therefore possible that the high energetic level characteristic of this oxidative burst may prevent membrane fusion by favouring the folded configuration.

The excretion of O₂⁻ from an open phagosome could be a characteristic of activated cells. This hypothesis is supported by the fact that the peripheral blood-derived monocyte, incapable of excreting O₂⁻ in our experimental conditions, is considered as being a quiescent cell. Moreover, the production of extracellular O₂⁻ by the macrophage has been shown to increase as a function of its activation state [32,33]. Besides having potentially microbicidal activity, the excreted O₂⁻ could constitute a specific signal of activation for other cellular or non-cellular systems occurring during inflammation, this however at the detriment of neighbouring structures. Indeed, the production of small quantities of O₂⁻ by non-phagocytic cells such as fibroblasts and endothelial cells has been observed [34] and we reported an oxidative burst in PMA-activated lymphocytes [35]. In such cases O₂⁻ cannot exert a microbicidal function, and its role remains to be elucidated. Since RA/VD-differentiated cells essentially produce extracellular O₂⁻, they may be a good model for studying the non-microbicidal functions of O₂⁻. Another interesting aspect of our cellular model is its contribution to a better understanding of the interaction between luminol and oxygen species. Our results show that luminol could be as efficient as lucigenin for the detection of extracellular O₂⁻ providing that the extracellular concentration of peroxidase was sufficient. These findings refute the generally accepted interpretation of the role of luminol as the preferential, if not exclusive, detector of H₂O₂ in the presence of peroxidase [26,36]. Our model also shows the limitations of the use of azide in the specific detection of an extracellular oxidative response. While Dahlgren’s system has proven efficacy in the case of neutrophils and monocytes, its use gives contradictory results in the case of RA/VD-differentiated U937 cells. However, the simultaneous measurement of lucigenin-enhanced chemiluminescence in order to specifically detect extracellular oxygen species can improve such a system of investigation.

RA/VD-differentiated U937 cells constitute, to
our knowledge, the first cellular model essentially able to produce $O_2^-$, This oxygen species is of particular interest since it is the first to be produced during the oxidative burst. Consequently, the detection of $O_2^-$ directly reflects the activation state of NADPH-oxidase whose hyperactivation could lead to severe oxidative injury. Very few molecules are now available to regulate NADPH-oxidase activity. Our model could allow the screening of such molecules in more physiological conditions, replacing the acellular generators of $O_2^-$ currently used.

References


