Automated Image Analysis for Monitoring Oxidative Burst in Macrophages

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OBJECTIVE: To evaluate oxidative bursts induced by phorbol myristate acetate in phagocytes at the single-cell level by automated image analysis.
STUDY DESIGN: The generation of reactive oxygen species was quantitatively expressed by means of histograms displaying the percentage of cells corresponding to each of the total optical densities measured.
RESULTS: Macrophage subpopulations were quantitatively defined. This method allows detailed analysis of the amount of formazan per cell and the sites of deposition of blue precipitate in each cell.
CONCLUSION: Image analysis is a reliable quantitative, single-cell assay for studying various cellular characteristics associated with macrophage functions. (Analyt Quant Cytol Histol 2000;22:423–427)

Keywords: image analysis, computed-assisted; oxidative burst; macrophages.

A characteristic feature of phagocytes is their ability to respond to appropriate stimuli by activation of a respiratory burst. This process has been described for all kinds of monocyte-derived cells.[1]

The respiratory burst involves increased production of reactive oxygen species (ROS), mainly superoxide anion ($O_2^{\cdot-}$), from reduction of O_2 by NADPH oxidase on the surface of the plasma membrane. The nitroblue tetrazolium (NBT) reduction test is one of the most commonly used assays to measure the superoxide anion produced by phagocytes during the respiratory burst.[2] NBT is a yellowish, water-soluble compound that enters freely into cells.[3] It is reduced stoichiometrically by superoxide anion, yielding intracellular insoluble formazan. This intracellular redox reduction product can be visualized in phagocytic vacuoles by light microscopy as dark blue granules.[4,5] This process

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can be experimentally induced by soluble agents, such as phorbol myristate acetate (PMA).[6] The most widely used method to evaluate this reaction involves assessment of NBT by spectrophotometry.[7] This method yields average cellular data and is not appropriate for single-cell detection because it lacks the accuracy needed to reveal the heterogeneity of macrophage populations. It also fails to detect heterogeneous deposition of formazan precipitates in each cell, an issue of particular importance in certain pathologic conditions.[8,9] Thus, detection and quantification of these oxygen species is of great interest to determine the association between elevated oxidative stress and pathologic conditions.

This study attempted to validate the use of cell digital image analysis to perform direct measurements of the amount of formazan precipitated as an expression of the $O_2^{\cdot-}$ generated during a respiratory burst on a per-cell basis. This methodology would contribute to the evaluation of other aspects of cell metabolism in which the individual cell production of active oxygen species is involved.

Materials and Methods

Chemicals

NBT, PMA, Dulbecco's buffered salt solution (DBSS), dimethylsulfoxide (DMSO), and polyvinylpyrrolidone (PVP) were purchased from Sigma Chemical Co., St. Louis, Missouri, U.S.A.

Animals

Wistar rats were bred at the breeding facility of the National Atomic Energy Commission and housed in a controlled environment. Food and drink were provided *ad libitum*.

Alveolar Macrophage (AM) Isolation

Pulmonary AMs from Wistar rats were harvested by broncheoalveolar lavage as described elsewhere.[10] The lungs were cannulated and lavaged repeatedly with cold DBSS. Lavage fluid was collected and centrifuged at 800 g for 10 minutes at 4°C. Cells were resuspended in DBSS, counted in a hemocytometer and separated into three fractions. Viability of AMs was determined by the trypan blue dye exclusion test (>95%).

Quantitative Measurement of $O_2^{\cdot-}$ Release

$O_2^{\cdot-}$ released during the respiratory burst was evaluated using the NBT reduction test as previously described.[11] Cell suspensions obtained from each animal were separated into three fractions:

Fraction 1 (control):	AMs + 1 mL DBSS.
Fraction 2 (nonstimulated cells):	AMs + 1 mL NBT + 10 µL acetone.
Fraction 3 (PMA-stimulated cells):	AMs + 1 mL NBT+10 µL PMA in acetone.

After 30 minutes of incubation, cells were processed for both cell digital image analysis and spectrophotometry, as described below.

Spectrophotometry

Two milliliter of HCl (0.8%) was added to all fractions for 20 minutes to stop the NBT/PMA reaction. Cell suspensions were immediately centrifuged and resuspended in DMSO to solubilize the formazan precipitate. The optical density (OD) of the final solution was determined at 570 nm against a DMSO reference in a double-beam GBC Scientific Equipment (Victoria, Australia) ultraviolet visible spectrophotometer.[7]

Cell Digital Image Analysis

A sample of 150 µL from fractions 1, 2 and 3 was fixed in glutaraldehyde (8% in DBSS), centrifuged and resuspended in 100 µL PVP (10% in DBSS). Cell smears were performed on glass slides, air dried, methanol fixed and mounted in glycerine. Samples were observed under an MPM 800 Carl Zeiss microscope (Jena, Germany) using an interferential filter (570 nm) and a plan achromat 40:1/0.75 objective coupled to a DK 7700 SXK camera (Hitachi, Hamburg, Germany). Images were digitized in 8 bits and analyzed with an image analyzer system (IBAS-Kontron, Jena, Germany). Ten fields of approximately 20 cells each were selected at random to evaluate 200 macrophages per slide. The light and background intensity was determined for each sample.

The software employed allows scanning and evaluating single cells. It affords data on the total OD (TOD) of preestablished areas obtained by adding the ODs for all pixels corresponding to that particular area. The optical system is configured in such a way that once the image is digitized, 1 μm^2 of the sample corresponds to an array of 4×4 pixels. Thus, the TOD value per cell corresponds to the sum of all OD values obtained from scanning the whole cell.

Results

Photomicrographs of stimulated and nonstimulated AMs sampled from fractions 1–3 are shown in

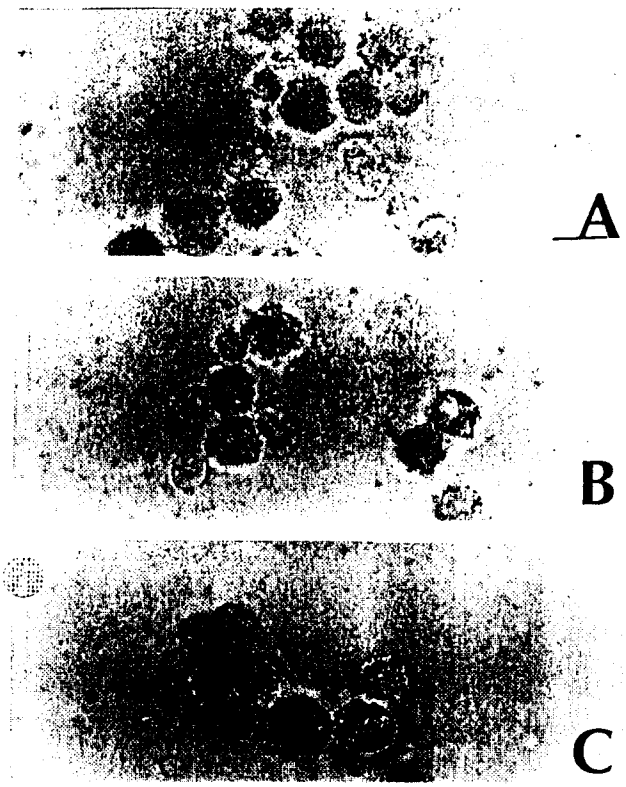


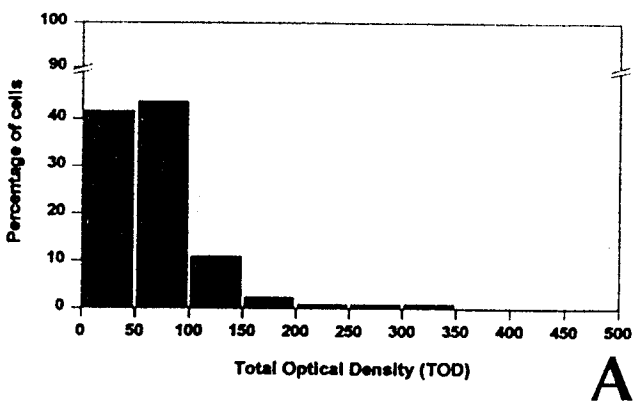
Figure 1 Light micrographs of AMs. (A) Fraction 1, control, nontreated macrophages. (B) Fraction 2, nonstimulated, NBT-treated macrophages showing a basal reaction. (C) Fraction 3, PMA-stimulated, NBT-treated macrophages confirming a heterogeneous population with varying degree of reaction. —= 10 μm.

Figure 1A, 1B and 1C, respectively. In Figure 1A, none of the cells exhibit a reaction. In Figure 1B most cells are nonreactive, although a few of them do exhibit scattered, light blue granules of formazan precipitate, which represents the basal reaction in the AM population in the lung. Figure 1C shows ROS generation in the PMA-stimulated population of AMs. This microphotograph shows that a larger proportion of cells responds to this stimulus and that the reaction is more intense. Moreover, not all the AMs population reacts to the same extent, as evidenced by the wide heterogeneity among cells in terms of formazan production.

The ROS generation per cell can be quantitatively expressed by digital image cytometry, as shown in Figure 2. Typical histograms display the percentage of cells in the sample corresponding to each TOD value. In the nonstimulated fraction almost all the macrophages were nonreactive, falling into the 0–100 TOD range (original TOD values used), herein considered a basal reaction (Figure 2A), whereas in PMA–stimulated macrophages, several subpopulations can be defined. They depict TOD values > 100 and < 500. However, a proportion of cells was nonreactive, showing TOD values < 100 (Figure 2B).

Digitized cell images of PMA-stimulated and -nonstimulated representative macrophages are displayed in Figure 3A and B, respectively. These images afford information on the heterogeneity of the cell reaction showing differences in OD over the whole cell and allowing the evaluation of singled-out areas.

$O_2^{\cdot-}$ generation, expressed as the amount of NBT reduction product, was evaluated in AMs by both digital image analysis and spectrophotometry. The mean TOD obtained by digital image analysis was plotted as a function of OD per cell obtained by



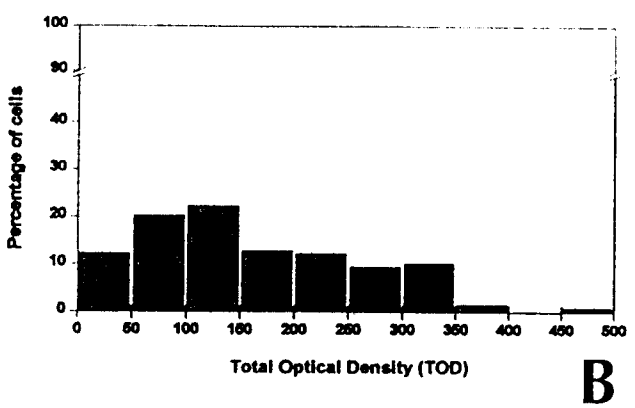


Figure 2 Histograms from digital image cytometry displaying the percentage of cells corresponding to each IOD range. (A) Nonstimulated macrophages (fraction 2 in Materials and Methods). (B) PMA-stimulated macrophages (fraction 3 in Materials and Methods).

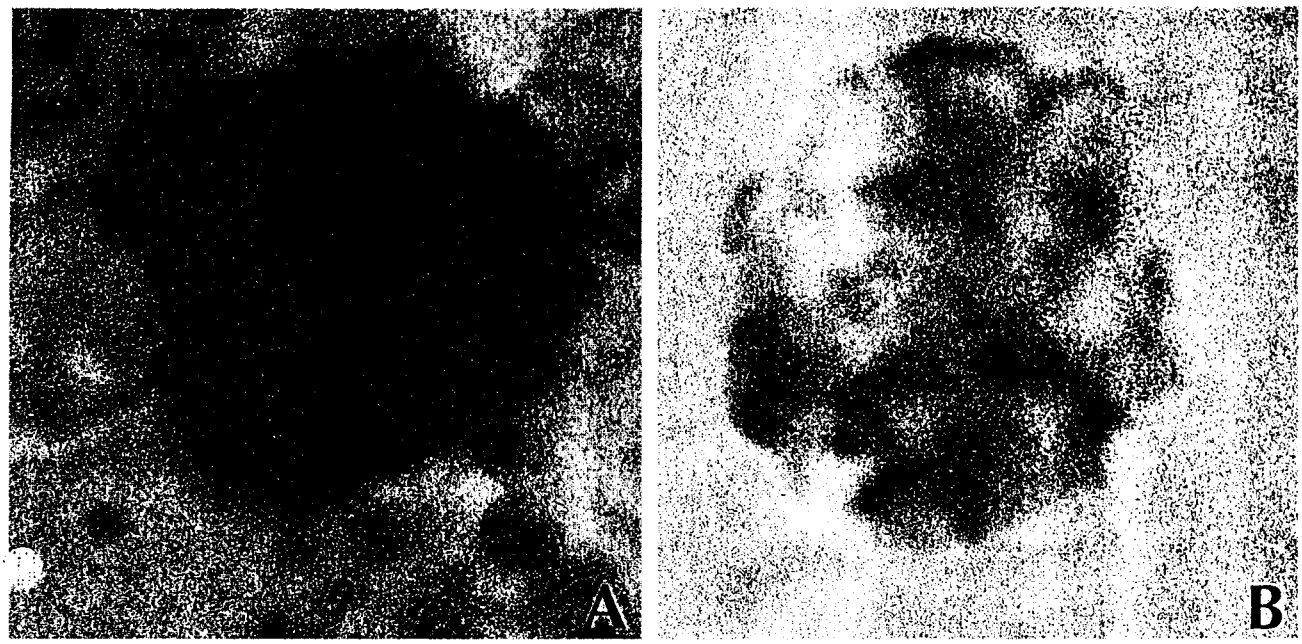


Figure 3 Graphic analysis of cell image digitalization of PMA-stimulated and -nonstimulated AMs. (A and B) Representative digitized macrophages from each experimental condition shown as they appear on the screen. Note the difference in cytoplasm density between (A) stimulated and (B) nonstimulated cells.

spectrophotometry. A significant correlation between both methods ($r = .92$, $P < .05$) indicates that digital image cytometry is a reliable quantitative method that allows analysis of different end points on a per-cell basis (Figure 4).

Discussion

It is well known that inflammatory and immunologic processes depend largely on the activation of the respiratory burst mechanism, which results in generation of $O_2^{\cdot-}$ and other active oxygen species.[12] The release of $O_2^{\cdot-}$ by cells can be revealed as the amount of formazan precipitated into the cell after tetrazolium reduction in the NBT test. As this method is based on a stoichiometrical reaction, it is possible to use it as a quantitative method to evaluate the $O_2^{\cdot-}$ generated during the respiratory burst. The magnitude of the $O_2^{\cdot-}$-generating capacity of phagocytic cells is physiologically regulated to produce an adequate effect without damaging the surrounding host tissues.[13,14]

Recent studies have demonstrated that within a cell population of phagocytes, individual cells respond to stimuli in a heterogeneous manner,[15] revealing the existence of functionally different subpopulations. In agreement with previous reports, we showed that the reduction of NBT to a formazan precipitate by macrophage populations is not homogeneous.

The quantitative assays of respiratory burst activity based on the release of $O_2^{\cdot-}$ are usually performed by spectrophotometry.[16] This technique affords information only on the average activity of a population of cells but fails to provide data on the reaction of each cell. Thus, these techniques are not appropriate to assess the production of oxidative species in individual cells. To accurately evaluate the different degrees of reaction at the single-cell level, a novel application of digital image cytometry is proposed here to quantify ROS generation during respiratory burst of AMs by means of NBT reduction.

The formazan precipitates into phagolysosomes,[17] which consequently do not distribute uniformly within the cell. When spectrophotometry is employed, the uneven distribution of the reaction within the cell becomes a problem because true OD values are underestimated. The digital cell analysis performed in this study clearly overcomes this drawback by calculating OD values pixel by pixel over the total cell area, thus minimizing distributional error by the high sampling rate. Furthermore,

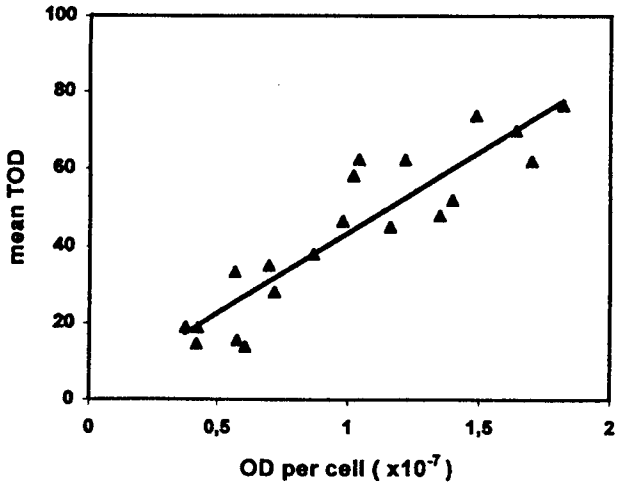


Figure 4 Mean TOD of 200 macrophages from each sample obtained by digital image analysis. OD per cell ($\times 10^{-7}$): OD obtained by spectrophotometry. OD is divided by the number of cells in that sample to obtain OD per cell. Thus, mean TOD as measured by digital analysis and OD per cell by spectrophotometry are obtained for macrophages from each animal for all experimental conditions. They are quantitative evaluations by two different methods that show a significant correlation coefficient ($r=.92$, $P<.05$).

the amount of stray light that can be estimated as approximately 1–3% in a videophotometer would affect pixels only with very high OD values. As in this model, only a few pixels have very high OD values; this issue implies a minor correction for the total OD of macrophages.

This method, based on image analysis of individual cells, allows discrimination of subpopulations of cells with varying degrees of reaction in a highly reproducible manner.[13] This technical approach proved to be more analytical in that it reveals responses that are masked when the analysis is carried out by means of averaging methodologies, such as spectrophotometry.

In conclusion, image analysis is a reliable automated, single-cell assay for the quantification of respiratory burst activity in macrophages as well as in other cells of mononuclear lineage. This technique allows the evaluation of oxidative metabolism of AMs both at the individual cell level and over the whole cell population, providing a useful method of studying cellular features associated with macrophage functions.

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