CHAPTER 12

Lysosomal Proton Pump Activity: Supravital Cell Staining with Acridine Orange Differentiates Leukocyte Subpopulations

Frank Traganos and Zbigniew Darzynkiewicz
Cancer Research Institute
New York Medical College
Valhalla, New York 10523

I. Introduction

Lysosomes are cellular organelles which store a variety of digestive enzymes, effectively segregating these potentially harmful hydrolytic constituents from the rest of the cell. This chapter will discuss the use of supravital cell staining with acridine orange to differentiate leukocyte subpopulations based on their lysosomal proton pump activity.
the rest of the cellular cytoplasm. Lysosomes are known to be internally acidic and to require metabolic energy to maintain an intralysosomal pH of about 4.8 (Ohkuma and Poole, 1978). It appears that an ATP-dependent proton (H⁺) pump is responsible for the internal acidic environment of lysosomes (Yamashiro et al., 1983).

Many uncharged, lipophilic substances are able to cross biological membranes by an unselective permeation mechanism (Rundquist et al., 1984). Some of these substances, such as the fluorescent dye acridine orange (AO), are weak bases. At acid pH, weak bases will accept a proton and be converted to a positively charged substance which is no longer capable of passing freely through cellular membranes (de Duve et al., 1974). Thus, AO, like other lysosomotropic agents, will accumulate and become trapped within the lysosomes of living cells (Allison and Young, 1964; Robbins and Marcus, 1963; Robbins et al., 1964; Rundquist et al., 1984; Zelenin, 1966).

Generally, little is known of AO's intracellular targets in living, nonpermeabilized cells. Nevertheless, nucleic acids within living cells exposed to relatively low concentrations of AO (<10⁻⁵ M) will bind the dye as monomers whereas lysosomes will concentrate the dye. The intralysosomal dye concentration is predominately determined by the pH gradient across the membrane (de Duve et al., 1974) which, in turn, is a reflection of the efficiency of the ATP-dependent proton pump (Yamashiro et al., 1983). The granular, bright red luminescence of AO concentrated in lysosomes of living cells is thought to be the result of the high concentration of dye generated in lysosomal granules which may form stacked dye aggregates. Aggregates of AO are known to luminesce red (Kapuscinski and Darzynkiewicz, 1984) as compared to the normal green fluorescence associated with the monomer form of the dye that would be expected to predominate at low AO concentrations (Darzynkiewicz and Kapuscinski, 1990). Under appropriate staining conditions, the intensity of AO red luminescence is a function of both total lysosomal volume and the capacity of the lysosomal membrane to maintain a proton gradient (Rundquist et al., 1984).

AO will also concentrate in the azurophilic granules of supravitally stained granulocytes (Abrams et al., 1983). These granules are similar to lysosomes, concentrate AO, and luminesce red when excited with blue light. In both instances, however, loss of membrane integrity, either occurring naturally as cells die or as a result of fixation or following permeabilization with detergents, abolishes the metachromatic staining of these organelles (Darzynkiewicz and Kapuscinski, 1990).

II. Applications

A. Cell Viability

Supravital staining of cells with AO to detect lysosomal proton pump activity can be used to test cell viability following exposure to a variety of environmental
insults and as a measure of general cellular integrity following treatment with toxic agents including chemotherapeutic drugs (e.g., Del Bino et al., 1991).

B. White Blood Cell Differential

Staining of leukocyte granules has been used to provide automated white cell differentials of lymphocytes, monocytes, and granulocytes from whole blood (Melamed et al., 1972a,b) and to assay neutrophil degranulation (Abrams et al., 1983).

C. Marker of Cell Differentiation

Lysosomal activity has been observed to vary with the degree of cell differentiation. For instance, both in the case of some granulocytic leukemias (Melamed et al., 1972b) and during acute bacterial infections (Melamed et al., 1974), large numbers of immature granulocytes appear in the peripheral blood. Such immature granulocyte elements had characteristically increased red luminescence compared to more mature (segmented) granulocytes typically observed in peripheral blood (Melamed et al., 1972b, 1974).

Lysosomal proton pump activity can also be used as a marker for the later stages of differentiation induced by various agents in some model systems. Thus, human myelogenous leukemic HL-60 cells differentiate into either granulocytes or monocyte/macrophages depending upon the type of inducer used. AO staining would be expected to differ depending upon which cell lineage was induced and the extent of differentiation within that lineage.

D. Functional Marker of Lysosomal Activity

Although largely unexplored, AO may be used to test the effects of various environmental stimuli on the activity of lysosomes. Thus, some drugs may affect lysosomal activity directly or indirectly. Lysosome activity is also likely to be affected by conditions which enhance or diminish cellular phagocytosis and/or pinocytosis. Some of these experiments have been carried out in isolated lysosomes in solution (Moriyama et al., 1982).

III. Materials

A stock solution of AO should be made up in distilled water at a concentration of 1 mg/ml. Because AO can contain up to 50% impurities, it is important to utilize a chromatographically purified form as is available from Polysciences, Inc. (Warrington, PA; catalog No. 4539). The stock solution is stable for several months at 4°C.
IV. Staining Procedures

A. Lysosomes

AO staining for lysosomal protein pump activity should be done under conditions of optimum cell growth. AO should be added to cells in growth medium plus serum at a concentration of 1–2 µg/ml by direct dilution from the stock solution. Cells can then be incubated at 37°C for 30 min or longer. It is neither necessary nor desirable to rinse cells with AO-free medium or balanced salt solution prior to measurement.

B. Leukocyte Differential

While it is possible to obtain differential staining of leukocyte populations using buffy coat or density gradient-separated leukocytes, the original staining reaction was performed with whole-blood preparations and remains simple and reproducible within relatively wide ranges of cell concentration (Melamed et al., 1972a).

A 1 mg/ml stock solution of AO is prepared as above. The dye is diluted to a concentration of 1 µg/ml in an isotonic buffer solution of pH 7.4 (Melamed et al., 1972a). One part whole blood is then added to 25 parts AO solution and the mixture allowed to stand at room temperature for 6–8 min before flow cytometric analysis.

V. Critical Aspects of the Procedure

A. Cell Viability

Generally, at low concentrations of AO (<10^{-5} M), only live cells accumulate the dye in lysosomes or azurophilic granules (which luminesce red) and dead cells stain uniformly green. At that low AO concentration, the green component probably reflects dye interaction with DNA and RNA (Darzynkiewicz and Kapuscinski, 1990). However, it should be stressed that, at higher AO concentrations (<10^{-4} M), dead cells stain rapidly and uniformly red (nucleus and cytoplasm) while live cells still have red lysosomes but green nuclei and cytoplasm. However, with time, as the intracellular concentration of AO increases in living cells they also stain uniformly red. Caution should therefore be exercised in interpreting data on supravital staining with AO. Clearly, to ensure appropriate staining of lysosomes to detect viable cells, the appropriate (low) AO concentration is important.

Unfortunately, the question of cell viability does not fit neatly into a two-compartment model, i.e., live or dead. Cells exposed to stress, be it as a
result of exposure to toxic chemicals, gases, or radiation or the withdrawal of necessary nutrients, may undergo a variety of physiological changes. Often these physiological changes represent a continuum ranging from no effect to immediate cell death. Cell death by necrosis means that the cell membranes have broken down. Such cells are no longer capable of concentrating dyes like AO in the lysosomal granules with the result that the remaining nuclei, with or without cytoplasmic tags attached, fluoresce green (see Section VIII, Results). In some instances, toxic agents (or removal of nutrients) will trigger a cascade of biochemical events leading toward specific morphological, biochemical, and molecular changes consistent with apoptosis (Darzynkiewicz et al., 1992). Early stages of the apoptotic process are often marked by activation of an endogenous protease and endonuclease which results in digestion of internucleosomal stretches of nuclear DNA (Arends et al., 1990). However, concomitant to nuclear DNA digestion, cellular membrane integrity, mitochondrial membrane potential, and lysosomal proton pump activity remain generally intact (Del Bino et al., 1991). Therefore, cells undergoing apoptosis will retain their ability to concentrate AO into red luminescing lysosomal granules, at least in the early stages of the process. As a result, populations deemed viable by supravital AO staining of lysosomes may contain some portion of cells which have no reproductive capability.

B. Leukocyte Differential

Supravital staining of leukocyte subpopulations with AO require appropriate adjustments to ensure that cell density is within an acceptable range. Generally a 1:25 dilution of whole blood into the staining solution is optimum when the typical leukocyte count is within the range of $5-25 \times 10^6$ mm$^3$. However, in instances in which leukemic blood is being stained, it may be necessary to first dilute the blood with a balanced salt solution to bring the cell count within the range noted above. It is also necessary, as will be illustrated below, to wait until the staining reaches equilibrium, which is invariably between 6 and 8 min. After reaching the initial staining equilibrium, the staining pattern is quite stable for up to about 20 min.

Spectral overlap between the green and red emission of the dye should be considered and use of appropriate dichroic mirrors and long-pass filters for the "red" PMT chosen accordingly. Since the green fluorescence is fairly weak in this procedure, its spillover into the red PMT channel is not as much a problem as with other AO staining techniques. However, most flow cytometers use a 590-nm dichroic mirror and 600-nm long-pass filter for the red PMT which is optimized for propidium iodide (DNA) or phycoerythrin (immunofluorescence). A more appropriate filter assembly would contain a dichroic filter reflecting (or transmitting) at 610 nm and a long-pass filter transmitting above 640 nm for optimum measurement of AO red luminescence.
VI. Controls and Standards

Controls and standards for lysosomal proton pump activity can take several forms. Normally, treated, exponentially growing cells will provide a positive staining control for AO red luminescence (see Section VIII, Results). Generally, such a population should be set at midway to two-thirds maximum on the red luminescence scale. This will leave room for the possibility that certain treatments actually increase lysosomal proton pump activity over basal levels. Alternatively, the low end of the red luminescence scale can be established with appropriately chosen fluorescent beads, with peripheral blood lymphocytes which tend to have little or no lysosomal activity when inactivated, or by the examination of the cell system following permeabilization of cellular membranes with a detergent (e.g., Triton X-100). Care should be exercised in using detergent-treated cells as controls if it is important to retain the cytoplasm since detergents dramatically increase the fragility of the cytoplasmic membrane. Alternatively, isolated nuclei would also provide an excellent control for the lower limit of red luminescence of a particular cell system.

Whole blood from a healthy donor provides an optimal control for supravital AO staining of peripheral blood leukocytes. Granulocytes which stain the brightest and are most variable in their AO red luminescence should be positioned slightly higher than midway along the red luminescence axis since (see Section VIII, Results), in some granulocytic leukemias and in instances of bacterial infections (Melamed et al., 1974), the red luminescence of the immature granulocyte population is skewed toward higher values.

VII. Instruments

Almost any flow cytometer with a light source capable of providing blue (~488 nm) excitation and able to simultaneously detect fluorescence emission at two separate wavelengths can be used for this assay, i.e., it has been successfully performed on Ortho Cytofluorographs with closed (nonsorting) channels, Ortho ICP 22s (utilizing a BG 38 filter to select the appropriate band of illumination from the mercury arc lamp), and a Becton–Dickinson FACscan. Generally, the shorter the distance traveled from the time the sample stream first comes into contact with the sheath flow and the intersection with the exciting light source, the easier and more straightforward the measurement. Often sorting channels because of their architecture tend to induce some interaction between sheath and sample streams which can adversely affect equilibrium staining with AO. Anything which affects (lowers) the dye concentration may result in too few dye molecules that, in turn, would inhibit formation of dye aggregates and/or dye-ligand condensation, diminishing or abolishing red luminescence.
An example of the use of the AO supravital staining technique to assay lysosomal function is displayed in Fig. 1. The human lymphocytic leukemia cell line (MOLT-4) was incubated for 30 min, at 37°C, with 1 μg/ml AO in RPMI 1640 medium supplemented with 10% fetal bovine serum as described previously (Traganos et al., 1993). The treated culture received 200 μM H7 (an isoquinoline sulfonamide from Seikagaku Kogyo Co., Tokyo) for 24 hr. From a 1 mg/ml stock solution, AO was added to each culture to achieve a final concentration of 2 μg/ml, and the cultures were returned to the CO2 incubator for 30 min at 37°C. Aliquots of cells were removed from the cultures at the end of the incubation and run on an Ortho Cytofluorograf System 30 flow cytometer. An air-cooled argon ion laser was used to provide blue (488 nm) light for excitation of the AO. Filters and a dichroic mirror separated the resultant emission into green (510–530 nm) and red (>640 nm) luminescence which was amplified, digitized, and recorded using the Acqcyte software from Phoenix Flow Systems (San Diego, CA). The green and red luminescence of 1 × 10⁴ cells was recorded and displayed as contour maps from control and H7-treated cultures. The approximate cell-cycle position of cells in control cultures is reflected in the green fluorescence whereas the red luminescence is proportional to the degree to which the cells were able to concentrate the stain in lysosomal granules. Treatment with H7 caused a decrease by more than 70% in the number of cells taking up AO intralysosomally.

Fig. 1 The AO lysosomal staining pattern of control, untreated and H7-treated MOLT-4 cells. The cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum as described previously (Traganos et al., 1993). The treated culture received 200 μM H7 (an isoquinoline sulfonamide from Seikagaku Kogyo Co., Tokyo) for 24 hr. From a 1 mg/ml stock solution, AO was added to each culture to achieve a final concentration of 2 μg/ml, and the cultures were returned to the CO2 incubator for 30 min at 37°C. Aliquots of cells were removed from the cultures at the end of the incubation and run on an Ortho Cytofluorograf System 30 flow cytometer. An air-cooled argon ion laser was used to provide blue (488 nm) light for excitation of the AO. Filters and a dichroic mirror separated the resultant emission into green (510–530 nm) and red (>640 nm) luminescence which was amplified, digitized, and recorded using the Acqcyte software from Phoenix Flow Systems (San Diego, CA). The green and red luminescence of 1 × 10⁴ cells was recorded and displayed as contour maps from control and H7-treated cultures. The approximate cell-cycle position of cells in control cultures is reflected in the green fluorescence whereas the red luminescence is proportional to the degree to which the cells were able to concentrate the stain in lysosomal granules. Treatment with H7 caused a decrease by more than 70% in the number of cells taking up AO intralysosomally.
complete tissue culture medium (RPMI 1640 plus 10% serum). The cells were removed directly from culture and their fluorescence was measured on an Ortho Cytofluorograf system 30. Dye excitation was provided by a 150-mW Omnichrome air-cooled argon ion laser. Green fluorescence and red luminescence of $2 \times 10^5$ cells were recorded using the Acqcyte program (Phoenix Flow Systems, San Diego, CA). The data were then plotted as contour maps of the correlated green fluorescence and red luminescence of the control (top) and treated (bottom) MOLT-4 cells. Treatment in this instance consisted of 24-hr exposure to 200 $\mu M$ H7, a serine/threonine kinase inhibitor which has been demonstrated to inhibit cell growth at lower concentrations (Traganos et al., 1993).

The green fluorescence distribution of control cultures actually provides information on the cell-cycle phase distribution. However, under these staining conditions, AO great fluorescence is not expected to represent DNA content as would be the case when using the AO staining reactions designed for permeabilized cells (Darzynkiewicz et al., 1975). Nevertheless, G0/G1 cells are expected to have the least green fluorescence, and as cells increase in size their green fluorescence is expected to increase. The red luminescence of untreated MOLT-4 cells should be proportional to the volume of the cellular lysosomal granules and the gradient across the lysosomal membrane. Treatment with H7, which in this instance resulted in complete loss of proliferative potential and a decrease in viable cell number, also caused a shift of AO red luminescence in 70% of the cells from the viable (V) compartment to the dead (D) cell compartment (Fig. 1). The remaining 30% of the treated cells had red luminescence values toward the low end of the viable cell compartment. Note that there is one single, though heterogenous, population. This is presumably the result of the fact that some cells have entirely lost their membrane integrity, some have leaky membranes, and a few cells are still intact and capable of concentrating AO in lysosomes. Such a response is not uncommon when dealing with agents which induce apoptosis. Thus, 100 $\mu M$ H7 caused cells to undergo apoptosis, which at 24 hr did not affect the ability of MOLT-4 cells to accumulate AO in lysosomes or exclude propidium iodide. It is not unusual that higher concentrations of agents which cause apoptosis induce necrosis in some or all cells depending on concentration and length of exposure. In this instance some 70–90% of the cells were nonviable based on AO and propidium iodide/rhodamine 123 cell staining, respectively (Traganos et al., 1993).

The use of the supravital AO staining technique to classify leukocyte populations from human peripheral blood is illustrated in Fig. 2. Whole blood from a healthy donor was stained as described above and the fluorescence recorded after incubation times were increased using an Ortho Cytofluorograf System 30.

As early as 3 min following addition of the blood to the staining solution it is possible to differentiate between the three main populations: lymphocytes with little AO red luminescence consistent with the presence of few lysosomes,
monocytes with a moderate amount of red luminescence, and granulocytes which have the highest and most variable amount of red luminescence. As the incubation time increased to 5 min, total separation was achieved for all three populations (Fig. 2). In addition, a subpopulation associated with the lymphocytes became more evident. This subpopulation has not been sorted but represents lymphocytes with a slight increase in cytoplasmic red (AO) luminescence that is often observed early during lymphocyte activation (Darzynkiewicz and Kapuscinski, 1990). Finally, by 7 min the increase in red luminescence plateaued.
and the separation between populations reached a maximum (Fig. 2). This pattern remained relatively stable for the next 10–15 min.

Acknowledgments

This work was supported in part by U.S. Public Health Service NCI Grants ROI CA28704 and R37 CA23296, as well as the Carl Inserra Fund and the “This Close” Foundation.

References