CHAPTER 25

Functional Measurements Using HL-60 Cells

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I. Introduction

In 1977 Collins *et al.* described a cell line that was derived from the peripheral blood of a patient with acute myelogenous leukemia (Collins *et al.*, 1977). These peripheral blood leukocytes, designated HL-60, are derived from the progenitors of granulocytes and monocytes which have undergone neoplastic transformation. Hence HL-60 cells resemble the blast cells of their lineage phenotypically (myeloblastic and promyelocytic cells with azurophilic granules) (De La Maza *et al.*, 1985; Harris and Ralph, 1985). However, more mature myeloid cells (myelocytes, metamyelocytes, bands, and segmented neutrophils) may also be seen (Collins *et al.*, 1977). HL-60 cell lines are heterogeneous, proliferate continuously in suspension culture, and exist in an arrested, yet pliant state of maturation (Dufer *et al.*, 1989). Proliferation is exponential with a doubling time of 34 hr up to a density of 3×10^6 cells/ml (Collins *et al.*, 1977; Cowen *et al.*, 1991).

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Exposure of HL-60 cells to a variety of agents induces terminal differentiation along either of two distinct pathways—one that bears many of the surface properties and functional characteristics of mature neutrophils and the other that resembles characteristics of monocytes/macrophages (Koeffler, 1983). This property of the cell line has been exploited by researchers in different laboratories to: (i) study differentiation of blast cells to granulocytes and macrophages, (ii) examine the genetic defects that may be responsible for the maturational arrest of leukemic cells *in vitro*, (iii) delineate the nature of their neoplastic transformation and define the conditions under which their malignancy can be reversed, and (iv) design therapy to induce maturation *in vivo* which might be expected to alter cell-cycle kinetics (Koeffler, 1983; Harris and Ralph, 1985; Ross, 1985; Thompson *et al.*, 1988; Dufer *et al.*, 1989).

Koeffler (1983) described many different types of maturation inducers. They can be classified as monocyte/macrophage-like differentiation inducers and granulocyte-like differentiation inducers. Agents that fall into the former category are phorbol diesters (Katagiri *et al.*, 1992), phospholipase C (PLC) (Cowen *et al.*, 1991; Madden *et al.*, 1992), teleocidins (Koeffler, 1983), cytokines, and vitamin D derivatives (Zhou *et al.*, 1991). Granulocyte-like differentiation inducers can be divided into physiologic and nonphysiologic inducers (Koeffler, 1983). The physiologic inducers are retinoids (Matzner *et al.*, 1987; Janick-Buckner *et al.*, 1991) and colony stimulating factors (CSFs) (Koeffler, 1983). The nonphysiologic inducers of differentiation are dimethyl sulfoxide (DMSO); purine and pyrimidine analogues; chemotherapeutic agents like actinomycin D, dibutyryl c-AMP bromodeoxyuridine, 5-azacytidine, 6-thioguanine, daunomycin, cytosine arabinoside, and vincristine; and P_2 purinergic receptor agonists (Koeffler, 1983; Matzner *et al.*, 1987; Thompson *et al.*, 1988; Cowen *et al.*, 1991). Calcium ionophores and phorbol diesters have been shown to act synergistically in inducing monocytic differentiation of HL-60 cells (Cowen *et al.*, 1991). Pilz *et al.* (1987) suggested that HL-60 differentiation can also be induced by starvation of a single essential amino acid. Granulocytic and monocytic differentiation inducers are known to increase the intracellular cAMP-dependent protein kinase C activity and decrease *c-myc* oncogene expression (Koeffler, 1983; Ross, 1985; Forsbeck *et al.*, 1985; Chaplinski and Niedel, 1986). Nitroprusside and $NaNO_2$, which activate cytosolic guanylate cyclase and increase the intracellular cGMP concentration, induce granulocytic differentiation of HL-60 cells (Boss, 1989).

II. Application

The properties described above can be used to establish a useful model for functional characterization of phagocytic cells. Parameters that are usually adopted as yardsticks of granulocyte differentiation are (i) increased oxidative

burst response to soluble as well as particulate stimuli, (ii) increased intracellular calcium mobilization in response to formylated chemotactic peptides, (iii) a reduction in cell size (see Fig. 1) with a decreased nuclear/cytoplasmic ratio, (iv) a sharply reduced rate of proliferation, (v) a reduction in the number of cells expressing transferrin receptors, (vi) increases in the percentage of cells expressing both type 1 (CR1) and type 3 (CD11b) complement receptor, and (vii) decreased esterase content. Changes suggestive of the monocyte/macrophage pathway are increased adherence, growth inhibition, decreased levels of *c-myc* mRNA, and expression of monocyte cell-surface markers (CD14) (Cowen *et al.*, 1991). Induced and uninduced HL-60 cell populations are heterogenous in their stages of differentiation. As induction causes a shift to a much higher proportion of mature cell types, all stages from promyelocyte to PMN are present in the culture, at each step of differentiation. Virtually all of the above functions can be relatively easily evaluated using flow cytometry.

III. Materials and Methods

The HL-60 cell line (ATCC) is maintained in RPMI medium 1640 (Sigma Chemical Company, St. Louis, MO). To make 200 ml total volume, the medium is supplemented with 5% fetal calf serum (Harlan Bioproducts for Science, Indianapolis, IN); 5% newborn calf serum (Sigma); 20,000 U penicillin, 20 mg streptomycin, and 50 μg amphotericin B (Sigma); and 2 ml 200 m*M* L-glutamine (Sigma) at 37°C, 5% CO_2, and cultured in 25-cm^2 flasks. Cell viability is assessed by trypan blue dye exclusion or by propidium iodide (PI) dye uptake using flow cytometry.

A. Estimation of Cellular Esterases

Enzymatic activities of cells or tissues can be determined with flow cytochemistry which offers some special advantages as a method of measuring myeloid maturation (Ross, 1986). With absorptive histochemical stains one can distinguish the various stages of myeloid maturation and differentiate between the monocytic and granulocytic maturation (Malin-Berdel and Valet, 1980; Thompson *et al.*, 1988).

Principle: The presence and approximate quantity of cellular esterases can be estimated by adding a dye such as carboxyfluorescein diacetate, which is rapidly hydrolyzed by the cellular esterases, and measuring the fluorescence of cells. The quantity of esterases is directly proportional to the stage of maturation of the HL-60 cells, especially when this cell line is induced to differentiate along the monocytic pathway. Cells induced to monocytic maturation have increased quantities of esterase. On the other hand, cells induced to differentiate along the granulocytic pathway have a reduced esterase content.

1. Reagents

Phosphate-buffered saline (PBS): Use fluorescent antibody (FA) Bacto buffer (Difco Laboratories, Detroit, MI), 100 g (bottle) and make up to 10 liters, pH 7.20.

Carboxyfluorescein diacetate (CFDA-AM) (Molecular Probes) (Cat. No. C-1354): Prepare a 12 m*M* stock solution of pure AR grade CFDA-AM in 1.0 ml spectrograde acetone (Watson, 1993). Keep this in the dark at −10°C. Add 50 μl stock CFDA-AM to 250 μl PBS. This is a 2 m*M* solution. Use 1 μl per 1 ml of cells for a final 2 μ*M* concentration.

Stock PBS gel:

EDTA (disodium salt) 0.2 *M*	7.604 g
Dextrose, 0.5 *M*	9.0 g
Gelatin, 10% (Difco)	10 g
Distilled water	100 ml.

To make PBS gel, heat water to 45–50°C and slowly add gelatin while mixing with a magnetic stirrer. Continue stirring and add EDTA and dextrose. Do not exceed 55°C because gelatin and glucose will "caramelize." Store in 1.2-ml aliquots at −20°C. Remove 1 ml to make 100 ml stock buffer.

Working solution PBS gel (make daily as needed): warm 1 ml gel to 45°C, add 95 ml warm PBS, and mix. Adjust pH to 7.4 and make up to 100 ml.

2. Instrumentation

Using appropriate beads calibrate linear green fluorescence (IGF) to the desired channel at a gain of 10 or 20. Collect forward light scatter (FALS) and IGF for each tube. Record settings each day. Select the population of interest using FALS-90° light scatter. Set the gates with unloaded cells (no CFDA-AM), then check the gates with CFDA-AM-loaded cells. Collect LOG green fluorescence if satisfactory log–lin conversions are available on the instrument. Excitation is 488 nm, and emission is collected with a 525-nm band pass filter.

3. Cell Preparation

Prepare a population of cells (1×10^6/ml) in PBS gel buffer. Warm the cells to the working temperature (37°C). Mix 1 ml of cells and 1 μl of dye and collect data for a specified time interval (e.g., 10 min).

4. Comments

When HL-60 cells are induced to granulocytic maturation, the number of cells that are positively stained for esterases will decrease daily. In contrast, their expression is upregulated during monocytic differentiation. Thus, ester-

ases are inducible and their measurement offers a useful marker of cell maturation.

B. Expression of CD11b Receptors

Studies on the regulation of expression of CD11b (MAC-1) during differentiation of HL-60 cells represent a valuable approach for characterization of the expression of leukocyte adhesion molecules during human myeloid differentiation. Surface changes that occur during the differentiation of myelomonocytic precursor cells to granulocytes and monocytes/macrophages can be visualized through the adoption of suitable *in vitro* models like HL-60 which can be induced to differentiate along the myelomonocytic pathway. The use of monoclonal antibodies directed against the surface antigens that show marked changes during differentiation enables separation of mature from immature cells (Perussia *et al.*, 1981; Janick-Buckner *et al.*, 1991; Back *et al.*, 1991) and provides a method to track the differentiation.

1. Principle

Differentiation of HL-60 cells along the granulocytic pathway results in enhanced CD11b surface antigen expression, consistent with high levels of expression of CD11b on human granulocytes. This CD11b molecule is noncovalently associated with a common β or CD18 unit which increases with differentiation along both granulocytic and macrophage pathways. The CD11b molecule is a membrane glycoprotein present on mature granulocytes and monocytes and is expressed by less than 10% of undifferentiated cells. These glycoproteins are thought to mediate binding and migration of leukocytes through the vascular endothelium, and attachment of C3bi-coated particles as in serum complement opsonized phagocytosis.

2. Reagents and Methods

OKM1 (Ortho Diagnostics, Raritan, NJ)

MO-1/FITC (Coulter Immunology, Hialeah, FL)

GAM-FITC (Goat-Anti-Mouse antiserum)(Caltag, San Francisco, CA)

Phosphate-buffered saline: To a 100-g bottle of Bacto buffer add distilled water to 10 liters, and adjust pH to 7.40

2% paraformaldehyde (PF): In a 500-ml flask, combine 10 g PF, 5 g (FA) Bacto buffer, and 400 ml distilled H_2O. Stir until solids dissolve. This may take several hours; the materials can be left on the stirrer overnight. When the solution has cleared, adjust pH to 7.3–7.4 and make up to 500 ml with distilled water. Place in a 500-ml bottle, cover with foil to protect from light, and store at 4°C.

3. Comment

Normally use PF with equal volumes of reagent to be fixed (final concentration of PF is 1%); however, we have found better phenotyping with HL-60 cells if the 2% PF is added directly to the vortexed cell pellet. This reagent is good for 1 month.

4. Indirect Immunophenotyping

After 48 hr of culture, obtain a cell count. Centrifuge cultures at 200*g* for 5 min at room temperature. Resuspend in PBS at 3–5 × 10^6/ml. Aliquot 100 μl cells into 12 × 75-mm tubes. Add 10 μl OKM1 to the cells and incubate at 4°C for 30 min. Add 10 μl of 1 : 10 dilution of GAM-FITC after the incubation. Add 2 ml of PBS to each tube. Spin at 250*g* for 10 min at 4°C. Aspirate supernatant, cap tubes, and vortex. Add 250 μl 2% PF while vortexing. Cover and store in refrigerator until ready to run on cytometer. Collect ungated list-mode data (FALS, 90° scatter, log FITC). Control samples should be labeled with GAM-FITC only, plus pooled human serum.

5. Direct Immunophenotyping

An antibody which is directly conjugated to a fluorescent label like FITC or PE can be used for this method. Add 10 μl of Mo1-FITC (1 : 8) (or similar antibody) to 100 μl of cells. Incubate for 30 min at 4°C. Add 2 ml PBS to the tube and spin at 250*g* for 10 min at 4°C. Aspirate supernatant EdE and vortex. Add 250 μl of 2% PF while vortexing to fix the samples. Cover and store in refrigerator until ready to run on cytometer. Proper isotypic controls should be used.

6. Instrumentation

Set up a two-parameter histogram (FALS-90° LS) to gate differentiated and undifferentiated cells. Set bitmaps on the population of interest. For instance, it might be important for drug screening studies to discriminate between differentiated and undifferentiated cells based on scatter (see Fig. 1). Set histograms for LOG FITC gated on FALS-90° scatter plot and collect 5000 events.

C. Oxidative Burst Measurements

Differentiated HL-60 cells are capable of demonstrating a respiratory burst after stimulation with PMA, FMLP, or other activating agents. Either H_2O_2 or O_2^- can be measured using standard flow cytometric methods. The techniques are described in detail in Chapter 28 in volume 41.

1. Materials

a. Hanks' balanced salt solution (HBSS)

i. Stock HBSS, 10× concentrated: NaCl, 40 g; KCl, 2.0 g; Na_2HPO_4, 0.5 g; $NaHCO_3$ 0.5 g; add distilled water to 500 ml.

ii. Stock Tris, 1.0 *M*: Tris base, 8.0 g; Tris–HCl, 68.5 g; add distilled water to 500 ml; adjust pH to 7.3.

iii. Preparation of 100 ml HBSS:

Stock HBSS, 10×	10 ml
Distilled water	80 ml
Tris, 1.0 *M*	2.75 ml
$CaCl_2$, 1.1 *M*	170 μl
$MgSO_4$, 0.4 *M*	200 μl
Dextrose	220 mg.

Adjust pH to 7.4 and add distilled water to 100 ml.

b. PBS gel (see previous description).

c. 2′,7′-Dichlorofluorescin diacetate (DCFH-DA, MW 487.2) (Molecular Probes Inc., Eugene, OR), 20 m*M* solution:

i. Weigh 2–9 mg of DCFH-DA and place in a foil-covered 12 × 75-mm tube.

ii. Add absolute ethanol in a milliliter volume equivalent to the weight in milligrams of the DCFH-DA divided by 9.74. For example, if you add 4 mg DCFH-DA add 4/9.74 = 402 μl of ethanol.

iii. Cap the tube, mix, cover in foil, and store at 4°C until use.

d. Hydroethidine (HE, MW 315) (Molecular Probes Inc., Eugene, OR), 10 m*M* solution: Stock solution 10 m*M* in dimethylformamide (3.15 mg/ml).

e. PMA (phorbol 12-myristate 13-acetate) (Sigma Chemical Co., St. Louis, MO): PMA is toxic and carcinogenic; additionally DMSO is readily absorbed through the skin. Wear gloves while handling solutions, prepare solutions in a hood, and be extremely cautious!

i. Stock PMA (2 mg/ml in DMSO): Mix well and aliquot 15–20 μl of stock PMA in small capped polypropylene bullets. Store at −20°C.

ii. Working PMA solution (make daily as needed): 5 μl PMA stock in 10 ml PBS gel (1000 ng/ml PMA solution). A final PMA concentration of 100 ng/ml will predictably result in maximal cell stimulation (for example, use 900 μl of cells in solution and 100 μl of working PMA solution). It is actually better to add a very small volume of activation reagent so as not to dilute the dye concentration. Thus it is preferable to add 1 μl/ml if possible. However, be aware that addition of very small volumes of fluorochromes may result in greater error.

2. DCFH-DA Assay

i. Centrifuge cells from one large flask (25 cm^2) to remove medium. Resuspend in PBS gel at 2×10^6/ml.
ii. Wash (250*g*) for 10 min and resuspend in HBSS plus 1% BSA. Final cell concentration should be 2.0×10^6 cells/ml.
iii. Add 1 μl 20 m*M* DCFH-DA per ml of cell suspension to be loaded.
iv. Incubate loaded cells at 37°C for 15 min.
v. Stimulate cells with PMA: add 100 μl PMA (working solution) to 900 μl of cell suspension (final PMA concentration 100 ng/ml). Reserve some loaded, unstimulated cell suspension for a control.
vi. Maintain cell sample at 37°C and run stimulated and unstimulated samples every 15 min on the cytometer for a total of 60 min.

3. Hydroethidine Assay

Procedures i and ii are identical to those above.

iii. Add 1 μl HE per ml of cell suspension to be loaded.
iv. Incubate loaded cells at 37°C for 5 min.

Procedures v and vi are identical to those above.

4. Combined DCFH-DA and Hydroethidine Assay

Procedures i and ii are identical to those above.

iii. Add 1 μl 20 m*M* DCFH-DA per ml of cell suspension to be loaded.
iv. Incubate loaded cells at 37°C for 15 min.
v. Add 1 μl HE per ml of cell suspension to be loaded.
vi. Incubate loaded cells at 37°C for an additional 5 min.
vii. Stimulate cells with PMA: add 100 μl PMA (working solution) to 900 μl of cell suspension (final PMA concentration 100 ng/ml). Reserve some loaded, unstimulated cell suspension for a control.
viii. Maintain cell sample at 37°C and run stimulated and unstimulated samples every 15 min on the cytometer for a total of 60 min. It is important to establish a standard procedure for running oxidative burst assays. This can be achieved by finding fluorescent beads which fall generally within the range of fluorescence of activated cells. These beads are then used to set up the flow cytometer each time, setting the high voltage of the photomultipliers based upon the bead fluorescence. If a full calibration is performed, the mean channel fluorescence can then be equated with the quantity of H_2O_2 formed per cell.

5. Instrumentation

All studies are carried out using a 15-mW argon laser operating at a wavelength of 488 nm. Optical filters, 488-nm dichroic, 488-nm laser blocking, 550-nm dichroic, 525-nm band pass (for DCFH-DA), and 610-nm long pass (for HE), are placed in the fluorescence collection pathway. Gated list-mode data are collected for FALS, 90° scatter (90° LS), log 90° light scatter (log 90), FITC, log FITC, EB, log EB, and TIME.

6. Comment

When using esterase-dependent dyes, it is better to add as small a concentration of activating substance as possible so as not to dilute the dye concentration. Although there are many instances where this will not alter the results, especially if correct controls are used, try to maintain a constant dye concentration if at all possible. Additionally, since the DCF fluorescence emission extends into the red spectrum, it is better to use a 610-nm band pass and ensure adequate color compensation for the DCF in the EB channel when the two fluorochromes are used together.

7. Critical Aspects

Several aspects of HL-60 cell culturing must be kept in mind while interpreting experiments using repeated samples from a continously maintained culture of HL-60 cells. HL-60 cells which are heterogenous to start with become more homogenous when passaged for a very long period of time. Multiple impairments in stimulus–response coupling and cell regulation develop in association with increased rate of cell replication. Repeated passages lead to the predominance of a subset of cells that proliferate more rapidly and are more functionally impaired. Alterations in doubling time, impairment in calcium mobilization, decreased response to differentiation inducers, and decreased oxidative burst have been observed in cells beyond passage 60. Hence it is clearly important that changes in cell function be related to the age of the culture or the passage number.

IV. Results and Discussion

HL-60 cells show some unique light scatter changes with differentiation. Figure 1a shows this change as there is a dramatic reduction in cell size and increase in 90° light scatter. Since spontaneous differentiation is continually occurring, some differentiated cells are always present in a so-called "undifferentiated" populations. The reduction in size of differentiating HL-60 cells over a period of 5 days can be seen in Fig. 1b. A rapid method for checking the

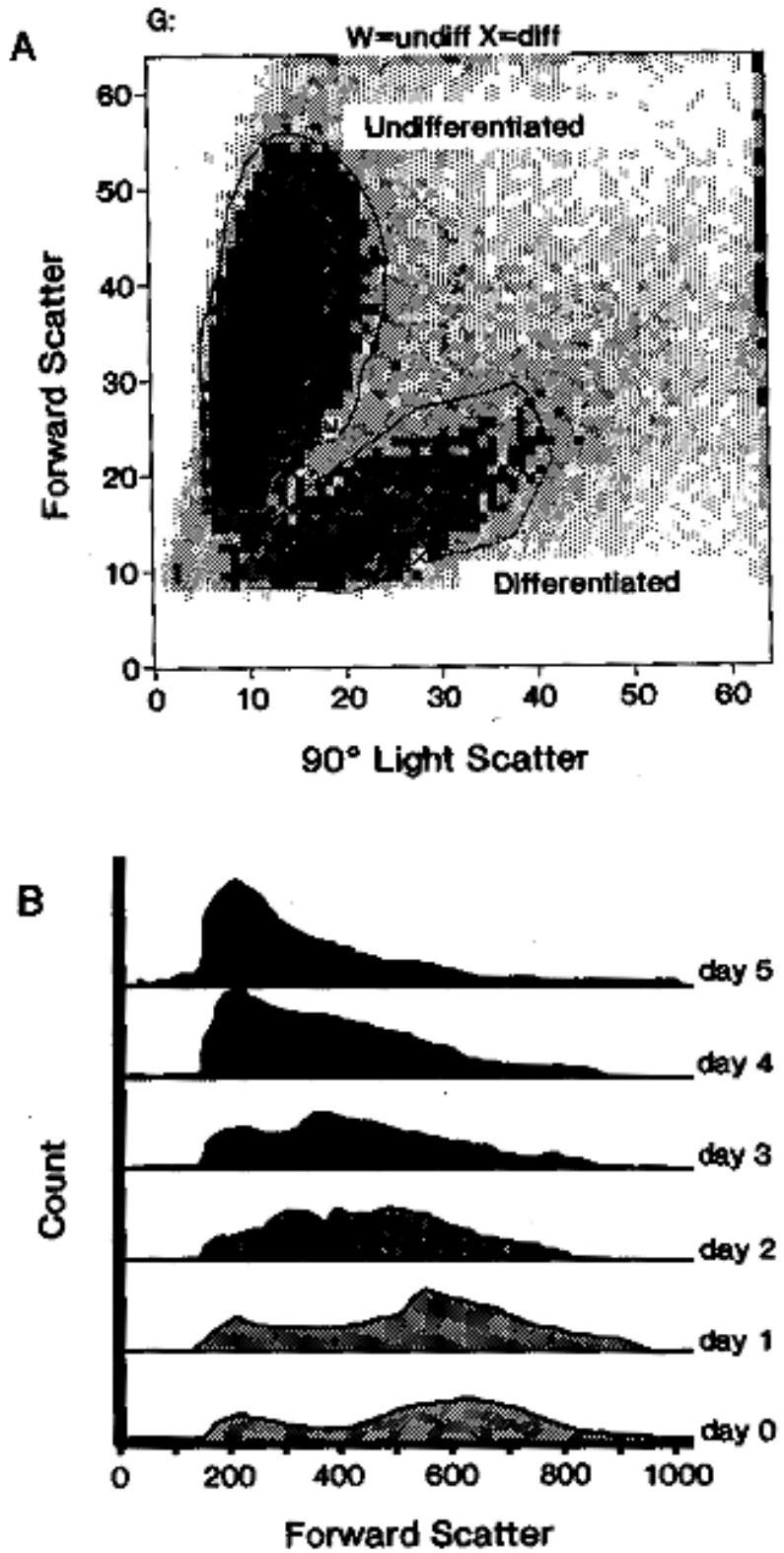

Fig. 1 A change in the scatter pattern of HL-60 cells when differentiated with DMSO. (a) Scatter plot of FALS versus 90° light scatter of both differentiated and undifferentiated HL-60 cells. Undifferentiated HL-60 cells (Day 0) have a significant cell population of large cells. This is mainly due to the large size of the immature promyelocytes and myeloblasts present in the culture. (b) On differentiation, cells become smaller and more granular (Day 5).

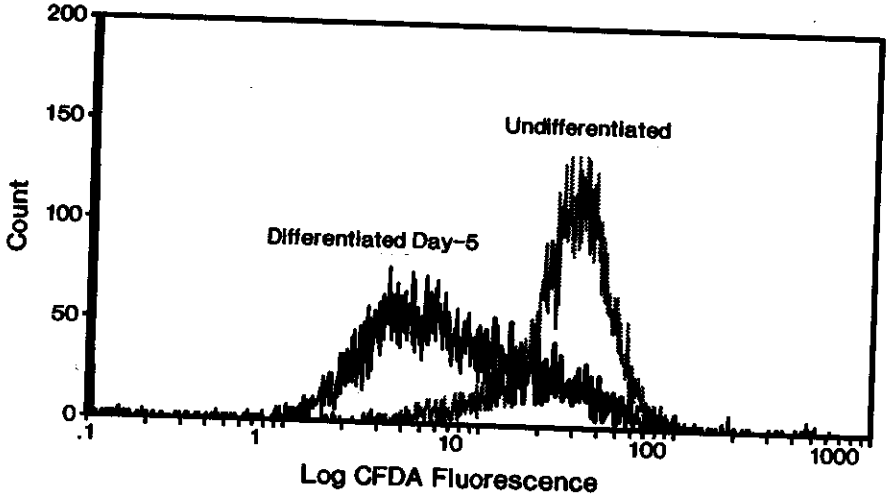

Fig. 2 Changes in esterase content of HL-60 cells when differentiated into granulocytes using DMSO. Undifferentiated HL-60 cells (Day 0) have high levels of esterase activity compared to differentiated cells. Fluorescence histograms (log CFDA fluorescence) were collected after the cells were incubated at 37°C for 30 min followed by flow cytometry.

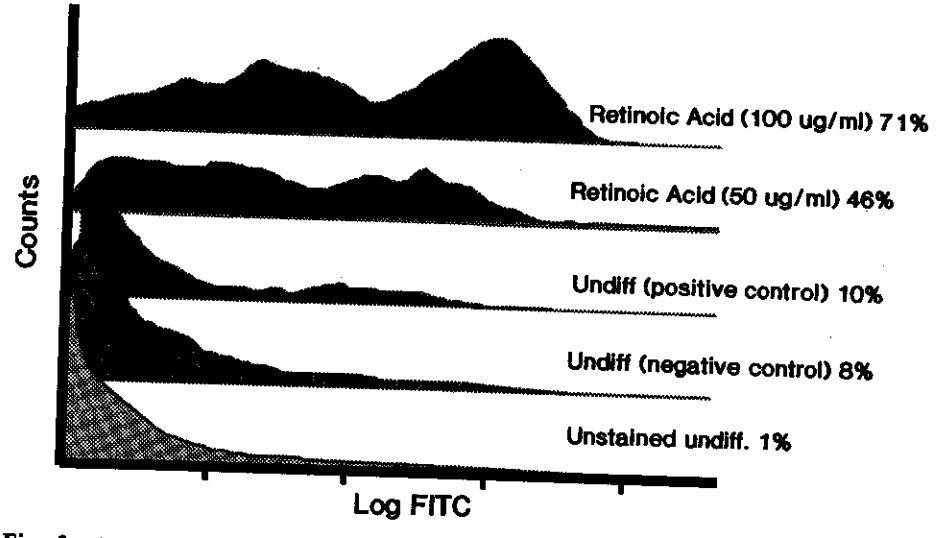

Fig. 3 A dose-dependent response of CD11b expression on HL-60 cells to retinoic acid (50–100 μg/ml). An increase in CD11b expression can be seen, with the highest dose of retinoic acid (100 μg/ml) eliciting maximum response. CD11b expression increases as HL-60 cells differentiate into mature granulocytes. Percentages of HL-60 cells expressing CD11b are shown beside each histogram. All measurements were made 48 hr after addition of differentiation agent.

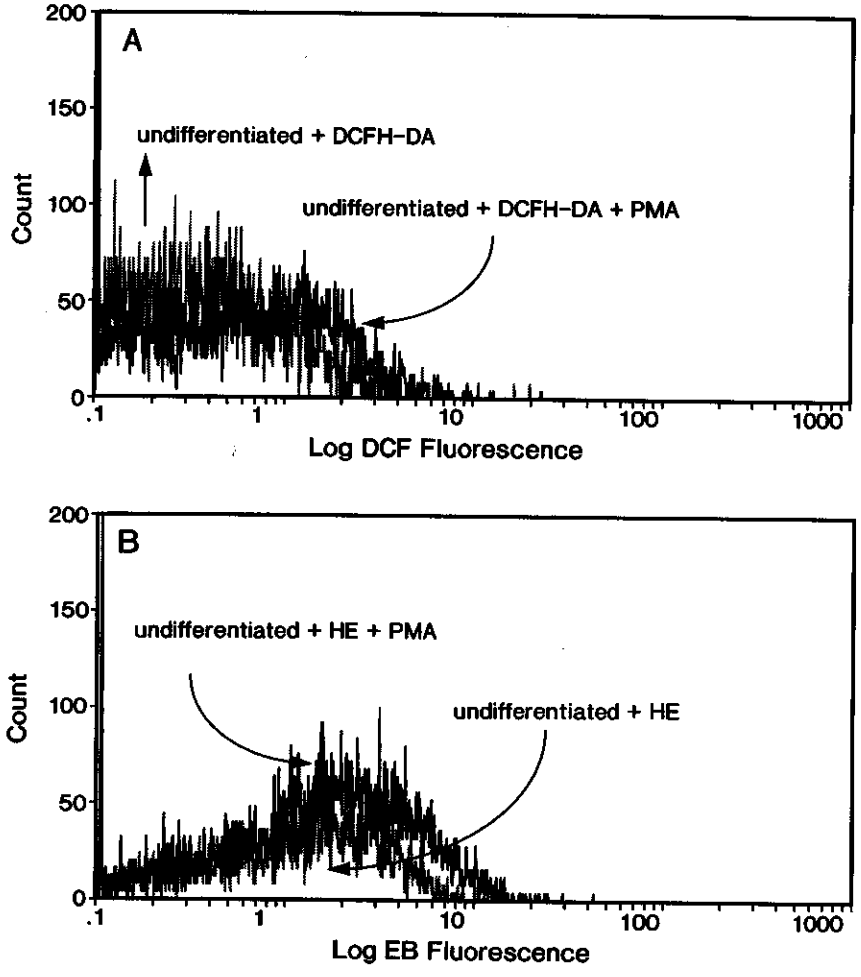

Fig. 4 Examples of PMA stimulation of HL-60 cells to produce H_2O_2 (DCF fluorescence) or O_2^- (EB fluorescence). Undifferentiated cells are shown for both the control (no stimulation) and the PMA-stimulated H_2O_2 and O_2^- responses. (A and B) H_2O_2 and O_2^- production, respectively, for undifferentiated HL-60 cells. (C and D) H_2O_2 and O_2^- production, respectively, for differentiated HL-60 cells (5 days). Responses were measured after 30 min stimulation with PMA (100 ng/ml).

differentiation status of HL-60 cells is to evaluate the esterase content as shown in Fig. 2. Undifferentiated cells have reasonably high esterase content, which decreases in granulocytic differentiation (Fig. 2) and increases in monocytic differentiation (not shown).

Differentiated HL-60 cells also express increased numbers of cell receptors such as CD11b. This is shown in Fig. 3, where the increased expression of CD11b is observed with maturation into granulocytic cells. This change in cell-

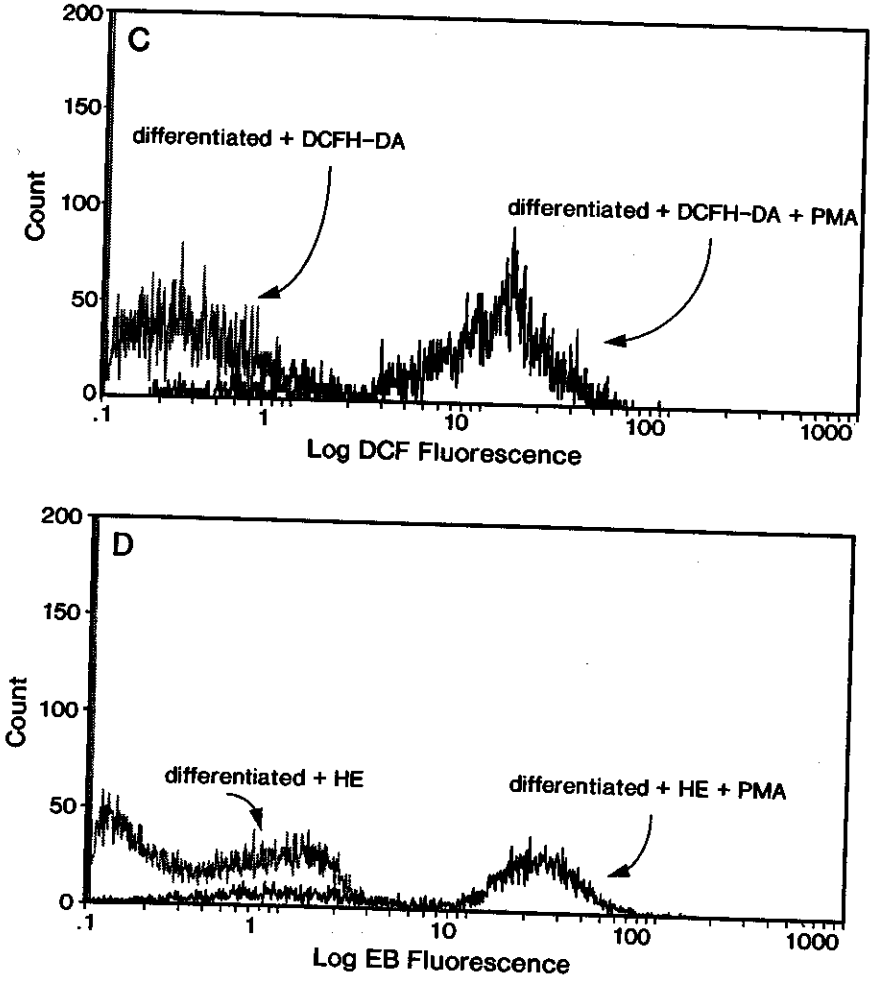

Fig. 4 Continued

surface expression is particularly useful for evaluation of chemicals thought to elicit an immune response, since screening for CD11b expression is a relatively simple assay. However, one must control for the high background fluorescence commonly observed in HL-60 cells.

An additional functional assay useful for evaluating HL-60 cells is the determination of their reactive oxygen species production. A substantial increase in both O_2^- and H_2O_2 production can be observed in differentiated HL-60 cells (Fig. 4) as they mature into granulocytic cells, indicative of the maturation of the respiratory burst components. Flow cytometry provides an excellent technique for monitoring this maturation. A significant advantage afforded by HL-60 cells is the ability to accurately reproduce differentiation conditions,

thereby creating an efficient model for studying phagocytic cell function. Care in interpretation of studies must of course be made since the HL-60 cell line is leukemic in origin.

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