Estimation of Cell Survival by Flow Cytometric Quantification of Fluorescein Diacetate/Propidium Iodide Viable Cell Number

Douglas D. Ross, Christopher C. Joneckis, José V. Ordóñez, Allison M. Sisk, Richard K. Wu, Anne W. Hamburger, and Richard E. Nora

Program of Oncology, University of Maryland Cancer Center [D. D. R., C. C. J., J. Y. O., A. M. S., R. K. W., A. W. H., R. E. N.], the Department of Medicine, Division of Hematology [D. D. R.], and the Department of Pathology [A. W. H.], University of Maryland School of Medicine, Baltimore, Maryland

ABSTRACT

We report a flow cytometric method to quantify the number of viable cells remaining in suspension culture following exposure to cytotoxic drugs. Cell viability is assessed by flow cytometric measurement of cellular fluorescence after staining with fluorescein diacetate and propidium iodide in isotonic solution. The number of viable cells per ml of culture is determined by a timed count of viable cells and from knowledge of the flow cytometer sample flow rate. P388 murine or HL-60 human leukemia cells in culture were used as model systems. This method can quantify accurately viable cell concentrations in suspension culture from 100 cells/ml to 1 million cells/ml. The sensitivity of the method as a cytotoxicity assay increases if, following brief (1-4-h) exposure to drug, greater time is allowed for cell death and lysis to occur prior to flow cytometric counting of viable cells. If the viability assessment is deferred for at least 72 h following drug (daunorubicin, actinomycin D, vincristine) exposure, results were obtained approximating those obtained from the soft agar clonogenic assay or the colorimetric 3-(4,5dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. In studying the cytotoxic effects of vincristine, actinomycin D, 1-beta-D-arabinofuranosylcytosine, and daunorubicin on P388 or HL-60 cells sensitive and resistant to these agents, reasonable results were obtained by flow cytometric counting of viable cell number. We have been able to perform this flow cytometric viability assay with ease using bone marrow blast cells obtained from patients with acute myelogenous leukemia. The method is facile, relatively rapid, and since it is ideal for studying cells in suspension culture, its potential as a predictor of chemotherapeutic response in leukemia warrants further evaluation.

INTRODUCTION

Recently, assays of chemosensitivity have been under intense investigation. The semiautomated colorimetric MTT assay has demonstrated much potential for in vitro chemotherapeutic drug screening (1, 2). However, with this assay, the best reproducibility is obtained with adherent cell lines, and optimal conditions must be determined for each cell line studied, particularly the number of cells plated per microwell (2). For these reasons, and because nonneoplastic cells can also reduce MTT, the MTT assay adapts poorly to the study of primary tumor samples, particularly those that exist in vitro in suspension culture, such as blast cells from patients with AML (2).

Clonogenic and self-renewal assays may have relevance to clinical outcome in AML (3-5). However, these assays are time consuming and very labor-intensive. Moreover, blast cell samples from 10 to 60% of patients with AML are unable to form colonies in vitro (6, 7), thus precluding the study of these patients.

Flow cytometry provides an ideal means of studying cells in suspension culture. In particular, for prospective studies of patient blast cells, irrelevant nontumor cells can be excluded from analysis by appropriate light scatter gating. We report here a facile and highly sensitive flow cytometric method for counting the number of viable cells per milliliter of suspension culture. Viable cells are identified by simultaneous assessment of PI and FDA fluorescence. The method adapts particularly well to assessing the effects of chemotherapeutic agents on cell survival in suspension culture.

MATERIALS AND METHODS

Chemicals and Pharmaceuticals. MTT, FDA, PI, and dimethyl sulfoxide are obtained from the Sigma Chemical Company, St. Louis, MO. Daunorubicin is obtained from Wyeth Laboratories, Philadelphia, PA. Vincristine is obtained from the Eli Lilly Pharmaceutical Corp., Indianapolis, IN. Actinomycin D is obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, MD.

Cell Culture Methods. HL-60 cells are grown in RPMI medium 1640 with 10% (v/v) heat inactivated (56°C for 30 min) fetal calf serum (GIBCO Inc., Grand Island, NY), 1-glutamine (2 mM), penicillin (50 IU/ml), and streptomycin (50 μg/ml), as described previously (8). P388/W cells and P388/ADR cells (9) obtained from Dr. Randall Johnson, Smith Kline and French Laboratories, Philadelphia, PA) are grown in RPMI medium 1640, as above, with the addition of 0.01 μM β-mercaptoethanol, with passage in culture as described previously (10). Cells are routinely tested to assure absence of contamination by mycoplasma (MycoTect, GIBCO, Inc., or Gen Probe, San Diego, CA). Routine cell counting is done by Coulter counter (Coulter Electronics, Hialeah, FL) or by Neubauer hemocytometer (Levy Ultra Plane, C. A. Hauser & Son, Philadelphia, PA). The Coulter counter gain and threshold settings are selected for each cell line used to provide results equal to those obtained by hemocytometer.

Flow Cytometric Assessment of Cell Viability. Following exposure of cells to drug for the times indicated (see below), cells are washed once with PBS (11), then resuspended in fresh growth medium at an initial cell density of 0.5 to 1 × 10⁶ cells/ml (for P388 cells) or 2 × 10⁵ cells/ml (for HL-60 cells). Cells are then incubated (37°C, 7.5% CO₂) in 25-cm² culture flasks (Corning, Inc., Corning, NY) for the time periods indicated (see below), after which assessment of viability is made.

At the time appropriate for assessment of viability, an aliquot (usually 2 ml) of each culture is placed in a test tube, and then FDA is added to each test tube to achieve a final concentration of 0.5 μg/ml. The tubes are incubated at room temperature in the dark for 30 min, and then PI in isotonic saline is added to each tube to achieve a final concentration of 50 μg/ml. The tubes are then immediately placed into ice, and kept ice-cold during flow cytometric analysis.

Viability is assessed by determining intracellular red and green fluorescence using a FACS IV flow cytometer (Becton Dickinson Immunocytometry Systems, Mountainview, CA). A 570-nm beam splitter is used for dual color fluorescence analysis, with a 625/35-nm filter used to collect red (PI) fluorescence, and a 530/30-nm filter used for green (fluorescein) fluorescence. Logarithmic amplification of red and
green fluorescence signals is used. Event triggering is based on forward light scatter. Viable cells are identified as those with high green fluorescence and low red fluorescence, as illustrated in Fig. 1.

Typically, we operate our FACS IV flow cytometer with a line pressure of 17 psi, sheath pressure of 13 psi, and relative sample flow of 50. Under these conditions, the sample flow rate of the FACS IV is determined by weighing the sample tube and contents before and after a timed sample run. Thus the weight of sample consumed per unit of time is determined. For this flow cytometry calibration procedure, ice-cold tissue culture medium is used. The measured sample flow in grams/min is converted to ml/min from knowledge of the density of the culture medium (1.008 g/ml, at 4°C). Typical FACS IV sample flow is 129.9 ± 6 µl/min, with minimal if any variation during any given operating day. A sample flow calibration as described above is performed prior to each experiment.

The number of FDA/PI viable cells per ml of culture is determined by making a timed count of FDA/PI viable cells (cells in area enclosed by the small rectangles in Fig. 1), then calculating viable cells/ml by dividing the number of viable cells per unit time by the sample flow rate. Usually, counts are made for one min; however, when cell numbers are very low, the number of viable cells are counted for a longer period of time (2-5 min). A correction is made for the dilution caused by addition of FDA and PI. Final cell survival data are expressed as number of viable cells/ml culture medium.

MTT Viability Assay. Underlayers (1.0 ml) consisting of 0.5% agar (Bacto-Agar, Difco Laboratories, Detroit, MI) in medium appropriate for P388 or HL-60 cells are placed in 35-mm petri dishes (Falcon Plastics division of Becton-Dickinson, Oxnard, CA). Medium containing 15% (v/v) heat-inactivated fetal calf serum is used for all clonogenic studies. Following exposure to drug, cells are added to medium containing 0.3% agar to achieve a final cell concentration of 8,000 or 80,000 cells/ml for P388, or 24,000 or 72,000 cells/ml for HL-60. One ml of this suspension is then placed on top of the underlayers in the petri dishes, and allowed to solidify. The petri dishes are incubated at 37°C, 7.5% CO2 for 7 days (P388) or 14 days (HL-60), after which the number of colonies (clusters greater than 50 cells) per plate are counted with the aid of an inverted microscope. We express cloning efficiency as the number of colonies per 1,000 cells plated.

MTT Viability Assay. A procedure similar to that of Carmichael et al. (7) is used. Briefly, following exposure to drug, 1,000 P388 or 5,000 HL-60 cells in 200 µl of growth medium are added per well of a flat-bottomed 96-well microtiter plate (Microtest III, Falcon Plastics). The microtiter plates are then incubated for 4 days at 37°C, 7.5% CO2, after which the viability assessment by MTT is done (see below). The number of cells (stated above) seeded per well is used because it was determined previously to lie on the linear portion of the curve of optical density (MTT formazan production) after 4 days culture versus number of cells seeded per well on Day 1. Six wells are filled per treatment condition.

At the time of viability assessment, 50 µl of a 2 mg/ml H2O solution of MTT is added per microwell. After incubation at 37°C for 4 h, the plates are centrifuged (500 x g, 10 min), then 200 µl of the supernatant solution are removed from each well by aspiration with a micropipet. 200 µl of dimethyl sulfoxide are added per well to solubilize the MTT formazan product. The microtiter plates are gently shaken, then placed at 37°C for 1 h, after which the plates are gently shaken again to mix the contents of the wells. The optical densities of the wells at 570 nm are read by a microelisa autoreader (MR580; Dynatech Laboratories, Inc., Alexandria, VA).

Isolation of Leukemic Blast Cells. After informed consent, bone marrow was collected from patients with AML and prepared for cell culture using a discontinuous Ficoll-hypaque density gradient (specific gravity, 1.077) exactly as described in a previous publication (12). Blast cells are cultured in medium identical to that used for HL-60 cells, as described above.

RESULTS

Flow Cytometric Identification and Quantification of Viable Cells. To identify viable cells by flow cytometry, we utilized FDA and isotonic PI (13-15). FDA is a nonpolar, nonfluorescent substance. It enters all cells freely. In viable cells, FDA is converted by intracellular esterases to highly fluorescent fluorescein (13–15). Being highly polar, fluorescein becomes trapped within cells that possess membrane integrity (viable cells). In cells lacking membrane integrity, fluorescein diffuses out of the cell. Thus with FDA, viable cells have bright green fluorescence when analyzed by flow cytometry, whereas nonviable cells are nonfluorescent.

PI is a polar, highly fluorescent (red color) compound which, under isotonic conditions, can only enter cells that lack membrane integrity. Thus, with flow cytometric analysis of cells in isotonic PI, nonviable cells have bright red fluorescence, viable cells are nonfluorescent.

Fig. 1 shows typical dot plots in log scale of green versus red fluorescence of cells in culture exposed to FDA and PI. In the example shown, P388 cells were used, with the viability assessment done 48 h after a 1-h exposure to daunorubicin. Similar results are obtained with HL-60 cells or AML blast cells (data not shown). The small rectangle within each plot outlines the area (or "window") that contains the viable cells (cells with high green fluorescence, low red fluorescence). In this typical experiment, when cells were obtained from the viable cell "window" by cell sorting, and were cultured in soft agar, the cloning efficiency was 48 ± 4%. In contrast, the cloning efficiency of all cells sorted from outside of the viable cell "window" was only 1.4 ± 0.07%. Note (Fig. 1) that as the concentration of daunorubicin increases, the number of viable cells decreases, whereas the number of nonviable cells (i.e., cells with high red fluorescence, low green fluorescence, or both) increases. Cellular debris (confirmed by cell sorting) appears in these dot plots as events with very low green fluorescence but with variable amounts of red fluorescence (linear group of events labeled "d" in Fig. 1). Note that as the concentration of daunorubicin increases, the amount of debris events increases. Note also that there is excellent separation of both nonviable cells and debris events from viable cells in the dot plots, thus confirming that no debris events or nonviable cells are counted in the viable cell "window."

By making a timed count of the cells in the viable cell "window," and from measurement of the sample flow rate (which we find to be very constant; see "Materials and Methods"), we are able to determine the absolute number of viable cells.
cells per milliliter of culture. All survival data obtained by the flow cytometric method presented in this paper are based on the number of viable cells/ml suspension culture.

Correlation of Flow Cytometric Cell Counting with Cell Counts Based on Coulter Counter or Hemocytometer. Using a Coulter Counter (calibrated by hemocytometer), cells in culture were adjusted to a density of precisely 10⁷ cells/ml. Then, serial 10-fold dilutions of this cell suspension were made to provide a number of cell suspensions of known cell concentration. For the original and each cell suspension created by dilution, the number of FDA/PI viable cells/ml was determined by flow cytometry. Fig. 2 shows the correlation of the results of flow cytometric cell counting with the known cell concentrations of the cell suspensions tested. Standard deviations for each point in Fig. 2 are <6% of the mean value. In the example shown (Fig. 2), P388 cells in log growth phase were used. This experiment has been repeated three times with similar results. The overall percentage of FDA/PI viable cells in this experiment was 90%. Note in Fig. 2 that excellent correlation of flow cytometric counts with expected counts is observed between 10⁵ cells/ml and 10⁷ cells/ml (slope = 0.935, r² = 0.99). Above 10⁸ cells/ml, flow cytometry underestimates viable cell number. Because of this, the experiments described below that monitor drug effects by this method are designed such that the cell concentration in control cultures at the time of viability assessment is equal to or less than 10⁶/ml.

Effects of the Time That Viability Assessment Is Made following Cellular Exposure to Cytotoxic Agents. The time following exposure to drug is crucial for allowing cell death (as assessed by FDA/PI) to become manifest. Fig. 3 shows the effects of making the viability assessment at various times following exposure of cells to drug. Multiple experiments of this type have been performed, with similar results. In this example (Fig. 3), HL-60 cells were exposed to daunorubicin for 1 h, then washed and returned to culture. Note that the sensitivity of the assay increases considerably if one defers viability assessment for 48 h compared to 24 h. Smaller increases in sensitivity compared to 48 h are obtained by assessing viability at 72 or 96 h. No benefit over 96 h is obtained by waiting 120 h (data not shown in Fig. 3). For this reason, we chose 72 h as a convenient endpoint in our standard flow-cytometric cytotoxicity assay.

Relation of Flow Cytometric Viable Cell Survival Assessment to Clonogenic Survival or MTT Survival Estimates. Fig. 4 shows examples of studies comparing cell survival estimates by flow cytometric viable cell counting with those of the MTT and clonogenic assays. P388 cells were exposed to daunorubicin for 1 h. For vincristine and actinomycin D, exposure time was 3 h. Following exposure and removal of drug by washing the cells, cells were either placed in soft agar culture, in multiwell plates for the MTT assay (see “Materials and Methods”), or returned to culture for 72 h, after which FDA/PI viable cell survival was determined by flow cytometry. The data shown are typical of those obtained from repeated experiments, using P388 or HL-60 cells. In general, the colony assay was slightly more sensitive than the flow cytometric or MTT assay (Fig. 4). For example, at higher daunorubicin concentrations, clonogenic survival fell to zero, whereas a small number of cells remained viable by the FDA/PI method. The MTT assay gave results approximately comparable to the flow cytometric assay, however, at high concentrations of daunorubicin (3 μM) or actinomycin D (1 μM), the MTT assay overestimated clonogenic or FDA/PI viable cell survival (Fig. 4). In comparing the flow cytometric FDA/PI method with the MTT assay, we find smaller standard deviations with the flow cytometric assay (SD less than 6% of mean versus 21% for the MTT assay; see Fig. 4, legend). Additionally, in our hands, the MTT assay only provides up to 2 logs dynamic range of cell survival, compared to up to 4 logs obtained by the flow cytometric method.

We studied the cloning efficiencies of P388 cells taken from the viable cell “window” following exposure to DNR, and compared this to overall clonogenic or FDA/PI survival (Table 1). Table 1 indicates that the cloning efficiency of cells in the
Fig. 4. Relationship of flow cytometric viable cell survival assessment with clonogenic survival or MTT survival estimates. P388 cells in log growth phase were exposed to daunorubicin for 1 h. For vincristine and actinomycin D, exposure time was 3 h. Following exposure to drug, cells were placed in drug-free medium either in suspension culture (for FDA/PI and MTT assays) or in soft agar culture for the clonogenic assay. The FDA/PI viability assessment was done 72 h after drug exposure. MTT viability assessment was done 96 h after drug exposure. III. FDA/PI viable cell survival; C, clonogenic cell survival; 1, MTT survival estimate. Control values for the daunorubicin, vincristine and actinomycin D studies were as follows: for FDA/PI viability, 9.9, 11.1, and 13.3 x 10^5 cells/ml respectively; for the clonogenic assay, 231, 621, and 599 colonies per 1000 cells plated; for the MTT assay, 1.126, 0.655, and 0.669 OD units, respectively. Average standard deviations (as percentage of mean value) of the experimental points shown are <6% for FDA/PI viable cell counting, 24% for the clonogenic assay, and 21% for the MTT assay. The data shown are typical of those obtained from multiple similar experiments, using P388 or HL-60 cells.

Table 1  Effects of daunorubicin on cloning efficiency of FDA/PI viable P388 cells obtained by cell sorting

P388 cells were exposed to DNR for 1 h, then placed in drug-free medium. Aliquots were removed at this time and placed in soft agar to determine cloning efficiency, as described in "Materials and Methods." These results are displayed under Column A. The remainder of the cells were incubated in liquid suspension culture for 72 h, at which time flow cytometric determination of FDA/PI viable cell number was made, the results of which are given under Column B. At the time of FDA/PI viability determination, cells within the "viable cell window" were collected by cell sorting, then placed in soft agar for determination of cloning efficiency, as described in "Materials and Methods." These results are shown under Column C.

<table>
<thead>
<tr>
<th>Daunorubicin concentration (µM)</th>
<th>A. Cloning efficiency, whole cell population (% control)</th>
<th>B. FDA/PI cell survival estimate (% control)</th>
<th>C. Cloning efficiency, sorted viable cells (% control)</th>
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<tr>
<td>0</td>
<td>100 ± 18*</td>
<td>100 ± 2</td>
<td>100 ± 8</td>
</tr>
<tr>
<td>0.03</td>
<td>105 ± 14</td>
<td>104 ± 2</td>
<td>169 ± 27</td>
</tr>
<tr>
<td>0.1</td>
<td>120 ± 27</td>
<td>102 ± 2</td>
<td>143 ± 30</td>
</tr>
<tr>
<td>0.3</td>
<td>38 ± 7</td>
<td>12 ± 1</td>
<td>74 ± 16</td>
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<tr>
<td>1</td>
<td>0.3 ± 0.12</td>
<td>1 ± 0</td>
<td>13 ± 0.4</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>&lt;0.1</td>
<td>ND*</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>&lt;0.1</td>
<td>ND*</td>
</tr>
</tbody>
</table>

* Data shown are percentage of control value, ± SD. Control values are as follows: Column A, 230 colonies per 1000 cells plated; Column B, 1 x 10^5 viable cells/ml; Column C, 470 colonies per 1000 cells plated.

FLOW CYTOMETRIC ASSESSMENT OF CELL SURVIVAL

of drug that decrease cell survival to 50 or 10% of control (i.e., IC50 or IC90 values), the flow cytometric and clonogenic assays are comparable.

Applications of the Flow Cytometric Measurement of Viable Cell Survival to Assess Cytotoxic Drug Effects. Fig. 5 illustrates the effects of daunorubicin, vincristine, and actinomycin D on viability of P388 wild-type cells (P388/W) and on the known multidrug-resistant line P388/ADR (9) using flow cytometric counting of viable surviving cells. Exposure to daunorubicin was for 1 h; exposure to vincristine or actinomycin D was for 3 h. Viability was assessed 72 h following exposure to drug in all cases. For daunorubicin, note a large difference in sensitivity between the resistant P388/ADR cells and the sensitive P388/W cells. The addition of 6.6 µM verapamil to P388/ADR cultures during and after exposure to daunorubicin enhanced approximately 10-fold the sensitivity of these cells to killing by daunorubicin, as determined by this method (Fig. 5, daunorubicin panel). For vincristine and actinomycin D, note the marked difference in sensitivity of P388/ADR to these agents compared to the wild-type cells (Fig. 5). Thus, in assessing drug effects in known sensitive and multidrug-resistant cells, flow cytometric determination of FDA/PI viable cell survival provides reasonable results, consistent with the original report of cross-resistance of P388/ADR using in vivo sensitivity testing (9). Moreover, Tsuruo et al. (16) found a 10-fold decrease in the IC50 value of ADR against P388/ADR in response to 6.6 µM verapamil, which is identical to our observed decrease in IC50 of DNR against P388/ADR caused by the same concentration of verapamil.

Fig. 6 illustrates the effects of continuous exposure to ara-C on the survival of FDA/PI viable HL-60 human leukemic cells. The viability assessment was made immediately after 24, 48, and 72 h of continuous exposure to drug. Note that a plateau in cell kill is observed at each time point. As indicated in Fig. 6, the plateau represents 60, 88, and 96% cell kill at 24, 48, and 72 h, respectively. Since ara-C is an S-phase-specific agent, such plateaus are typical of ara-C cytotoxicity curves, and represent the fraction of cells that were in S-phase during the exposure for a time period sufficient to enable cell killing by ara-C. Such plateaus in ara-C cytotoxicity curves have been observed by Major et al. (17) for HL-60 cells, using a clonogenic cytotoxicity assay. HL-60 cells resistant to ara-C (18) were also viable cell “window” does decrease with DNR treatment, but to a lesser extent (approximately one order of magnitude less) than does the cloning efficiency of the whole cell population. For example, after 1 µM DNR, clonogenic survival was 0.3% (Column A), whereas the FDA/PI survival estimate was 1% (Column B). Of these 1% surviving FDA/PI “viable” cells, the cloning efficiency was reduced to 13% of the control (untreated) value (Column C). Similarly, for 0.3 µM DNR, the 12% of cells that remained viable by FDA/PI criteria had cloning efficiency reduced to only 74% of control value. At this concentration, cloning slightly overestimated FDA/PI survival (38% versus 12%). Thus, in the range of 1 log cell kill, the FDA/PI assay is in good agreement with the clonogenic assay. However, at high drug concentrations (causing over one log of cell kill), the flow cytometric assay may overestimate clonogenic survival, and one must be cautioned in this regard. However, we feel that this overestimation does not at all invalidate the flow cytometric assay, since for the practical estimation of the concentrations.
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Fig. 5. Effects of daunorubicin, vincristine, and actinomycin D on drug-sensitive P388/W cells (•) and on the multidrug-resistant subline P388/ADR (O) (9) as assessed by flow cytometric determination of FDA/PI viable cell survival. For daunorubicin, cells were exposed for 1 h, and viability assessment was made 72 h after drug exposure. Certain cultures of P388/ADR cells were exposed to verapamil (6.6 μM) during and following exposure to daunorubicin (C). For vincristine and actinomycin D, cells were exposed for 3 h and viability was assessed 72 h following drug exposure. The data shown are typical of those obtained from repeated experiments. Standard deviations of the experimental points are <7% of the mean values.

Fig. 6. Effects of ara-C on HL-60 cells as assessed by flow cytometric determination of FDA/PI viable cell survival. HL-60 cells in log growth phase were exposed to the concentrations of ara-C indicated. After 24 (•), 48 (O), or 72 (△) h of exposure, cells were removed from culture for FDA/PI viability assessment. The data shown are typical of those obtained from replicate experiments. Standard deviations of the experimental points shown are <8% of the mean values.

studied, and displayed minimal cytotoxicity under these conditions (data not shown).

Flow cytometric determination of viable cell number can also be used to study the growth kinetics of cells in culture (Fig. 7). In this example, we inoculated P388 cells in fresh culture medium at an initial cell density of 5,000 cells per ml, based on Coulter counter counts. Vincristine (0, 0.01, and 0.1 μM) was added to separate cultures of cells. Then, the FDA/PI viable cell number was determined by flow cytometry, after which the cultures were placed in the incubator. Aliquots of cells were removed from the cultures daily for 6 successive days for determination of FDA/PI viable cell number. With the low initial cell concentration (5,000 cells/ml), control cultures of cells remained in logarithmic growth phase for 5 days, with a doubling time of 13 h for growth between Days 1 and 5. Note that the continuous presence of vincristine diminished viable cell numbers in a dose-dependent manner. Fig. 7 illustrates the broad range of cell concentrations that can be monitored by this flow cytometric procedure for counting viable cell number.

Studies of AML Blast Cells. Fig. 8 shows typical responses of AML patient blast cells following a 4-h exposure to daunorubicin. The flow cytometric FDA/PI viability assessment was made 24 h after drug exposure. Despite our data that the optimal time for assessment of cell viability is 48–72 h after drug exposure (see above), the 24-h time point was chosen because AML blast cells survive poorly in simple in vitro culture in the absence of hemopoietic growth factors. For example, after 24 h, the overall FDA/PI viability of control cultures ranged from 15 to 77%, with a median of 35%. All of the patients shown in Fig. 8 were clinically refractory to daunorubicin treatment with the exception of patient M. S., who obtained a complete remission. These data are insufficient to make any conclusions in regard to the relevance of the assay to clinical outcome; they are shown simply to illustrate that this flow cytometric method adapts well to the study of blast cells obtained from AML patients and to indicate the feasibility of further studies to determine the relevance of the assay to predicting clinical outcome.

DISCUSSION

The present studies demonstrate that flow cytometry provides a highly sensitive and accurate means of quantifying viable cells in suspension culture over a broad range of cell concentrations (100–10⁶ cells/ml). This method is easy to perform, is repro-
very small numbers of surviving viable cells. In the range of counting and clonogenicity lies mostly in treated cultures with cells exposed to 1 MMDNR (13%/0.3% = 43). Thus, our data show clearly that the discrepancy between FDA/PI viable cell (e.g., LUM, Table 1), of the small fraction (1%) of cells that (Table 1). This can be explained by the fact that assays of cell indicators of cell death. Thus, at higher DNR concentrations fraction of surviving FDA/PI viable cells was <10% of control did decrease in DNR-treated samples, particularly when the residual intact cells. Despite this, 72 h after exposure to drug, the cloning efficiency of cells in the “viable cell window” is due both to cell lysis and to loss of membrane integrity of and intact nonviable cells is excellent (Fig. 1), thus ensuring that cellular debris and nonviable cells are completely excluded following a cytotoxic event, and not from simply distinguishing to cell lines grown as suspension cultures.

Much of the power of this flow cytometric assay lies in the counting of the number of intact viable cells surviving in culture following a cytotoxic event, and not from simply distinguishing viable from nonviable cells per se. The ability of the flow cytometer to distinguish intact viable cells from cellular debris and intact nonviable cells is excellent (Fig. 1), thus ensuring that cellular debris and nonviable cells are completely excluded from the quantification of viable cell number. The increased sensitivity of the assay obtained by allowing a longer period of time to elapse between drug exposure and viability assessment is due both to cell lysis and to loss of membrane integrity of the residual intact cells. Despite this, 72 h after exposure to drug, the cloning efficiency of cells in the “viable cell window” did decrease in DNR-treated samples, particularly when the fraction of surviving FDA/PI viable cells was <10% of control (Table 1). This can be explained by the fact that assays of cell membrane integrity, such as FDA and PI, are relatively late indicators of cell death. Thus, at higher DNR concentrations (e.g., 1 µM, Table 1), of the small fraction (1%) of cells that remained intact and still manifested membrane integrity as assessed by FDA/PI, only 13% were clonogenic. Still, this represents a 43-fold enrichment in clonogenic cells in the “viable cell window” compared to the overall cloning efficiency of cells exposed to 1 µM DNR (13%/0.3% = 43). Thus, our data show clearly that the discrepancy between FDA/PI viable cell counting and clonogenicity lies mostly in treated cultures with very small numbers of surviving viable cells. In the range of practical estimators of cell survival (e.g., IC_{50} and IC_{90} values) the flow cytometric (at 72 h) and clonogenic assays are comparable.

Jones & Senft (15) used FDA/PI to identify viable cells air-dried on microscope slides and found that these dyes give more consistent results than did trypan blue. We used dual labeling with FDA and PI to enhance flow cytometric discrimination of viable cells compared to single label discrimination. Cells are considered viable only if they meet both FDA and PI viability criteria. Of the two labels, FDA provides the most power of discrimination, with greater separation of mean fluorescence of viable cells from that of nonviable cells and cellular debris than obtained with PI (see Fig. 1). One reason for selecting PI as a second discriminant is the work of Prosperi et al. (19), who reported that prior to complete disruption of membrane integrity, daunorubicin decreases FDA efflux from cells, causing an increase in intracellular fluorescence. Such an effect is evident in Fig. 1 (0.03 and 0.3 µM daunorubicin panels), where a population of cells with green fluorescence higher than that of control viable cells appears. However, the use of dual labeling enables us to classify these cells as nonviable since these cells have high PI fluorescence.

Funa et al. (20) reported a flow cytometric method for quantifying cell survival adapted from the microscope-based dye exclusion method of Weisenthal (21, 22), using PI to identify viable cells. Similarly, Ellwart et al. (23) recently described a flow cytometric vitality assay using FDA and ethidium bromide. These methods quantify viable tumor cells by adding an exact number of acetaldehyde-fixed duck erythrocytes (20) or fluorescent microbeads (23) to a given number of tumor cells in suspension culture, with surviving cells expressed as the ratio of viable tumor cells:marker particles (duck erythrocytes or microspheres). Based on the magnitude of separation of mean fluorescence between viable and nonviable cell/debris populations, we feel that dual labeling with FDA and PI is superior to single labeling with PI in discriminating viable cells, but is probably comparable to dual labeling with FDA/ethidium bromide. Moreover, we feel that our method of quantifying viable cell number by timed viable cell count and calibration of sample flow rate is an improvement over methods in which duck erythrocytes or fluorescent microspheres are added. Our method ensures that the possibility of error in viable cell counts caused by outliers of the erythrocyte or microbead population falling within the viable cell window is eliminated. Such an error could be important in estimating low numbers of residual viable cells. The dynamic range of cell survival we report (approximately 4 logs) is greater than the 2 logs reported previously (20).

The flow cytometric assay of cell survival may provide a means of estimating the relative drug sensitivity in blast cells from patients with AML. However, to do this under the most optimal assay conditions (assess viability 72 h after drug exposure), in vitro culture conditions for AML blast cells must be improved. Use of recently available human recombinant growth factors (e.g., granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, interleukin 3) that stimulate leukemic cell growth (2) may accomplish this. Miyachi et al. (24) have reported that AML blast cells in culture respond to the above growth factors by both self renewal and differentiation; cells from all six patients showed net growth after 7 days in culture in response to one or more of the growth factors. Since such in vitro culture of AML blast cells promotes the growth of cells with self-renewal capacity (24), cytotoxicity studies done using growth-factor stimulated AML blast cells...
may reflect more accurately the drug sensitivity of cells clinically relevant to the in vivo perpetuation of the leukemic cells. Moreover, the ability of flow cytometry to distinguish blast cells from differentiated cells (e.g., polymorphonuclear cells, RBCs) can be utilized to ensure that the FDA/PI viability estimate is made only on blast cells. Flow cytometric estimation of leukemic cell drug sensitivity can be obtained for all patients because of insufficient colony growth in control cultures. In contrast to the MTT assay, for reasons mentioned earlier, the flow cytometric method adapts better to the study of primary suspension cultures of blast cells obtained from AML patients. Further research into the clinical relevance of the flow cytometric estimation of leukemic cell survival following in vitro drug treatment is ongoing in our laboratory.

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REFERENCES

Estimation of Cell Survival by Flow Cytometric Quantification of Fluorescein Diacetate/Propidium Iodide Viable Cell Number

Douglas D. Ross, Christopher C. Joneckis, José V. Ordóñez, et al.


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