**Comparison of Dye Exclusion Assays with a Clonogenic Assay in the Determination of Drug-induced Cytotoxicity**

Larry M. Weisenthal, Patricia L. Dill, Nathaniel B. Kurnick, and Marc E. Lippman

ABSTRACT

The following factors must be considered when dye exclusion assays are interpreted. (a) It may require several days for lethally damaged cells to lose their membrane integrity following a cytotoxic insult. (b) During this time, the “surviving” cells may continue to proliferate. (c) Also during this time, some lethally damaged cells may undergo an early disintegration, so that they are not present to be stained with dye at the end of the culture period. Factors b and c may cause an underestimate of cell kill when the results of the assay are based upon the traditional “percent viability” expression. In order to overcome these problems, an internal standard was developed and tested. This was based upon the addition of a constant number of permanently fixed duck erythrocytes to the cultures of cells from two different established tumor cell lines. Results were based upon comparisons of the ratios of “viable” tumor cells to duck erythrocytes on permanent cytocentrifuge slides prepared from the cultures. This novel “ratio” method was found to be a more sensitive index of drug-induced cell kill than the traditional percent viability method. A standard agar cloning assay gave somewhat higher estimates of cell kill than the ratio method, although both assays were in qualitative agreement for the drugs tested. All three assays demonstrated a clear dose-effect relationship for most of the drugs tested. Dye exclusion assays may have a useful role in chemosensitivity testing in vitro.

INTRODUCTION

The ability of cells to exclude vital dyes such as eosin, nigrosin, trypan blue, and erythrosin B has been used frequently as an index of cell viability (5, 18, 19, 24, 28). Several authors have used dye exclusion techniques to estimate cell kill following exposure to cytotoxic drugs (12, 31). In one of these studies (12), the results of an in vitro test for cell kill based on dye exclusion correlated perfectly with the in vivo clinical response of lymphoma patients to cytotoxic chemotherapy with the same drugs used in the in vitro test. However, other authors (5, 18, 25, 26, 35, 36) have reported poor correlation between dye exclusion tests and other in vitro tests which measured the reproductive capacity of cells in culture following exposure to drug or enzymatic insults. As a result of these latter studies, it has been stated that “the ‘classically’ used dye exclusion test should be finally relegated to the historical niche it deserves and should not be used to evaluate agents intended for controlling the reproduction of proliferating cell populations” (26).

We have studied several factors which may influence the interpretation of dye exclusion assays. Based on these studies, we conclude that dye exclusion assays are useful in estimating drug-induced cell lethality, provided that certain technical pitfalls are recognized. The failure to recognize these pitfalls may have been responsible for the poor correlations noted in some of the previous investigations (5, 18, 25, 26, 35, 36). Dye exclusion tests may be especially valuable in assessing drug-induced cytotoxicity in nondividing cells and in cells which may not be evaluable by other means (e.g., lack of ability to clone).

MATERIALS AND METHODS

Cell Lines. HL-60 human promyelocytic leukemia cells (6) were a gift from Dr. Stephen Collins of the Seattle, Wash. Veterans Administration Medical Center. MDAY-D2 cells (14, 20) were early-in vitro-passage cells grown in suspension culture following removal from a DBA/2 mouse ascites tumor. This latter tumor is an undifferentiated metastatic neoplasm derived by pasaging a methylcholanthrene-induced A strain sarcoma in DBA/2 mice. The phenotypic, karyotypic, and antigenic characteristics of these cells have been described (14, 20). They were a gift of Dr. Philip Frost of the Long Beach Veterans Administration Medical Center. Both cell lines were maintained in liquid culture with RPMI 1640 containing 15% fetal calf serum and 5% horse serum.

Drugs. All drugs were obtained in powder form from the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. Drugs were dissolved in 0.15 M NaCl at 10 times the desired final concentration and stored at −70°C until use, except for 1,3-bis(2-chloroethyl)-1-nitrosourea, which was freshly prepared for each experiment by dissolving in ethanol and then diluting in 0.15 M NaCl.

Drug Incubations and Cell Counting. Asynchronous cells were suspended in fresh complete medium (containing serum) at a concentration of 10⁶ cells/ml and incubated for either 1 hr or continuously at the drug concentrations indicated in “Results.” Cells were counted at the indicated times using a Model A Coulter Counter (Coulter Electronics, Hialeah, Fla.). Dye exclusion assays were performed using a novel procedure described elsewhere (33).Briefly, cells were cultured for 1 to 6 days in liquid RPMI 1640 plus 15% fetal calf serum and 5% horse serum. Cells were cultured in polypropylene tubes which prevented fibroblast growth in the early-passage MDAY-D2 cells. Cells were then stained for 10 min with 1% Fast green dye, sedimented onto microscope slides using a Cytospin centrifuge (1200 rpm, 7 min), and counterstained with a modified hematoxylin-eosin technique. The slides, fresh from the cytocentrifuge, were placed in a standard histological staining rack and stained, without prefixing, in Harris formula hematoxylin solution (Harleco, Gibbstown, N.H. 08024) to which glacial acetic acid was added in a final concentration of 1.9%. After 90 sec, the staining rack was removed and transferred to a solution of 7.5% ethyl alcohol for 2 quick dips, lasting less than 5 sec. Following this,

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2 To whom requests for reprints should be addressed.
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the rack was quickly dipped twice in 3 additional changes of 7.5% ethanol to remove the excess hematoxylin before eosin staining. Following the ethanol washes, the slides were placed in eosin solution (100 ml 1% eosin, 10 ml 1% phloxine, 780 ml 95% ethanol, and 4 ml glacial acetic acid) for precisely 30 sec, during which time fixation took place. The slides were then briefly dehydrated by 2 successive quick dips in 95% alcohol, 100% alcohol, and xylene, respectively. The slides were then coverslipped using mounting balsam. “Living” cells stained pink with hematoxylin:eosin, and “dead” cells stained green. The ratio of living cells over living cells plus dead cells (percent viability) was determined for each cytocentrifuge slide. This result was then expressed as a percentage of control (culture without drug). The classic percent viability method of expressing the results of a dye exclusion assay may be dramatically altered by (a) continued proliferation of the living cells during the culture period and by (b) early disintegration of the dead cells, so that they are no longer present to be stained with dye at the end of the culture period. We hypothesized that direct comparisons between the numbers of “living” cells present in the control and drug-treated cultures might therefore give a more sensitive estimate of drug effect than the classic percent viability method. An internal standard was developed to allow these comparisons to be made.

Internal Standard of Fixed Duck Erythrocytes. A 4-kg white duck was purchased from a local farm and then released to a local pond after removal of 10 ml of blood from a large wing vein. The blood was heparinized, and the DRBC was washed 3 times with 0.15 M NaCl. They were then resuspended in 2% acetaldehyde in phosphate-buffered saline by dropwise addition of the packed DRBC into the acetaldehyde buffer with continuous stirring on a magnetic stirring plate. Cells were maintained in this buffer for 10 days and were then "sterilized" by soaking in 99% ethanol overnight, followed by extensive washing with sterile 0.15 M NaCl. The cells were finally suspended in 0.15 M NaCl containing streptomycin (100 µg/ml), penicillin (100 units/ml), and amphotericin B (1 µg/ml). The cells were adjusted to a hematocrit of about 2.5% (volume of DRBC over total volume of DRBC plus suspending medium). This "working suspension" could be stored indefinitely at 4° and was of sufficient quantity for hundreds of assays. Prior to use in assay, this working suspension was diluted 1:40 to approximately 7 million DRBC/ml. One-half ml of this suspension was added to 20 ml of 2% Fast green in 0.15 M NaCl. In preparing the cytocentrifuge slides, 0.2 ml of tumor cell suspension was added to 0.2 ml of the Fast green:DRBC suspension, and the entire 0.4-ml mixture was sedimented after 10 min onto microscope slides with the Cytospin centrifuge. The DRBC were easily identifiable as nucleated microelliptocytes (Fig. 1). For quantification of results, the ratio of living (stained with hematoxylin:eosin, not Fast green) tumor cells over simultaneously counted DRBC was determined and expressed as a percentage of control (culture without drug). All assays were performed in triplicate, and the results reported represent the mean of the triplicate cultures.

To illustrate how this type of a comparison may give a more valid estimate of cell kill than the traditional percent viability measurement, consider the following example.

At time 0, replicate cultures containing 100 viable tumor cells are exposed to either physiological culture medium or to Drug A. The generation time of surviving cells is 24 hr. The physiological culture medium kills 0% of the cells, and Drug A kills 90% of the cells. Of the 90% of the cells which are killed, presume that one-half of the cells disintegrate immediately, while one-half gradually lose their membrane integrity over 4 days and stain with dye on Day 4. On Day 4, there will be 1600 viable cells in the control culture (100 × 2 × 2 × 2 × 2), which do not stain with the dye. The percent viability [100 times the number of viable cells/(number of viable cells plus nonviable cells)] will be 100% for the control cells. At the same time, there will be 160 viable cells in the drug-treated cultures (10 × 2 × 2 × 2 × 2). There will also be 45 nonviable cells, which have not disintegrated and remain in the cultures to be stained with the dye. Thus, the percent viability will be 100 × 160/(160 + 45), or 78%. This would obviously be an underestimate of cell kill. If, on the other hand, a fixed number of inert DRBC were added to the cultures, the assay could be based upon the relative ratios of viable cells to DRBC. In the above example, 100 DRBC might be added to each culture. In the control cultures, there would be a ratio of 1600 viable cells to 100 DRBC or 16 viable cells to one DRBC. In the drug-treated cultures, there would be 160 viable cells to 100 DRBC or 1.6 viable cells to one DRBC. Based upon a percentage of control, the surviving fraction of viable cells would be 1.6 to 16 or 10%. Thus, the use of the DRBC internal standard allows for a correction of early disintegration of some lethally damaged cells and continued proliferation of the surviving cells during the time required for some of the remaining lethally damaged cells to lose their membrane integrity.

Clonogenic Assay. The standard agar cloning assay as described by Ogawa et al. (22) was utilized. Cells were either incubated with drugs for 1 hr in liquid medium and then plated in agar, or else drugs were incorporated into the agar medium for continuous exposure. Cells were plated at 25,000 cells/ml (MDAY-D2 cells) or 50,000 cells/ml (HL-60 cells). Cells were plated in an upper layer of 0.3% agar over a base layer of 0.5% agar in a 35-mm plastic Petri dish as described by Park et al. (23), except that daily feedings with fresh medium were not used. The medium used in both the top and bottom layers was the same enriched RPMI 1640 (described above) used in the liquid cell cultures. Colonies were counted at 7 days (MDAY-D2 cells) or at 10 to 14 days (HL-60 cells). Cloning efficiencies were 10% for the MDAY-D2 cells and 6% for the HL-60 cells.

RESULTS

Time Course of Cell Kill after Drug Exposure. To determine the optimum time which should be allowed to elapse following drug exposure before testing with Fast green, HL-60 cells were exposed to a variety of drugs and then incubated for varying periods of time in the absence of drugs before the staining-counterstaining procedure was carried out (Chart 1). A potential concern was that the surviving cells would continue to proliferate during the period between drug incubation and the performance of the test. This would obscure by dilution the number of cells which were killed by the drug. For this reason, this experiment was carried out in the presence of only 2.5% fetal calf serum, which reduced cell multiplication during the culture period. The results indicate an increasing loss of viability after exposure to 4 of the drugs which continued for at least 6 days following drug exposure. With a normal (i.e., 20%) serum concentration, the control cultures “outgrew” the medium during the 6-day culture period, and their viability spontaneously diminished. For this reason, a 4-day culture period was generally used in the subsequent dose-response experiments.

Testing of the Duck Erythrocyte Internal Standard. In the first experiment (Chart 2), MDAY-D2 cells were exposed to doxorubicin for 1 hr and then placed back in liquid culture without drug for 6 days. On each day, the percent viability (living cells divided by living plus dead cells) was determined, as well as the ratio of living cells divided by simultaneously counted DRBC. The total cell count, as determined by a Coulter Counter, was also measured. In Chart 2, the ratio of viable cells to DRBC is normalized to the same scale as the cell counts. This chart shows that there was a typical log phase-plateau phase growth pattern in the control cells. This pattern is mimicked remarkably by the viable cell:DRBC ratio, except that there is a decline after the third day. As the plateau phase is probably secondary to nutrient depletion and/or acidity in

JANUARY 1983
these suspension cultures, this decline in the viable cell:DRBC ratio probably represented an actual loss of viable cells in the later stages of the cultures. Thus, the viable cell:DRBC ratio may be a more accurate representation of the relative number of viable cells than that represented by the Coulter Counter counts. In the drug-treated cultures, there was a 3-day lag in the growth of the cultures, followed by a resumption in log growth. This may represent either a cell cycle arrest, unassociated with cell kill, or it may represent a plateau balance arising from ongoing cell lysis balanced by continued proliferation of the surviving cells. This effect of drug treatment was again closely mimicked by the ratio of living tumor cells to DRBC. The percent viability of the control cultures declined after 3 days, probably because of nutrient depletion. The percent viability of the drug-treated cultures declined more precipitously for 4 days but then increased. This occurred presumably because the dead cells were being diluted by the continued proliferation of the surviving cells observed between Days 4 and 6.

Chart 3 illustrates the results of a similar experiment in which doxorubicin was maintained continuously during the culture period. With this greater drug exposure, there was much greater cell kill. This greater cell kill was more dramatically reflected in the ratio of viable cells and in the percent viability than in the electronic particle counts, presumably because the latter technique detected incompletely lysed dead cells which stained with Fast green.

Comparison of Dye Exclusion Techniques with Clonogenic Assay. As the above results (Charts 2 and 3) indicated that the viable cell:DRBC ratio might be a more sensitive index of cell kill than the percentage of viability, both of these techniques were compared to an agar clonogenic assay. A number of standard antineoplastic agents were tested at several concentrations. Cells were exposed to the drugs for 1 hr and then cultured for 4 days (for the dye exclusion studies) or for 7 to 14 days (for the colony formation studies), as described in "Materials and Methods."

With several of the drugs tested, the clonogenic assay gave a somewhat lower estimate for cell survival at some concentrations than did the viable cell:DRBC ratio method (Charts 4, A, E, and F, and 5, A, E, and F). Conversely, the ratio method

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**Chart 1.** Time course for cell kill as measured by Fast green dye exclusion in HL-60 cells. Cells were incubated in low serum (2.5%) medium following drug exposure in order to limit cell proliferation. The control cells had a percent viability of greater than 95% during the 6-day culture period. All results are expressed on the ordinate as a percentage of control. Estimates of cell kill increased for at least 6 days with several drugs, as judged by Fast green staining. 5FU, 5-fluorouracil; MTX, methotrexate; DOXO, doxorubicin; LPAM, L-phenylalanine mustard; HN2, nitrogen mustard; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea.

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**Chart 2.** Effect of doxorubicin (0.12 µg/ml, 1 hr) on MDAY-D2 cells as assessed by 3 different techniques: Coulter Counter particle counts (----) (Y axis units are cells/ml × 10^-4); ratio of living tumor cells to DRBC, normalized to the same scale as the Counter counts (---); and percent viability (-----) [(Y axis units are percent viability (living cells/living + dead cells, expressed as a percentage)]. ○, control cultures; □, doxorubicin-treated cultures.

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**Chart 3.** Effect of doxorubicin (0.12 µg/ml, continuous exposure) on MDAY-D2 cells as assessed by 3 different techniques. Symbols, lines, and Y axis units are as described in the legend to Chart 2.
gave a lower estimate for cell survival for methotrexate than did the clonogenic assay (Charts 4H and 5G). Both methods demonstrated a clear dose-response relationship for most of the drugs, however, and the estimates of drug efficacy were qualitatively similar for both methods (Charts 4 and 5). In contrast, the traditional percent viability method showed a dose-response relationship only for nitrogen mustard, 1,3-bis(2-chloroethyl)-1-nitrosourea, and doxorubicin over the concentrations tested in the MDAY-D2 cells (Chart 4). This method failed to detect effects by 5-fluorouracil, DDP, and methotrexate, in contrast with the other methods (Chart 4, B, D, and H).

In order to determine if a greater drug exposure would allow detection of a dose-response effect, 4 drugs were tested using continuous drug exposure, rather than a 1-hr exposure (Chart 6, A to D). A clear dose-effect relationship was observed for vinblastine, 5-fluorouracil, and DDP, but not for methotrexate. Both the clonogenic and ratio assays gave generally lower estimates of cell survival than did the percent viability assay. The clonogenic assay gave the lowest estimates of cell survival for vinblastine and DDP, while the ratio assay gave the lowest estimates of cell survival for concentrations of methotrexate below 20 \( \mu g/ml \) (Chart 6, A to D).

**DISCUSSION**

On the basis of the studies described here, we conclude that dye exclusion assays may have a role in the assessment of in vitro cell kill caused by cytotoxic drugs. However, several potential pitfalls must be considered.

Sufficient time must elapse following drug treatment for lethally damaged cells to lose their membrane integrity. Other investigators reported that dye exclusion assays gave no indication of neuraminidase-induced cytotoxicity in murine lung cancer cells (35). This conclusion was based on a retained ability of the cells to exclude dyes when they were assayed...
immediately after a 1-hr exposure to the enzyme. Still other authors reported that dye exclusion assays performed immediately after drug exposure underestimated cell kill in 2 different cell lines (5). It is not obvious that a loss of membrane integrity must coincide temporally with an injury that is eventually lethal. Our data strongly imply that some lethally damaged cells may not take up a stain until several days after exposure to a cytotoxic drug (Chart 1).

Cell cycle-specific drugs may inhibit cell growth without being cytotoxic, and this might not be detected with a method based on cell kill.

The surviving cell fraction may continue to proliferate during the time required for the lethally damaged fraction to lose membrane integrity. Thus, by the time the killed cells fail to exclude dye, the surviving cells may have proliferated to such an extent that the apparent percent viability of the drug-treated cells is misleadingly high.

Some lethally damaged cells may undergo early disintegration, so that they are not present to be stained with dye at the time the assay is to be performed.

Our method of using a constant number of DRBC as an "internal standard" allows for a direct comparison of the number of surviving cells present in control and drug-treated cultures and increases the sensitivity of the dye exclusion method in detecting cell kill. We have given a theoretical example in "Materials and Methods" of the manner in which this internal standard works to correct for the above pitfalls.

It must be noted, however, that additional potential pitfalls remain. For example, (a) it is possible that different types of cells may require different periods of time for a loss of membrane integrity to occur following lethal injury. We have chosen a 4-day post-drug exposure period before the addition of the Fast green stain as a practical interval to be tested in clinical chemosensitivity assays. However, if a single cell line is to be assayed repeatedly, it would be appropriate to perform preliminary experiments to determine the optimum interval between drug exposure and Fast green dye staining.

(b) It is possible that some drugs may induce cell cycle delays not associated with cell kill (25). This could produce differences between the ratios of living tumor cells to DRBC in rapidly proliferating cells which would be indistinguishable from differences caused by actual cell kill. However, this objection should be evaluated in light of the following considerations. A 90% cell kill (1-"log" cell kill) is probably the minimum cell kill which translates into an in vivo response (34). In the absence of any cell kill, it would take a complete cell cycle arrest maintained over 3 to 4 generation times in 100% of the drug-treated cells to produce a log difference in the cell numbers between the control and treated cultures. Therefore, the question is, to what extent does a drug-induced cell cycle arrest occur in the absence of a correspondingly great cell kill? This question has been addressed in several elegant studies (1--4, 8--10, 30). Although the data are very complex, the general findings have been that cell kills of less than 1 log are not associated with cell cycle delays great enough to cause false-positive cytotoxicity assays, based on the criterion for a positive assay of a 1-log difference in the number of proliferating cells in control and drug-treated cultures. It should also be noted that a cell cycle delay in the absence of cell kill would result in a progressively decreasing estimate of cell kill in a colony assay if control and drug-tested cultures were serially counted over time. This phenomenon has not, to our knowledge, been reported to occur in drug studies utilizing clonogenic assays (possibly because a systematic study to examine this potential problem has not been carried out).

(c) It is possible that some drugs may cause a lethal injury which might not become manifest until several cell generations had passed after the lethal injury. If an investigator were to wait for 6 generation times before counting colonies (the time required for a 64-cell colony to form), then the killing of a clone which died after 3 generation times would be detected in a clonogenic assay. However, this cell kill would not be detected if the investigator had performed a nonclonogenic assay within the first 3 generation times. This argument has been used to support the theoretical superiority of clonogenic assays (25). This issue of "delayed death" is very complex, however. It has been shown that either (a) none of the clone, (b) a part of the clone, or (c) all of the clone may undergo delayed death after a cytotoxic insult (29). With delayed death of a part of the clone, the investigator would overestimate cell kill by counting colonies "early" (before the surviving cells of the partially destroyed clone could divide enough to permit the clone to be scored as a colony) and underestimate cell kill by counting colonies "late" (when the killing of a part of the clone would go undetected). Although both clonogenic and nonclonogenic assays would be subject to pitfalls associated with the delayed death phenomenon, these pitfalls would be of practical significance only if the fraction of cells undergoing delayed death after a cytotoxic insult were large compared to the fraction undergoing early death (within the first generation following the insult). Additional studies would be needed to determine whether or not this potential problem is of practical importance.

Another potential pitfall is that, in monolayer cell systems, nonviable cells tend to detach from the culture flask (17) and may be washed away during cell harvesting. This would leave behind only viable cells to test with dye. Roper and Drewinko used a cell-harvesting technique which would be expected to wash away nonviable cells (11, 25), and this potential artifact may partially explain the poor correlation between cell kill and dye exclusion reported by these authors (25, 26). Our "DRBC ratio" method does not depend on the counting of nonviable
cells and would not be affected by this potential pitfall.

In the present study, we compared the dye exclusion techniques to a standard agar cloning assay. In general, the cloning assay gave somewhat lower estimates of cell survival than did the assay based upon the ratio of viable cells to DRBC, although both assays were in qualitative agreement for the drugs tested. Both assays gave substantially lower estimates of cell survival than did the traditional dye exclusion method based upon percent viability. Other authors have also noted that traditional dye exclusion methods were relatively insensitive compared to clonogenic assays (5, 25, 26).

When 2 in vitro assays do not precisely agree, it is appropriate to consider which of the assays is "correct." It is possible that the more sensitive assay may overestimate cell kill or that the less sensitive assay may underestimate cell kill. For example, the stress of plating cells in agar or at low cell densities may reduce the capacity of the cells to repair what would only by sublethal damage if the cells were maintained in their usual mass culture environment after drug exposure. This might cause clonogenic assays to underestimate cell damage. We have found that doxorubicin at 0.12 µg/ml caused 0% cell survival in MDAY-D2 cells as assessed by the clonogenic assay but 30% cell survival when assessed by the dye exclusion ratio assay (Chart 4F). The 30% surviving cell fraction continued to proliferate logarithmically when the cultures were refed with fresh medium, indicating that these cells retained their capacity for self-renewal (data not shown). Other experiments, based on phosphate incorporation, also indicated that agar cloning assays may overestimate cell kill in HL-60 cells.*

REFERENCES

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L. M. Weisenthal et al.


Fig. 1. Appearance of cytocentrifuge slide preparation after Fast green staining but before H & E counterstaining. Prefixed DRBC have been added as described in "Materials and Methods." 1. "living" cells, which are clear (nonstained) before H & E and which stain reddish pink upon H & E counterstaining (not shown); 2. "dead" cells, which stain a brilliant green (black in this figure) with Fast green and which retain this stain following H & E counterstaining (i.e., they remain green and do not turn reddish pink); 3. degenerating "ghost" cells, which stain pale green; 4. DRBC, appearing as highly refractile nucleated microellipocytes.