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Drug Testing in Established Cell Lines by Flow Cytometric Vitality Measurements *versus* Clonogenic Assay

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ABSTRACT

The clonogenic assay is widely considered to be the most valid test for predicting tumor cell sensitivity to cytostatic drugs. In this study it was compared with early growth curves of human leukemic cell lines (HL-60, K562, Reh) after treatment with different types of cytostatic drugs (Adriamycin, *cis*-diamminedichloroplatinum(II):, 1-β-D-arabinofuranosyl-cytosine, and 5-fluorouracil) for 1 and 24 h. Following drug treatment two parallel cultures were started: a soft agar culture for the clonogenic assay; and a liquid suspension culture for vital cell counting by measuring esterase activity with fluorescein diacetate at different time points. The latter was recorded using flow cytometry during the following 3 days in 12-h intervals. For each drug concentration a survival factor was calculated from the growth curve between 24 and 72 h. This survival factor takes into account both the *y* intercept of the extrapolated growth curve and the slope of the growth curve. The dose-response curves resulting from either the survival factors or the clonogenic assay were always nearly identical. The results demonstrate that in established cell lines flow cytometric determination of vital cell increase rates provides a convenient alternative to the clonogenic assay for drug testing.

INTRODUCTION

In the past decade the clonogenic assay has been widely accepted as the most reliable test for predicting the response of human tumor cells to cytostatic drugs (1). Despite the long time needed for its performance, its technical limitations (2, 3), and conceptual objections (4) the success of the human tumor stem cell assay is based on its sound theoretical concept. According to this concept, each tumor contains a compartment of cells with unlimited self-renewal which is the proper compartment to be assayed (2, 3).

Still faster assays are nevertheless desirable for drug sensitivity testing. For studying cell survival, dye exclusion measurements performed immediately after drug treatment are the fastest. However, when simple alive:dead ratios or percentages of vital cells were compared with the clonogenic assay, survival was generally found to be overestimated (5–7). More relevant information on cell survival following drug exposure is provided by counting absolute numbers of vital cells. Whereas poorly corresponding results were obtained when the number of vital cells was determined 1 day after drug treatment (8), a better correspondence resulted when the counts were taken 3 or 4 days later (9–13). Among these latter studies the degree of correspondence between the two methods differed considerably.

In another type of approach towards the prediction of drug response, growth curves of tumor cells were established after complete recovery from the injury had occurred (14). The authors of this study obtained survival values comparable to the colony assay when they extrapolated the growth curves about 10 days back. This predictive test, however, is almost as complicated and time consuming as is the clonogenic assay. Roper and Drewinko (6) compared the clonogenic assay with changes in cell numbers instead of using absolute numbers during the first 5 posttreatment days. The obtained cell doubling times or slopes showed a dose dependence; however, they failed to correlate with results from the colony assay.

In view of these different results reported on permanent cell lines in the literature it may be speculated that correspondence between vitality measurements and clonogenic cell counts after drug exposure may primarily depend on the choice of an appropriate parameter for the vital cells. In the present study the number of vital cells from established tumor cell lines has been investigated during the first 3 posttreatment days and compared with the clonogenic assay. Two variables were considered in this approach: (*a*) the change in absolute vital cell numbers following drug exposure, and (*b*) the change with time in vital cell numbers as compared to the untreated controls. For a precise monitoring of vital and nonvital cells esterase activity was measured by using flow cytometry.

MATERIALS AND METHODS

Cells. The experiments were performed with 3 established human leukemic cell lines, the lymphoblastic Reh (15), the pluripotent K562 (16), and the promyelocytic HL-60 (17) cells. The cells were grown in suspension in RPMI 1640 (Gibco, Karlsruhe, Federal Republic of Germany) supplemented with 10% inactivated fetal calf serum (Gibco) at 37°C in a humidified atmosphere with 5% CO_2 and 95% air. Fifty units/ml penicillin, 50 μg/ml streptomycin (Seromed, West Berlin) and 2 mM L-glutamine (Seromed) were added. Twenty-four h prior to drug incubation the cells were suspended in fresh medium at a concentration of about 20,000 (K562), 50,000 (HL-60), or 200,000 (Reh) cells/ml and divided into equal 30-ml cultures (80 cm^2 culture flasks; Nunc, Roskilde, Denmark). At the beginning of drug incubation, the cells were growing exponentially with a population-doubling time of 19 h (K562), 22 h (HL-60), and 36 h (Reh). From 1 to 2% of the cells were dead.

Drug Exposure. Without cooling down of the prepared cell suspensions, Adriamycin (Farmitalia Carlo Erba GmbH, Freiburg, Federal Republic of Germany), *cis*-diamminedichloroplatinum(II) (Rhone-Poulenc Pharma GmbH, Norderstedt, Federal Republic of Germany), 1-β-D-arabinofuranosylcytosine (Heinrich Mack Nachf., Illertissen, Federal Republic of Germany), or 5-fluorouracil (Farmitalia) were added to give 6 exponentially increasing final concentrations of each drug. The suspensions with the drugs including 2 control suspensions were incubated at 37°C either in a shaking water bath for 1 h or in a CO_2 incubator for 24 h. The drug exposure was stopped by washing the cells twice with 4°C cold medium. Each sample was resuspended in 30 ml medium prewarmed to 37°C and cultured in a CO_2 incubator for enumeration of vital cell increase with time.

Vitality Measurements. Two ml of cell suspension were taken from the liquid cultures at 12-h intervals. Without any further treatment 50 μl of fluorescent beads (multicolored Fluorospheres; Coulter Electronics GmbH, Krefeld, Federal Republic of Germany) kept at a constant concentration throughout all experiments were added. The cells were stained using 100 × stock solutions of FD[2] dissolved in acetone and EB dissolved in distilled water. FD (Serva, Heidelberg, Federal Republic of Germany) and EB (Serva) were used at final concentrations of 10 and 40 μg/ml, respectively. FD is converted by unspecific esterases of vital cells to fluorescein which has a bright green fluorescence. The polar fluorescein is trapped in cells with intact surface membranes. EB

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[2] The abbreviations used are: FD, fluorescein diacetate; EB, ethidium bromide.

stains the nuclei of dead cells red. The samples were measured 2 min later by a 2-parametrical flow cytometer (FACS II; Becton Dickinson, Mountain View, CA) at 488 nm excitation wavelength. The vital cell population was recognized by its bright green fluorescence in the biparametric fluorescence distributions (18) as illustrated in Fig. 1.

Analysis of Flow Data. After logarithmic amplification of the fluorescence signals the pulses were digitized in a FDAS II flow data analyzer (Ahrens, Bargteheide, Federal Republic of Germany). The flow data lists were converted into 2-dimensional histograms by a PDP-11 computer. The number of vital cells and beads was determined interactively on a graphic terminal equipped with a light pen. A relative number of vital cells in each sample was derived by dividing the constant number of beads into the number of vital cells.

Interpretation of Data. From the relative numbers of vital cells at different times after drug treatment, a survival curve was obtained for each drug concentration. The survival curves with logarithmic ordinates were approximated between 24 and 72 h by straight lines of the form $Y = a + b \times X$. The control was approximated by $Y = a(\text{control}) + b(\text{control}) \times X$. From each survival curve based on one drug concentration a survival factor f was calculated using an empirically derived formula:

$$f = 10^{a}/10^{a(\text{control})} \times b/b(\text{control})$$

Dose-response curves resulted from survival factors at different drug concentrations. The concentrations which inhibit cell growth by 90% were calculated from the dose-response curves.

Colony Assay. In parallel with the liquid suspension cultures a standard agar colony assay (19) was started. Tumor colony forming units were cultured in Dulbecco's modified Eagle's medium (Gibco) supplemented with 0.3% agar (Difco Bacto-agar; Difco Laboratories, Detroit, MI) and 20% fetal calf serum (Flow Laboratories GmbH, Meckenheim, Federal Republic of Germany). The cultures were performed in 1-ml volumes using 35-mm plastic Petri dishes (Greiner, Nürtingen, Federal Republic of Germany) and incubated at 37°C in a fully humidified atmosphere containing 10% CO_2 in air. Colonies (greater than 40 cells) were scored after 10–20 days using a dissecting microscope at ×32. Plating efficiencies amounted to 65% for K562, 48% for HL-60, and 37% for the Reh cells. A linear relation of the number of cells plated and colonies could be established. Concentrations of cells seeded were chosen so as to yield about 150 colonies/untreated control plate. The survival of clonogenic cells was expressed as ratio between the mean number per plate of colonies surviving on triplicate plates and the mean number per plate of colonies growing on two triplicate control plates multiplied by 100.

RESULTS

In the FD/EB assay a group of nonvital cells after drug treatment was observable earlier than with other vitality measurements, *e.g.*, with erythrosin B and forward scatter or acridine orange and EB (data not shown). Cells which had lost esterase activity and still excluded EB from the nucleus (nonvital cells; *a* in Fig. 1) immediately appeared after drug treatment. This intermediate population of damaged cells without esterase activity became stainable with EB (nonvital cells; *b* in Fig. 1 between 12 and 60 h. After 72 h only very few dead or damaged cells could yet be seen.

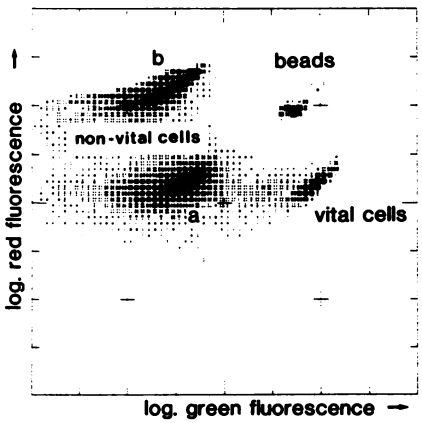

Fig. 1. Fluorescence distribution of drug-treated Reh cells after adding fluorescein diacetate and ethidium bromide to the cell suspension. The exponentially growing cells were incubated with 5 mg/liter Adriamycin for 1 h and thereafter grown without drugs in a liquid suspension culture for another 21 h. A constant number of multispectral fluorescent beads was added to the cell suspension for the calculation of the relative number of vital cells.

Figs. 2*A*, 3*A*, and 4*A* show examples of survival curves measured by the FD/EB vital test over 3 days at about 12-h intervals. The *ordinates* represent the logarithms of the relative number of vital cells obtained from 2-dimensional distributions. By semilogarithmic representation of the survival curves a linear approximation could be performed. This, however, proved to be satisfactory only after a lag time of 24 h. Three types of straight lines were observable and will be demonstrated by examples: type 1, in Fig. 2*A* all survival curves from HL-60 cells treated with various Adriamycin concentrations for 1 h show a common intersection with the ordinate ($t = 0$) but have different slopes; type 2, doses of 1-β-D-arabinofuranosylcytosine between 1.25 and 1280 mg/liter administered for 1 h equally decreased the number of vital K562 cells by a constant factor of about 0.6 at observation times beyond 24 h (Fig. 3*A*); type 3, the survival curves of Reh cells after 24 h of 1-β-D-arabinofuranosylcytosine treatment had different slopes and different intersections with the ordinate (Fig. 4*A*). In most experiments survival curves of type 1 were observed.

Dose-response curves were constructed by inserting a survival factor for each drug concentration. Examples are shown in Figs. 2*B*, 3*B*, and 4*B* in which they were compared with the results from the clonogenic assay. Both types of survival curves showed an almost identical pattern. In the case of a plateau with low drug concentrations such as in Fig. 4*B*, both methods produced an identical extension of this part of the curve. When there was a negative slope in the growth curve (Fig. 2*A*, 0.32 mg/liter) no colonies could be found in the clonogenic assay (Fig. 2*B*, 0.32 mg/liter). Furthermore, both types of assay identically recorded dose-response curves that showed no decrease with increasing drug concentration (Fig. 3*B*).

With both methods in Reh cells were the most sensitive ones among the three cell lines (Table 1). Coincidentally the Reh cells needed only 1/2 mg/liter 1-β-D-arabinofuranosylcytosine and an incubation time of 1 h to decrease the survival to 10%, whereas with both methods the HL-60 and K562 cells needed more than 1000 mg/liter. Furthermore, both methods equally showed that 5-fluorouracil had almost no effect on any cell line when administered for only 1 h.

DISCUSSION

In this study it has been demonstrated using suspended human leukemic cell lines that an assay based on the counting of vital cells between 24 and 72 h after drug exposure is suitable for establishing dose-response curves identical to those obtained by the clonogenic assay. The critical parameter derived from the data of cell counting is the survival factor f. It is composed of 2 mathematical factors. The first one is the extrapolated linear ordinate value representing the intersection of growth curve with the time at which drug exposure is terminated ($t = 0$). The second one is the slope of the growth curve on a semilogarithmic scale. Both components are expressed in relation to the respective conditions in control cultures.

After termination of drug exposure a series of cell irritations occurs such as centrifugation, washing, and refeeding of the

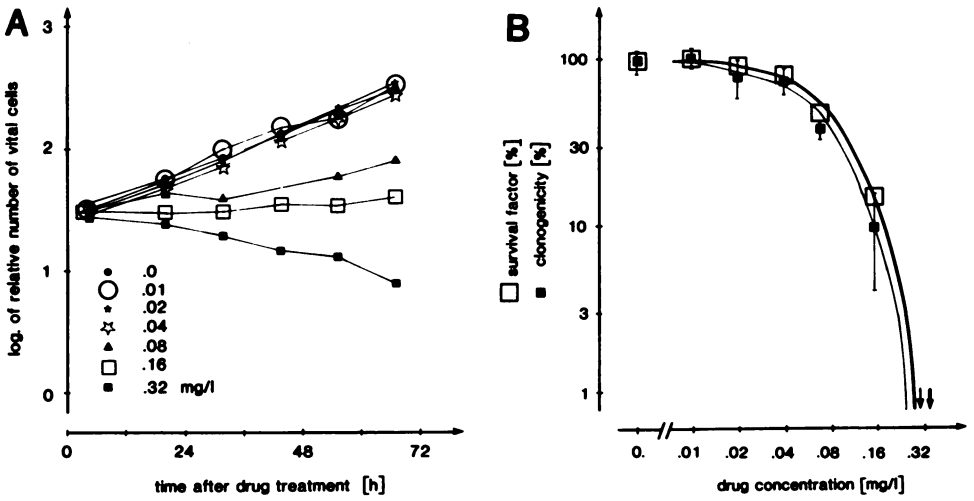

Fig. 2. *A*, survival curves of HL-60 cells which had been treated with different concentrations of Adriamycin for 1 h. The relative numbers of vital cells were determined by flow cytometric measurement of esterase activity. *B*, comparison of dose response curves measured by the increase with time of vital cells (□) and by the clonogenic assay (■). The survival factors were calculated from the survival curves in *A*. *Bars*, SD.

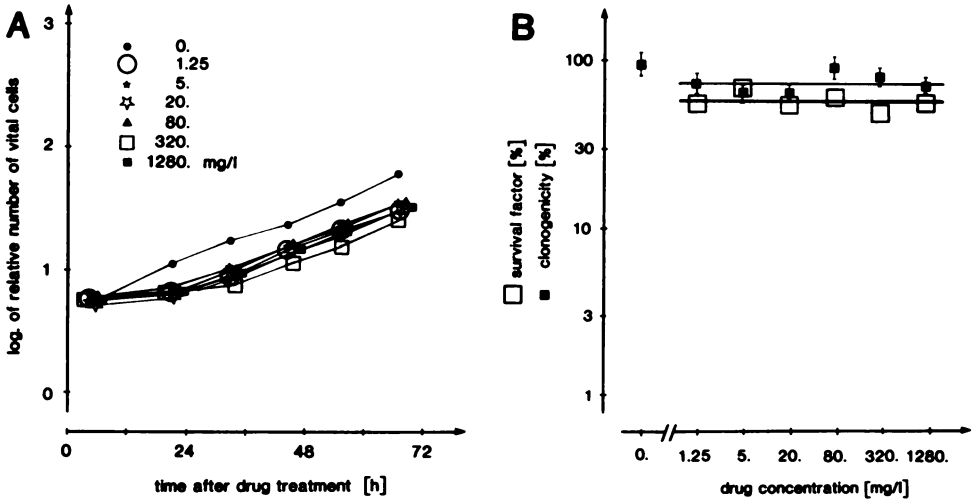

Fig. 3. *A*, survival curves of K562 cells which had been treated with different concentrations of 1-β-D-arabinofuronosylcytosine for 1 h. *B*, comparison of dose-response curves measured by the increase with time of vital cells (□) and by clonogenic assay (■). The survival factors were calculated from the survival curves in *A*.

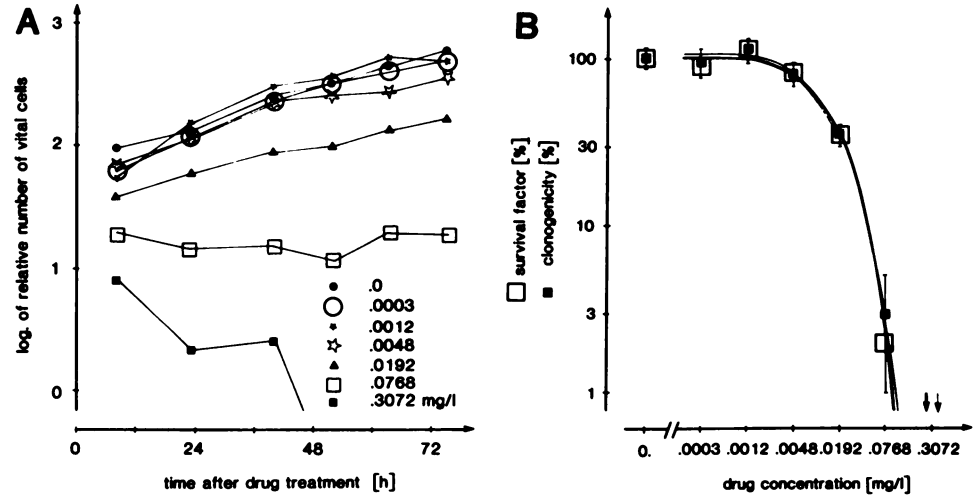

Fig. 4. *A*, survival curves of Reh cells which had been treated with different concentrations of 1-β-D-arabinofuranosylcytosine for 24 h. *B*, comparison of dose-response curves measured by the increase with time of vital cells (□) and by the clonogenic assay (■). The survival factors were calculated from the survival curves in *A*.

cells. Furthermore, due to drug effects, cell cycle rates and membrane integrity may be transiently altered. Therefore, according to our experience, linear extrapolation of the growth curve of vital tumor cells should not be started earlier than 24 h after termination of drug exposure. With long exposure times or very high doses of drugs a larger fraction of cells may already be inhibited or damaged at $t = 0$. The reduced fraction is taken into account by the survival factor by using the quotient of numbers of treated and untreated cells at $t = 0$. On the other hand, delayed drug effects on the rates of proliferation, cell loss, or recovery are included by using the quotient of the slopes of treated and untreated vital cell increase.

With the FD/EB assay an earlier and higher cell kill after drug treatment was observed than with other vitality stains such as EB alone or erythrosin B (data not shown). However, when survival factors were calculated based on these other vital stains,

Table 1 *Comparison of the clonogenic assay with the rate of vital cell increase on the basis of drug concentrations reducing survival by 90% (ID_{90})*

Drug (incubation time)	ID_{90}[a] (mg/liter) HL-60	Reh	K562
Adriamycin (1 h)	0.2	0.1	0.1
	0.2	0.1	0.2
cis-Platinum (1 h)	4.0	1.0	5.0
	4.0	1.0	8.0
1-β-D-Arabinofuranosylcytosine (1 h)	>1000.0	0.5	>1000.0
	>1000.0	0.4	>1000.0
1-β-D-Arabinofuranosylcytosine (24 h)	ND[b]	0.05	0.02
	ND	0.05	0.04
5-Fluorouracil (1 h)	>3000.0	200.0	>3000.0
	>3000.0	200.0	>3000.0
5-Fluorouracil (24 h)	ND	20.0	30.0
	ND	10.0	30.0

[a] The upper concentrations refer to the clonogenic assay, the lower to the flow cytometrically determined rate of vital cell increase. The ID_{90} values were calculated as mean values from up to 4 independent dose response curves.
[b] ND, not determined.

Table 2 *Cell survival assays based on techniques of vital cell counting*

Method and ref.	Observation time or period (days)	*y* intercept[a]	Slope[a]	Comparison with colony test
Vital cell number at a fixed day (9)	4	*i*	*i*	Somewhat lower cell kill
Early slope of growth curve (6)	1–5	–	+	Not proportional
y-intercept after back extrapolation of the growth curve after complete recovery (14)	7–12	+	*i*	Very similar
Growth factor *f*	2–3	+	+	Very similar

[a] Comprises influence of *y* intercept and slope of the linearized growth curve in a semilogarithmic plot, *i* taken into account indirectly or partially.

very similar results were obtained. This novel approach of quantifying cell survival depends on a survival factor which can be easily calculated from the growth curve of a drug-treated cell culture and its untreated control. Previously described vitality assays based on cell counting techniques tended to produce qualitative similarities with, however, quantitative differences from the clonogenic assay. These previous efforts measured either slopes of growth curves when the doubling time was determined (6) or a mixture of slope and intercept with the *y*-axis when the vital cell count was taken at a fixed time point after drug exposure (9–13). Theoretically, an accurate estimate of cell kill may be obtained by extrapolating that part of the growth curve to $t = 0$ which results after complete recovery from drug injury has taken place and which will parallel the log phase of the control culture (14). Since recovery may last longer than 1 week, refeeding of the cells will become necessary and the assay may become as time consuming as the clonogenic assay. For the matter of comparison, the various types of vitality assays have been compiled in Table 2.

The concentrations which inhibit cell growth by 90% calculated from the results of either the clonogenic assay or the assay based on vital cell counting maximally differed by a factor of 2 (Table 1). Such differences are negligible with regard to the purpose of the assays to indicate the response of a tumor cell line within an applicable dose range. With our assay based on vital cell counting no systematic underestimate of the percentage of cell kill was observed like with many other assays that include nonclonogenic cells (5–9). In our experiments the differences between results from 2 independent clonogenic assays or vital cell counting tests using identical conditions mostly were larger than differences by the 2 types of assays performed on the same sample of treated cells. In agreement with others (4, 9) we therefore conclude that in our leukemic cell lines the drug response of clonogenic cells was also represented by the nonclonogenic population.

Besides the ease, a further advantage of measuring the vital cell number at different times after cytostatic drug exposure is the possibility to distingush between immediate and delayed cell death. in Fig. 3*A*, 1-β-D-arabinofuranosylcytosine has killed or arrested about 40% of the K562 cells by a 1-h incubation. After 24 h the vital cells increased in number at the same rate as the control cells. This means that there was no delayed cell kill or proliferative inhibition indicating that recovery had already been terminated. On the other hand, such delayed effects are obvious in Fig. 2*A*. At concentrations exceeding 0.04 mg/liter Adriamycin showed a long-term effect lasting for the entire observation period. If recruitment of dormant cells into the cell cycle would be operative *in vitro* a steeper slope of vital cell increase than seen in the respective control experiment might be expected to occur. This however, was not the case in the cell lines presently studied.

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