Estimating the number of viable animal cells in multi-well cultures based on their lactate dehydrogenase activities

Gale Haslam, Diane Wyatt & Paul A. Kitos*

Department of Biochemistry, The University of Kansas, Lawrence, KS 66045, U.S.A.

(*Author for correspondence, e-mail: kitos@falcon.cc.ukans.edu)

Received 27 October 1998; accepted 29 June 1999

Key words: indirect cell count, lactate dehydrogenase, LDH, tetrazolium reduction, toxicity assay

Abstract

A method is described for estimating the numbers of animal cells in multi-well culture by simultaneously measuring the lactate dehydrogenase activity of the total culture and the medium. The difference between the two reflects the dehydrogenase content of the cells and correlates with cell number. This LDH/INT method was tested using several lines of normal and transformed suspension and adherent cells. The lactate dehydrogenase activities of duplicate cultures were determined colourimetrically using reaction cocktails containing lactate, NAD^+ , diaphorase, and p-iodonitrotetrazolium violet, with and without Triton X-100. The difference in absorbance at 490 nm ($\Delta A_{490} = A_{490, \text{test}} - A_{490, \text{control}}$) was used to calculate the lactate dehydrogenase activity of the total culture (+ Triton) and the medium (– Triton). The cellular lactate dehydrogenase activity (difference between **total** and **medium** dehydrogenase activities) was proportional to viable cell number. The effects on cell growth of four metabolic inhibitors, sodium azide, actinomycin D, cycloheximide, and taxol, were determined using the LDH/INT assay and direct cell counting. The inhibitor concentrations that caused decreases in the LDH activity and cell number by 50% were similar. The LDH/INT assay is quick and sensitive, works equally well for adherent and suspension cells, and provides information about LDH activities of both the medium and cells. It is particularly useful for screening potential cell-growth inhibitors.

Abbreviations: Dase, diaphorase; D/F, 50% DME, 50% Hams F12; FBS, foetal bovine serum; IC_{50} , concentration of inhibitor that causes a 50% decrease in cell count or INT reduction; INT, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride, also called p-iodonitrotetrazolium violet; LDH, lactate dehydrogenase; Tris, tris hydroxymethylaminomethane; TX, Triton X-100.

Introduction

There are many reasons for screening the numbers of viable eukaryotic cells in culture and many methods of doing it, each with advantages and disadvantages (Doyle and Griffiths, 1998; Morris et al., 1997; Wilson, 1986; Patterson, 1979). Some methods are suitable for large numbers of cultures and others not, some for adherent but not for suspension cells, some for clumps of cells, and some are more sensitive than others. Counting by haemocytometer, using a vital stain such as trypan blue, provides information about both cell number and viability but for large numbers of

samples is labourious and fraught with personal error (Doyle and Griffiths, 1998; Falkenhain et al., 1998; Morris et al., 1997; Cook and Mitchell, 1989; Freshney, 1987; Patterson, 1979; Asher, 1973; Phillips, 1973). Some models of Coulter[®] and Casy[®] 1 electronic particle counters can be used to estimate both cell number and viability but are inconvenient for large numbers of samples and inaccurate with clumped cells (Falkenhain et al., 1998; Winkelmeyer et al., 1993; Harris, 1973).

Indirect estimation of cell numbers involves measuring a cell-associated characteristic that correlates with the cell population. For example, the amount

of cellular protein in a culture is generally a function of the number of cells, and several methods are predicated on this relationship (Skehan et al., 1990; Babich and Borenfreund, 1987; Hakala and Rustrum, 1979; Oyama and Eagle, 1956). The sulforhodamine B (SRB) method of Skehan et al. (1990) stains proteins in the cell layer in multi-well plates and quantifies the stain. It works well for attached cells, is sensitive, and gives a linear dye-binding response over a broad population range but involves a lot of operator handling and is unreliable for cells in suspension culture. The neutral red (NR) assay measures NR trapped by the lysosomes of viable cells but likewise is not useful for large numbers of suspension culture samples (Babich and Borenfreund, 1990, 1992). The binding of a fluorescent dye to the DNA of permeabilized cells followed by fluorescence measurement of the bound fluorophore has been used to determine the number of cells in culture, but this procedure is not readily applicable to large numbers of suspension cell cultures (Falkenhain et al., 1998; Loontjens et al., 1991; CyQuant® method, Molecular Probes, Eugene, OR). The MTT assay (Morris et al., 1997; Al-Rubeai and Spier, 1989; Carmichael et al., 1987; Denizot and Lang, 1986; Mossman, 1983) is useful for both adherent and suspension cells and can be completed within an hour, especially if the number of cells per well is large (Doyle and Griffiths, 1998). Methods that involve the uptake of radioactive substances by the cells are handicapped by problems of isotope handling and disposal, radioactivity monitoring, and interpretation of results (Doyle and Griffiths, 1998; Racher et al., 1990; Wilson, 1986; Friedman and Glaubiger, 1982; Freshney and Morgan, 1978). Even nutrient consumption from, and metabolite accumulation in the medium have been used to determine cell numbers but the basic assumption of these methods is metabolic constancy under possibly inconstant environmental circumstances, not necessarily a safe premise (Morris et al., 1997; Pelletier et al., 1994; Familletti and Wardwell Swanson, 1988).

Here we describe a simple method for determining cell numbers in multi-well plates based on endogenous LDH activities of the cells. Several variations of this approach, in which the activities of constitutive enzymes such as LDH, acid phosphatase, and cytoplasmic esterases are measured, are currently in use (Morris et al., 1997; Ihrig et al., 1994). Some of them can be used to estimate the extent of induced or natural cell damage (Merten et al., 1992; Marc et al., 1991; Decker and Lohmann-Matthes, 1988; Korzeniewski

and Callewaert, 1983; Szekeres et al., 1981). Endogenous LDH and phosphatases in damaged or dead cells gain access to the medium wherein their catalytic activities can be easily monitored. A modification of this approach, the LDH/INT assay, is used here to determine simultaneously the LDH activities of both the total culture and the medium. Two reaction systems are used, one with Triton X-100 (TX) and the other without. Both cocktails contain lactate, NAD^+ , a redox dye (p-iodonitrotetrazolium violet [INT]), and diaphorase (Dase). LDH in the liquid phase of the culture catalyzes the energetically unfavourable conversion of lactate and NAD^+ to pyruvate and NADH. Dase catalyzes the favourable conversion of NADH and INT to NAD^+ and INT-formazan. In this test system, LDH activity is rate limiting. Without TX, the LDH activity is due to LDH of the medium plus LDH released from the cells during the culture period. In the presence of TX the LDH activity is the sum of that in the medium plus that released from the cells by the action of the detergent. The difference in activity between the '+TX' and '-TX' assays is equal to the intracellular LDH activity.

Materials and methods

Cells

The following two cell lines were used in most of the studies reported here: **CHO-K1** (Chinese Hamster Ovary cells, ATCC CCL-61, adherent); and **L1210** (mouse lymphocytic leukaemia cells, ATCC CCL-219, suspension). Five other cell lines, HF-B1 (human foreskin fibroblast cells, low passage number, adherent, normal human cells isolated in this laboratory), B16 F1 (mouse melanoma cells, ATCC CRL-6323, adherent), CCRF-CEM cells (human acute lymphoblastic leukaemia cells, ATCC CCL-119, suspension), MDCK (canine kidney cells, ATCC CCL-34.1, adherent), and BHK-21 (Syrian Hamster Kidney cells, ATCC CCL-10, adherent) were also used. The cells were cultured in D/F medium (1:1 mixture of DME and Ham's F12) containing 2 g l^{-1} of NaHCO_3 and supplemented with 5% foetal bovine serum (FBS) and antibiotics (100 units penicillin G and $100 \mu\text{g}$ streptomycin per ml of medium). Inocula for the experiments were taken from either sub-confluent, adherent cell cultures or low-density suspension cultures that had been fed with fresh, 5% FBS-supplemented medium one day prior to seeding. Before inoculation, the

adherent cells were suspended using trypsin/EDTA (0.25% trypsin 1:250, 0.1% EDTA in PBS w/o Ca^{++} or Mg^{++} , pH 7.2). The cultures were incubated at 37.5 °C in humidified 5% CO_2 /95% air.

Reagents

The following reagents were purchased from the Sigma Chemical Co., St. Louis, MO: Triton X-100, laboratory grade; diaphorase (Dase, EC 1.8.1.4) from *Clostridium kluyveri*; lactate dehydrogenase (EC 1.1.1.27), type II, purified from rabbit muscle; p-iodonitrotetrazolium violet (INT); L(+)-lactic acid, lithium salt; β -NAD⁺, 98%, from yeast; oxamic acid, sodium salt; dithiothreitol; dithioerythritol, glutathione; β -mercaptoethanol; 6-thioguanine; L-cysteine; cycloheximide; actinomycin D; and sodium azide. Taxol was a generous gift from Dr. Richard Himes (Department of Molecular Biosciences, Kansas University).

Assay solutions (see Table 1)

[1] 2 M Tris, pH 7.5, made up in water containing 0.05% sodium azide, stored at room temperature. [2] 0.2 M Li lactate in water, stored at -20 °C. [3] 60 mM NAD⁺ in water, stored at -20 °C. [4] 15 mM INT; 10 ml of this solution was prepared by suspending the powder in 1 ml of water and adding 1 ml of acetone with stirring until the INT was dissolved. Then the solution was gradually diluted to 10 ml with water so that it was 10% (v/v) acetone. The solution was filtered through a 0.45 μm pore-size nylon membrane and stored shielded from light at room temperature. [5] 20% (w/v) TX in water, stored at room temperature. [6] 100 unit ml^{-1} Dase in 75 mM Tris, pH 7.5, stored at -20 °C in 50 μl aliquots. Just prior to beginning the assay, a portion of this solution was diluted 1:100 with 75 mM Tris, pH 7.5. [7] 150 mM sodium oxamate, made up in phosphate buffered saline w/o calcium or magnesium (Biowhittaker Inc., Walkersville, MD), stored at 4 °C.

Assay procedure

The assays were carried out in 96-well, flat-bottom, polystyrene, tissue culture plates (Corning Glass Works, Corning, NY; surface area of a well, 0.32 cm^2). Reaction cocktails A and B, enough for 48 wells, were made up just prior to the assay from stock solutions according to the information in Table 1.

Culture setup

To each well of columns 2 to 12 of a 96-well plate was added 150 μl of fresh culture medium containing the desired number of cells and amount of test substance. The wells of column 1 received 150 μl of medium without cells or test substance. The cultures were incubated at 37.5 °C in a humidified CO_2 -incubator with 5% CO_2 /95% air for the desired period of time, 3 to 7 days depending on the cell type and the cells' doubling time. For example, in D/F + 5% FBS, CHO-K1 cells had a doubling time (t_d) of approximately 14 hr. Beginning with 500 cells per well there would be approximately 18 000 cells at 72 hr of culture, a number within the useful range of the LDH/INT assay. In contrast, CCRF-CEM cells (t_d = 26 hr) would take 134 hr to reach 18 000 cells. At 72 hr the CCRF-CEM cultures would be rather sparse (3400 cells) and at 134 hr the CHO-K1 cells would be too overgrown (380 000 cells) for the assay.

LDH/INT assay

After the culture period, 50 μl of reaction cocktail A was added to rows a to d, columns 1 to 12, and 50 μl of cocktail B to rows e to h, columns 1 to 12. The plates were carefully vortexed for 10 s at low speed on a vortex mixer with plate adapter (Fisher Scientific, St. Louis, MO). *It was important to avoid spillage and foam formation at this or any other step; foam interfered with colour measurement on the plate reader. Foam bubbles can be disrupted by touching them with the tip of a sharpened lead pencil.* The plates were then incubated at room temperature for the desired time, usually 1 hr or less. If colour development was slow, the incubation period was increased, and vice-versa. To terminate the LDH-catalyzed reaction, 20 μl of the oxamate solution was added by multi-channel pipetter to each well and the plate vortexed at low speed for 10 s. If colour development was monitored as a function of time, the oxamate solution was not added; rather, the same plate was read at consecutive time points. The absorbance at 490 nm was determined on a plate reader (model EL 311 or EL_x 800, Bio-Tek Instruments, Inc.) at the specified times, or as soon as possible after oxamate addition. The net absorbance (ΔA_{490} , i.e., absorbance in the test well (columns 2 to 12) minus that in the corresponding control well (column 1)) was calculated and converted to nmol of INT reduced and/or mU of LDH activity. To calculate the nmol of INT reduced in the assay, the conversion multiplicand was 90 for the +TX wells and

Table 1. Stock solutions and reagent concentrations

Stock solutions	Volume used		Solute concentration	
	A	B	In A and/or B	In reaction system
Water	0.84 ml	0.90 ml	–	–
2 M Tris, pH 7.5	1 ml	1 ml	333 mM	83 mM
0.2 M Li lactate	1 ml	1 ml	33 mM	8.3 mM
60 mM NAD ⁺	0.1 ml	0.1 ml	1.0 mM	0.25 mM
15 mM INT	2 ml	2 ml	5.0 mM	1.25 mM
20% Triton X-100	62 μ l	–	0.208%	0.05%
	–	0	0%	0%
1 U ml ⁻¹ Dase	1 ml	1 ml	167 mU ml ⁻¹	42 mU ml ⁻¹
Total Volume	6.0 ml	6.0 ml	–	–

Table 2. INT reduction; dependence on LDH/INT assay components

Dase	nmol INT reduced hr ⁻¹ ^a	Lactate	nmol INT reduced hr ⁻¹ ^a	NAD	nmol INT reduced hr ⁻¹ ^b
mU well ⁻¹	($\pm$ s.d.) n = 3	mM	($\pm$ s.d.) n = 8	mM	($\pm$ s.d.) n = 3
0	0.19 (0.03)	0.26	0.60 (0.15)	0	0.00 (0.01)
6.3*	6.33 (0.14)	1.04	1.04 (0.15)	0.125	1.74 (0.20)
12.6	7.64 (0.44)	4.16	2.46 (0.17)	0.25*	3.04 (0.32)
25.2	6.90 (0.61)	8.32*	4.12 (0.61)	0.50	4.10 (0.30)
		16.65	6.41 (0.63)	1.00	4.86 (0.38)
		33.3	9.62 (0.61)	2.00	5.01 (0.42)

* Standard concentration used in the assay.

^a Per 1000 L1210 cells.^b Per mU rabbit LDH.

102 for the –TX wells. These factors were determined from the molar absorbance of formazan at 490 nm under the assay conditions. In this assay system, 1 mU of LDH corresponds to 2.7 nmol of INT reduced hr⁻¹. Therefore, to calculate mU of LDH in a +TX culture, the $\Delta\alpha_{490}$ value was multiplied by ‘2000/assay time in minutes’. In the –TX culture the factor was ‘2267/assay time in minutes’.

Direct estimations of cell number

To estimate cell number by electronic particle counting, the cells in the wells of a 96-well plate were suspended in Isoton[®] (Coulter Instrument Co., Hialeah, FL). If the cells were attached to the substratum, the medium was removed by aspiration, 0.2 ml of trypsin/EDTA solution was added to each well, and the plate was incubated at 37 °C until the cells detached. The cells in each column of 8 wells were pooled in Isoton using a multi-channel pipetter and counted on either a model Z_{B1} Coulter Counter[®] (Coulter Med-

ical Electronics, Hialeah, FL) or Casy[®] 1 model TTC Cell Counter + Analyzer System (Schärfe System GmbH, Reutlingen, Germany). From the electronic count data the average number of cells per well was calculated (the standard deviation could not be determined because the contents of the 8 wells had been pooled for the count). With suspension culture cells, the entire well contents were added directly to Isoton and counted on the electronic particle counter.

Results

Pyruvate and NADH are formed from lactate and NAD⁺. This reaction, catalyzed by LDH, can be coupled to the diaphorase-catalyzed re-oxidation of NADH by INT to regenerate NAD⁺ and facilitate further conversion of lactate to pyruvate. Reaction coupling is central to these LDH activity determinations.

Table 3. Effect of Triton X-100 on trypan blue staining of 72 hr L1210 cells

	Triton X-100 (%, w/v)	Trypan blue-stained cells % of total ($\pm$ s.d.), n = 2
1	0	6.8 (2.5)
2	0.0065	4.1 (3.4)
3	0.013	74.5 (26.7)
4	0.026	98.8 (0.4)
5	0.052	100 (0)
6	0.104	100 (0)

Conc. of TX used in LDH/INT assay = 0.05%.

Concentrations of reagents in the LDH/INT reaction cocktail

Concentrations of the principal reaction cocktail ingredients were varied independently to establish the levels to be used in the assay. Without lactate, NAD^+ , or Dase there was no measurable INT reduction. The Dase concentration selected for use was optimal for INT reduction (Table 2). The selected concentration of NAD^+ was about 60% of that needed for maximal dye reduction. The concentration of lactate was arrived at operationally, avoiding the maximal rate of INT reduction. Because of the limited solubility of the formazan product, the lower lactate concentration was preferred. The most suitable concentration of TX was determined from its effects on both INT reduction and cell permeability; cell permeability was monitored by trypan blue staining. The lowest concentration of TX that permeabilized all the cells and caused the release of LDH to the medium was approximately 0.05% (w/v) (Table 3); concentrations up to 0.83% permitted maximal LDH activity. Tween 40, another non-ionic detergent, was found to be a less effective membrane permeant. In the reaction mixture the concentration of acetone needed to solubilize the INT (0.83%) was not sufficient to perforate the plasma membrane during a 1-hr assay at room temperature. This was established by incubating 24-hr CHO-K1 cell cultures with various concentrations of acetone, from 0% to 6.4%, for 1-hr and then determining the smallest acetone concentration that caused an increase in the proportion of trypan blue-stainable cells. Even at 1.6% acetone, 98% of the cells were not stained by trypan blue.

Dependence of INT reduction on LDH

Purified rabbit LDH was used to determine the dependence of INT reduction on LDH concentration.

Table 4. LDH activity of animal sera

Serum type	Special features	LDH activity mU ml^{-1} serum ($\pm$ s.d.), n = 8	Vendor code ^a
FBS		79 (23)	1
FBS		101 (11)	3
FBS		110 (20)	5
FBS	dialyzed	26 (18)	11
FBS	dialyzed	23 (23)	4
Calf	donor	163 (17)	1
Calf	newborn	92 (25)	2
Calf		196 (17)	6
Calf	heat-inactiv. newborn	107 (8)	3
Horse		64 (20)	7
Horse		87 (15)	3
Horse	donor	75 (14)	8
Horse	plasma-derived	76 (14)	9
Rabbit		47 (11)	1
Chicken		65 (10)	10

^a Vendor not identified by name.

The LDH activity was determined using D/F medium containing 5% of the indicated serum. The reported activity values are corrected to 100% serum. The analyses were carried out using the LDH/INT assay.

INT reduction was found to be essentially proportional to both the LDH concentration (Figure 1a) and length of the assay (Figure 1b).

LDH in animal sera

There are two potential sources of LDH in culture media: serum and membrane-damaged or dead cells (Marc et al., 1991). The LDH activities of sera from different animal species, ages, modes of preparation and commercial sources were measured using the LDH/INT assay and found to be variable, without obvious correlation with the serum source or its processing (Table 4). Moreover, the LDH activity of FBS in D/F medium was proportional to the serum concentration (Figures 2a and b). The LDH of human and canine sera is quite stable, showing no change in activity for up to 4 days at room temperature and for up to a week at 0 to 5 °C (Wroblewski and LaDue, 1955). The stability of LDH released from animal cells in culture may vary from one cell type to another (Goergen et al., 1993; Marc et al., 1991). In hybridoma cultures, Marc et al. (1991) observed a loss of only 1% per day of the released LDH activity.

Figure 1. INT reduction in the LDH/INT assay. (a) As a function of LDH activity: Purified rabbit muscle LDH at the indicated activities per well in D/F medium containing 5% FBS was incubated at room temperature for 31 min and then the A_{490} values were determined. Each ΔA_{490} value was calculated and plotted as a function of the LDH activity in the well. The difference between the two lines reflects the difference in molar absorbance of the formazan in the presence and absence of 0.05% Triton $\times 100$. Vertical bars at the data points: $\pm$ s.d., $n = 4$, single plate. At several of the data points the range of the s.d. falls within the dimensions of the point. (b) Kinetics: purified rabbit muscle LDH, at the indicated activities per well in D/F + 5% FBS, was assayed as a function of time by the LDH/INT assay. The assays were conducted in the presence and absence of 0.05% Triton $\times 100$. The ΔA_{490} values were converted to and reported as nmol of INT reduced per well. Vertical bars at the data points: $\pm$ s.d., $n = 4$, single plate. At several of the data points the range of the s.d. falls within the dimensions of the point.

LDH activities of cell cultures

The contribution of cells in culture to the LDH activity of the culture was examined using several different cell lines (Table 5). Duplicate 96-well plates were set up with 150 μ l of medium and the cells serially diluted from a high of 1500 per well to a low of 0. The plates were incubated long enough to permit at least 3 cell doublings. The cells in one of the plates were counted using a Coulter or Casy 1 particle counter; INT reduction was assayed in the duplicate plate. Because the control and test wells contained the same amount of FBS the contribution of LDH in the serum to the ΔA_{490} values was automatically eliminated. Without TX in the assay mixture, the ΔA_{490} measured the LDH released from the cells while they were in culture, commonly viewed as an index of cell damage or death (Doyle and Griffiths, 1998; Goergen et al., 1993; Wagner et al., 1992; Merten et al., 1992; Marc et al., 1991; Racher et al., 1990; Bour et al., 1989; Cook

and Mitchell, 1989). TX lysed the cells, releasing their LDH into the medium; the ΔA_{490} monitored the total LDH of the culture, cells plus medium, but minus that of the serum.

L1210 mouse lymphocytic leukaemia cells

L1210 mouse lymphocytic leukaemia cells are often used in screens for potential anti-cancer agents. They were cultured for 72 hr and then monitored by the LDH/INT assay. Figure 3 shows three sets of LDH activities: that for *medium + cells* ('+TX' assay); for *medium alone* ('-TX' assay); and for *cells alone* (difference between '+TX' and '-TX'). The net LDH activity in the medium was only about 8% of that from the cells. In the presence of TX, the LDH activity was found to be proportional to the cell number up to about 40 000 per well; above 40 000 per well the response was not proportional. Therefore, starting with 1000 or fewer L1210 cells per well, the cell population re-

Figure 2. LDH in FBS. (a) Kinetics of INT reduction: fresh D/F media containing the indicated concentrations of FBS were analyzed by the LDH/INT assay as a function of time. The ΔA_{490} values were converted to and reported as nmol of INT reduced per well under the specified conditions. Vertical bars: $\pm$ s.d., $n = 4$, single plate. The range of the s.d. in most cases is smaller than the dimensions of the data points. (b) LDH activity, function of serum content of the medium: D/F containing FBS at the indicated concentrations was analyzed for LDH activity in a 30-min LDH/INT assay. The LDH activities were calculated and plotted as a function of the FBS content of the medium. The vertical bars at the data points are $\pm$ s.d., $n = 4$, single plate.

maintained within the linear range of the LDH/INT assay for at least 3 days. The calculations were done on a suitably programmed template in Microsoft Excel[®] and the graphs prepared using Jandel Sigma Plot[®]. By light microscopy the cells in the -TX wells at the end of the assay period seemed to be intact and healthy (round, plump, and clearly 3-dimensional) while those in the +TX wells were flat (looked only 2-dimensional) and granular, probably dead.

CHO-K1 cells

CHO-K1 cells are rapidly growing, adherent cells, modified forms of which are often used for transfection and production of genetically engineered proteins. They were seeded at 1000 or fewer cells per well and analyzed as described above. The linear portion of the LDH activity response extended from 0 to a maximum of 20 000 cells per well (Figure 4a). An inoculum of up to 500 CHO-K1 cells per well gave an INT-reduction response at day-3 that was proportional to cell number. Time-dependent increases in cell number and LDH activity are shown in Figure 4b.

Inhibition of cell proliferation

The LDH/INT assay was used to test substances for their anti-proliferative activities. The substances were diluted serially in D/F + 5% FBS and added to the cell suspension in two 96-well plates to give a constant inoculum in a final volume of 150 μ l per well. Four different growth inhibitors were used: sodium azide, which inhibits respiration; actinomycin D, which inhibits nucleic acid synthesis; cycloheximide, which inhibits protein synthesis; and taxol, which inhibits microtubule dynamics.

The effects of cycloheximide on L1210 cell proliferation, determined by direct cell count and the LDH/INT assay, are shown in Figures 5a and b. The IC_{50} values obtained by direct cell count and by the LDH/INT assay in the presence of TX were 6.2 and 13.0 nM, respectively. By INT reduction, the IC_{50} value for 'cells alone' (10.0 nM) was close to that for 'medium + cells'. More precise inhibition values are obtainable by narrowing the inhibitor concentration range.

L1210 cells were also used in inhibition studies with actinomycin D and sodium azide; CHO-K1 cells

Table 5. Cell characteristics

1	2	3	4	5	6	7	8
Cell line	Doubling time [t_d] ^a , hr ($\pm$ s.d.), n	Cell vol. ^b $\mu\text{l} \times 10^9$ ($\pm$ s.d.), n	Cell diam. ^c μm	LDH activity mU/cell ($\times 10^3$)			LDH activity mU/ μl cell vol. ^d ($\times 10^6$)
				medium + cell (+TX)	medium (-TX)	cells (difference)	
L1210 ^e	14.3 (2.6), 11	751 (270), 4	11.28	1.37	0.09	1.29	1.72
CHO K1	14.1 (1.2), 12	1190, 1	13.14	3.19	0.15	3.04	2.56
CCRF...	26.3 (3.9), 4	1615 (160), 4	14.56	4.78	0.22	4.56	2.82
BHK	17.1 (3.6), 6	1913 (448), 4	15.40	4.74	0.24	4.50	2.35
MDCK	20.6 (5.6), 4	2086 (658), 4	15.85	4.89	0.22	4.67	2.24
B16	19.9 (2.9), 6	2135 (262), 5	15.98	3.41	0.21	3.20	1.50
HF	49.1 (10.9), 6	2430 (470), 2	16.68	7.11	0.59	6.52	2.68

^a Calculated as follows: t_d , hr = ((incub. time, hr) ($\log_{10} 2$))/ $\log_{10} (N_t/N_0)$, where N_0 = cell number at time 0 and N_t = cell number at time t.

^b Determined using the Casy 1 TTC electronic particle counter and sizer.

^c Calculated from cell volume, column 3.

^d Column 7 divided by column 3.

^e All cells used in this study were from 72-hr cultures except HF which were 168 hr.

For cell count and size determinations, the cells from 8 wells were pooled. The LDH values +TX and -TX were from 4 separate determinations by the LDH/INT method.

Figure 3. LDH activities of L1210 cell cultures. L1210 cells were seeded in D/F + 5% FBS at 0 to 1500 cells per well in duplicate 96-well plates and incubated for 72 hr. The number of cells per well was then determined by Coulter count using one of the plates and the LDH activity per well measured by LDH/INT assay using the other plate. The LDH assays were done $\pm 0.05\%$ TX and the mean ΔA_{490} values converted to mU of LDH and plotted as a function of the final number of cells per well. The +TX assays show the LDH activities of 'medium + cells' minus any contribution of the FBS; the -TX values show the LDH activities of 'medium' minus that of the FBS; the difference between the two is the activity of the cells alone. Vertical bars at the data points: $\pm$ the s.d., $n = 4$.

Figure 4. LDH activities of CHO-K1 cell cultures. (a) Relative to cell number: CHO-K1 cell cultures were inoculated with 0 to 800 cells per well in duplicate 96-well plates. One of them was assayed 72 hr later by the LDH/INT method as described in the legend for Figure 3 and the other was used to obtain cell counts using the Coulter Counter. Length of the LDH/INT assay: 60 min. Vertical bars at the data points: $\pm$ s.d., $n = 4$. For some of the data points the range of the s.d. is within the dimensions of the data point. (b) Relative to the period of cell growth: Cell cultures were set up at 500 cells per well in replicate plates and incubated at 37.5 °C. Representative plates were removed for LDH/INT analyses and cell counts at 24, 48 and 72 hr. The left ordinate gives the LDH activity values determined by the LDH/INT assay and the right ordinate gives the cell numbers, determined by Coulter counting. Vertical bars at the data points: $\pm$ s.d., $n = 4$. For several of the time points the range of the s.d. is within the dimensions of the data points.

were used in studies of taxol and sodium azide. The IC_{50} values are reported in Table 6.

Discussion

The LDH activity of the serum in a culture medium is variable and can affect the INT-reduction results (Table 4). The greater the serum content of the medium, the greater the background LDH activity (Figures 2a and b).

INT, used as the electron acceptor in the LDH assay, was colourless and its formazan, coloured. Enough INT was used to minimize the possibility of it

becoming rate limiting as the reaction progressed. Its reduction was irreversible and, in excess, INT served to drive the cycle. It did not dissolve readily in water but did go into solution adequately as described in section Materials and Methods. INT-formazan was less water-soluble than INT itself. At high concentration, the formazan crystallized in the culture vessels, preventing its direct quantification. Hence, levels of LDH that produced large amounts of formazan were deliberately avoided. Pyruvate accumulated as the reaction progressed. It binds LDH tightly (Holbrook et al., 1975), decreasing the rate of lactate conversion to pyruvate and, hence, INT reduction. The inhibitory effect of pyruvate also meant that if it were a

Figure 5. Inhibition of the proliferation of L1210 cells by cycloheximide. (a) LDH/INT assay: L1210 cells were seeded in duplicate 96-well plates at 1000 cells per well in D/F + 5% FBS containing 0 to 10 μM cycloheximide and incubated for 72 hr. Then, the LDH activity per well was determined with one of the plates in a 35-min LDH/INT assay. The IC_{50} value was the concentration of inhibitor causing 50% reduction in LDH activity per well. Three sets of values were plotted as functions of cycloheximide concentration: 1) LDH activity of 'medium + cells' (+TX); 2) LDH activity of 'medium alone' (-TX); and 3) LDH activity of 'cells alone': (difference between 1 and 2). Vertical bars at each point: $\pm$ s.d., $n = 4$. Arrows indicate the IC_{50} values determined from the 'medium + cells' and 'cells alone' curves. (b) Cell counts: using the second plate from part (a), above, the number of cells at each concentration of cycloheximide was determined by Coulter counting and plotted as a function of the cycloheximide concentration. There are no s.d. values. The arrow indicates the IC_{50} value by this method.

component of the nutrient medium, the sensitivity of the assay could be decreased, depending upon how completely it had been metabolized during the culture period. The spectrum of INT-formazan was altered slightly by 0.05% TX (i.e., the A_{max} in the presence of TX was 490 nm; without TX it was 505 nm). The difference in molar absorbance at 490 nm was compensated for in the factors used to convert the ΔA_{490} absorbance values to nmoles of INT reduced (i.e., 90 in the presence of TX and 102 in its absence). Phenol red, a pH-sensitive dye often used in culture media, also absorbed at 490 nm. At the pH of the assay, 7.5, the contribution of phenol red to the A_{490} was constant and automatically eliminated by the calculations.

INT was reduced non-enzymically by strong reducing agents. For example, ascorbate, β -mercaptoethanol, dithiothreitol and dithioerythritol in the reaction mixture strongly reduced INT. If any of them were included in the nutrient medium and persisted in their reduced form at the time of the LDH/INT assay, they would produce misleading results. Consequently, the LDH/INT assay cannot be used for such cultures. The same restriction would apply to any similar assay system using tetrazolium or

certain other redox dyes (E.G., 2,6-dichlorophenol indophenol) as the ultimate electron acceptor. Cysteine, glutathione and 6-thioguanine, much weaker reducing agents, do not interfere with the assay (Table 7).

The LDH/INT assay was applied to several different animal cell lines in culture. As long as the cell population was not too large, the LDH activity was proportional to cell number (Figures 3 and 4a). Every cell line that was tested within its linear range showed a direct correlation between the amount of dye reduced and the number of cells present in the culture. However, the maximal number of cells giving a linear response differed from one line to the next. For example, in a 1-hr assay, up to 40 000 L1210 cells per well (Figure 3) and 20 000 CHO-K1 cells per well (Figure 4a) gave linear INT-reduction responses. At larger cell numbers, the curves deviated from linearity. In these studies, the upper limit of the linear INT-reduction response was close to 160 nmol of INT reduced per well (equivalent to the INT reduced per hour in the presence of 60 mU of purified rabbit LDH). Differences in the amounts of LDH per cell could be partly a function of cell size. This possibility is indicated in Table 5 in which the mean cell volumes of the 7

Table 6. IC₅₀ values by direct cell count and LDH/INT assay

Inhibitor	Cell line	IC ₅₀ , M	
		By electronic particle count	By LDH/INT assay, cells alone
Na azide	CHO-K1	0.15×10^{-3}	0.43×10^{-3}
Na azide	L1210	0.15×10^{-3}	0.22×10^{-3}
Cycloheximide	L1210	6.20×10^{-9}	13.00×10^{-9}
Actinomycin D	L1210	0.27×10^{-9}	0.40×10^{-9}
Taxol	CHO-K1	37.00×10^{-9}	100.00×10^{-9}

Table 7. INT reduction by ascorbate and some commonly used thiols

nmol of INT reduced per hour by the indicated reducing agents ^a , ($\pm$ s.d.), n = 4						
DTT, 0.1 mM	β -ME, 0.1 mM	DTE, 0.1 mM	Ascorbate, 0.1 mM	Cysteine, 100 mM	GSH, 100 mM	6-TG, 10 μ m
100 (12)	11.2 (0.8)	71 (2)	97 (3)	5.0 (0.6)	10.1 (0.7)	16.2 (6.9)

^a: DTT: dithiothreitol; β -ME: β -mercaptoethanol; DTE: dithioerythritol; GSH: glutathione (reduced form); 6-TG: 6-thioguanine. The assays were carried out in a 96 well plate in 0.20 ml reaction volume, 0.05 ml of which was the LDH/INT assay mix. The reaction mixture contained TX but not FBS.

cell lines used here are recorded along with their LDH activities. The correlation between LDH activity and cell size is not perfect but there is a trend from low activity with small cells (L1210) to high activity with large cells (HF). Within a factor of 2 the LDH activity per unit cell volume is the same for all 7 lines tested (Table 5, column 8). Changes in the culture state during incubation, such as glucose, glutamine, ammonia and lactate concentrations, oxygen partial pressure and pH, may affect the LDH content of cells which, in turn, would make it difficult to translate INT reduction to cell number (Kurokawa et al., 1994).

The validity of the results in the LDH/INT assay depends in part on the relative magnitudes of the A₄₉₀ values for the test sample and the cell-free control (due to serum). In calculating the ΔA_{490} values, the latter A₄₉₀ (control) is subtracted from the former. For meaningful results, the latter value should not be a large portion of the former. The validity of a result depends on several factors, including the amount of serum used in the medium and the number of cells in the culture. The greater the serum content of the medium, the greater its contribution to the A₄₉₀ of the test sample. The greater the cell number, the greater the difference between its A₄₉₀ and that of the cell-free control. The study reported in Figure 5a can be used to illustrate how the number of cells in a test sample affects the validity of the test result. This study, like most

of the others reported here, used medium containing 5% FBS. The A₄₉₀ values in the '+TX' samples were: 1) 0.275 ± 0.002 (cell-free control); 2) 0.711 ± 0.030 (with cells but no inhibitor; at the time of the test there were 31,280 cells in this culture); and 3) 0.344 ± 0.012 (with cells and 100 nM cycloheximide; this concentration of inhibitor was selected because it caused strong, but not complete inhibition; at the time of the test there were 3300 cells in the culture). According to the Student t-test, each of the latter two results (2 and 3) differ from that of the control with significance greater than 0.001. The A₄₉₀ values in the '-TX' samples were: 4) 0.241 ± 0.002 (cell-free control); 5) 0.310 ± 0.004 (with cells but no inhibitor); and 6) 0.243 ± 0.005 (with cells and 100 nM cycloheximide). Number 5 differs from 4 with significance greater than 0.001 but 6 does not differ significantly from 4. In this case, 4 is a large portion of 6.

INT reduction was used to determine the effects of 4 inhibitors on cell growth: azide, cycloheximide, actinomycin D, and taxol. Generally the IC₅₀ values obtained by cell counts were slightly smaller than those by the LDH/INT assay (Table 6). The IC₅₀ for taxol, an anti-mitotic agent, was much smaller by direct cell count than by the LDH/INT assay. Although taxol inhibits cell division, the cells continue to increase in size. The greater amount of LDH in the taxol-inhibited cells may be a function of the greater

amount of cytoplasm (Table 5), consistent with the larger IC₅₀ by INT-reduction than by cell count. This type of problem would exist for most indirect methods of estimating cell numbers.

Acknowledgement

The authors gratefully acknowledge the support of Wyatt Cytoconsulting in these studies.

References

- Asher M (1973) Hemocytometer counting, In: Kruse PF and Patterson MK (eds) *Tissue Culture. Methods and Applications*. Academic Press, New York, pp. 395–397.
- Babich H and Borenfreund E (1987) Structure-activity relationship (SAR) models established *in vitro* with the neutral red cytotoxicity assay. *Toxicology in Vitro* 1: 3–9.
- Babich H and Borenfreund E (1990) Application of neutral red assay to *in vitro* toxicology. *Alternatives to Laboratory Animals* 18: 129–144.
- Babich H and Borenfreund E (1992) 'Neutral red assay for toxicology *in vitro*', In: Watson RR (Ed) *In Vitro Methods in Toxicology* (Chapt. 17) CRC Press, Boca Raton, FL, pp. 237–251.
- Bour JM, Maugras M, Capiamont J, Dousset B, Straczek J, Gelot MA and Nabet P (1989) Marqueurs biochimiques de la prolifération et de la mort des hybridomes en culture. *BioSciences* 7: 35–40.
- Carmichael J, DeGraff WG, Gazdar AF, Minna JD and Mitcoll JB (1987) Evaluation of a tetrazolium-based semiautomatic colorimetric test: assessment of chemosensitivity testing. *Cancer Res* 47: 936–942.
- Cook JR and Mitchell JB (1989) Viability measurements in mammalian cell systems. *Anal Biochem* 179: 1–7.
- Decker T and Lohmann-Matthes M-L (1988) A quick and simple method for quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. *J Immunol Methods* 15: 61–69.
- Denizot F and Lang R (1986) Modifications of the tetrazolium dye procedure giving improved sensitivity and reliability. *J Immunol Methods* 89: 271–277.
- Doyle A and Griffiths JB (Eds) (1998) 'Cell and tissue culture. Laboratory procedures in biotechnology', John Wiley & Sons, New York, pp. 55–81.
- Falkenhain A, Lorenz Th, Behrendt U and Lehmann J (1998) 'Dead cell estimation – A comparison of different methods', In Merten O-W, Perrin P and Griffiths JB (Eds) *New developments and new applications in animal cell technology*, Kluwer Academic Publishers, Boston, pp. 333–336.
- Familletti PC and Wardwell-Swanson JA (1988) A novel approach to bioassays. *Biotechnology* 6: 1169–1172.
- Freshney RI and Morgan D (1978) Radioisotopic quantitation in microtitration plates by an autofluorographic method. *Cell Biol Int Rep* 2: 375–380.
- Freshney RI (1987) *Culture of Animal Cells – A Manual of Basic Technique*. 2nd ed, Alan R. Liss, Inc., New York, p. 229.
- Friedman HM and Glaubiger DL (1982) Assessment of *in vitro* drug sensitivity of human tumor cells using [³H]thymidine incorporation in a modified human tumor stem cell. *Cancer Res* 42: 4683–4689.
- Goergen JL, Marc A and Engasser JM (1993) Determination of cell lysis and death kinetics in continuous hybridoma cultures from the measurement of lactate dehydrogenase release. *Cytotechnology* 11: 189–195.
- Hakala MT and Rustrum YM (1979) In: DeVita VT and Busch H (eds) *Methods in Cancer Research: Cancer Drug Development*. Part A, Academic Press, New York, p. 47.
- Harris M (1973) 'Electronic enumeration and sizing of cells. B. Tissue culture cells', In: Kruse PF and Patterson MK (eds) *Tissue Culture, Methods and Applications*, Academic Press, New York, pp. 400–405.
- Holbrook JJ, Liljas R, Steindel SJ and Rossman MG (1975) Lactate dehydrogenase, In: Boyer PD (ed) *The Enzymes*. Vol. XI, part A, 3rd ed, Academic Press, New York, pp. 191–292.
- Ihrig T, Tsao M, Hilton M, Jacobson F and Sliwkowski MB (1994) Cell culture raw materials screening by calcein-AM fluorescence using a 96 well plate format, In: Spier RE, Griffiths JB and Berthold W (eds) *Animal Cell Technology: Products of Today, Prospects for Tomorrow*. Butterworth-Heinemann Ltd., Oxford, UK, pp. 110–114.
- Kurokawa H, Park YS, Lijima S and Kobayashi T (1994) Growth characteristics in fed-batch culture of hybridoma cells with control of glucose and glutamine concentrations. *Biotechnol Bioeng* 44: 95–103.
- Korzeniewski C and Callewaert DM (1983) An enzyme release assay for natural cytotoxicity. *J Immunol Methods* 64: 313–320.
- Loontjens FG, McLaughlin LW, Diekmann S and Clegg RM (1991) Binding of Hoechst 33528 and 4',6-diaminophenylindole to self-complementary decaoxynucleotides with modified exocyclic base substituents. *Biochemistry* 30: 182–189.
- Marc A, Wagner A, Martial A, Goergen JL, Engasser JM, Geaugey V and Pinton H (1991) Potential and pitfalls of using LDH release for the evaluation of animal cell death kinetics, In: Spier RE et al. (eds) *Production of Biologicals from Animal Cells in Culture*, Oxford: Butterworth, UK, pp. 569–575.
- Morris C, Griffiths JB, Warburton S, West CML, Al-Rubeai M, Clarke JB, Simone F and Doyle A (1997) *Core Techniques*, In: Doyle A and Griffiths JB (eds) *Mammalian Cell Culture: Essential Techniques*, John Wiley & Sons, New York, pp. 47–62.
- Merten O-W, Keller H, Cabanie L, van Kan Martin C and Moeurs D (1992) Release of cellular enzymes for evaluating the dead cell number in bioreactor cultures, In: Spier RE, Griffiths JB and McDonald C (eds) *Animal Cell Technology: Developments, Processes and products*. Oxford: Butterworth, London, pp. 319–324.
- Mossman T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 65: 55–63.
- Oyama VI and Eagle H (1956) Measurement of cell growth in tissue culture with a phenol reagent (Folin-Ciocalteu). *Proc Soc Exp Biol Med* 91: 305–307.
- Patterson MK (1979) Measurement of growth and viability in culture, In: Jacoby WB and Pastan IH (eds) *Methods in Enzymology, Cell Culture*. Vol. 58, Academic Press, New York, pp. 141–152.
- Pelletier F, Fonteix C, Lourenço da Silva A, Goergen JL, Marc A and Engasser JM (1994) Software sensors for the monitoring of high-density hybridoma perfusion cultures, In: Spier RE, Griffiths JB and Berthold W (eds) *Animal Cell Tech-*

- nology: Products of Today, Prospects for Tomorrow. Oxford: Butterworth, London, UK, pp. 379–382.
- Phillips HJ (1973) Dye exclusion tests for cell viability, In: Kruse PF and Patterson MK (eds) Tissue Culture: Methods and Applications. Academic Press, New York, pp. 406–408.
- Racher AJ, Looby D and Griffiths JB (1990) Use of LDH release to assess changes in culture viability. Cytotechnology 3: 301–307.
- Roper PR and Drewinko B (1976) Comparison of *in vitro* methods to determine drug-induced cell lethality. Cancer Res 36: 2182–2188.
- Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren JT, Bokesch H, Kenney S and Boyd MR (1990) New colorimetric cytotoxicity assay for anticancer-drug screening. J Natl Cancer Instit 82: 1107–1112.
- Szekeres J, Pacsa AS and Pejtsik B (1981) Measurement of lymphocyte cytotoxicity by assessing endogenous alkaline phosphatase activity of the target cells. J Immunol Methods 40: 151–154.
- Wagner A, Marc A and Engasser J-M (1992) The use of lactate dehydrogenase (LDH) release kinetics for the evaluation of death and growth of mammalian cells in perfusion reactors. Biotechnol Bioeng 39: 320–326.
- Wilson AP (1986) Cytotoxicity and viability assays, In: Freshney RI (ed) Animal Cell Culture, a Practical Approach. IRL Press, Washington, DC, pp. 183–216.
- Winkelmeyer P, Glauner B and Lindl T (1993) Quantification of cytotoxicity by cell volume and cell proliferation. Atla 21: 269–280.
- Wroblewski F and LaDue JS (1955) Lactate dehydrogenase activity in blood. Proc Soc Exptl Biol Med 90: 210–213.