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IMPROVED DETECTION OF DRUG CYTOTOXICITY IN THE SOFT AGAR COLONY FORMATION ASSAY THROUGH USE OF A METABOLIZABLE TETRAZOLIUM SALT

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Summary

Use of a metabolizable tetrazolium salt was observed to facilitate assessments of tumor cell drug sensitivity in the soft-agar colony formation assay. Enzyme-mediated staining permits discrimination between viable and non-viable groups of cells so that drug-induced cytotoxicity is clearly identifiable by visual inspection as well as by computerized image analysis. The technique appears to be especially useful in the evaluation of primary tumor cell cultures which often contain substantial numbers of non-viable cellular aggregates.

Introduction

In vitro assessment of tumor cell drug sensitivities has become an integral part of cancer research. Our laboratory has evaluated the soft-agar colony formation assay of Hamburger and Salmon [1] in a large series of primary tumor cell cultures [2,3]. Enumeration of colonies by either visual inspection or computerized image analysis designed specifically for cell cultures [4] represents the endpoint of the assay. One of our concerns has been that the presence of non-viable cellular aggregates and debris within the soft-agar matrix may impair recognition of active cell proliferation as well as lead to significant artifactual colony counts. Therefore, in the present study we investigated whether use of the metabolizable vital dye, 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT), could facilitate detection of valid colonies. By enzymatic reduction, viable cells transform this water-soluble tetrazolium salt into a formazan product which is water-insoluble and dark red [5]. Previously INT has been employed to increase the efficiency of macroscopic counting [6,7,8] and image analysis [9] of cultured cell lines. In the present study the use of INT as a substrate for soft-agar cultures of human tumor cells was observed to permit instrumental distinction of colony-forming units from non-viable cellular aggregates and debris. This differentiation was observed to improve substantially *in vitro* detection of the cytotoxicity of cancer chemotherapeutic agents.

Materials and Methods

Reagents. Sterile aliquots of stock doxorubicin hydrochloride (Adria Laboratories, Inc.), cisplatin (National Cancer Institute), mitomycin C (Sigma Chemical Co.), and sodium azide (Sigma Chemical Co.) were stored at -20°C.

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Appropriate drug dilutions were performed in standard culture media (see below) within one-half hour of application to cultures. Stock INT (Aldrich Chemical Co.) was prepared fresh weekly at a concentration of 1 mg/ml distilled water as described previously [7]. Optimal staining of colonies was achieved by adding 1 ml of INT solution to the surface of soft agar cultures followed by reincubation at 37°C, 5% CO_2, and 100% relative humidity for 20-44 hours. In general, cultures were analyzed immediately following INT re-incubation; a few cultures were evaluated following storage at 4°C for an additional 48 hours. Staining of cultures with trypan blue (Grand Island Biological Co.) was performed by applying 1 ml of sterile 0.4% to each culture surface for 20 minutes at room temperature followed by removal of excess liquid, application of 1 ml sterile water and incubation for 2-4 hours at 4°C. Acridine orange/ethidium bromide (Sigma Chemical Co.) was prepared and utilized as described previously [10,11]. For viability assessment, each culture (2 ml) was incubated in the dark at 4°C for 30 minutes with 1 ml of phosphate-buffered saline containing 3 µg of each dye.

Cultures of Human Cancer Cell Lines. Dulbecco's modified Eagle medium (DMEM), calf serum, penicillin-streptomycin, trypsin/EDTA, and HEPES were purchased from Grand Island Biological Co. Tissue culture dishes (35 mm, Falcon Plastics) and flasks (75 cm^2, Falcon Plastics) were purchased from Curtin Matheson Scientific. Seaplaque agarose was purchased from the FMC Corporation. Melanoma (A101D), rhabdomyosarcoma (A204), lung carcinoma (A549), and bladder carcinoma (A1663) cell lines were obtained from the Frederick Cancer Research Center. CaKi-1 was provided by the Sloan-Kettering Institute for Cancer Research. Each cell line was mycoplasma-free (verified by culture, Virology Laboratory, Mayo Clinic) and maintained as bulk culture monolayers in multiple 75 cm^2 flasks containing standard culture media: DMEM supplemented with 10% calf serum, 100 units penicillin/ml, 100 µg streptomycin/ml, 2 mM L-glutamine and 25 mM HEPES. Media was changed twice per week and cells were passaged each week for a maximum of 15 weeks. Subsequent bulk cultures were re-established from cells stored in liquid nitrogen.

All soft-agar cultures were performed in similar fashion: each 35 mm culture dish contained a base layer consisting of 0.5 ml standard culture media (0.5% agarose). On day 0 cells in bulk culture were dissociated with trypsin/EDTA, washed once in standard culture media, and subcultured by layering 1 x 10^4 viable cells in 0.5 ml standard culture media (0.3% agarose) over each base layer. Cultures were examined with the aid of an inverted stage microscope: only cultures containing uniformly distributed single cell suspensions (< ten 30 µ diameter cell clusters) were considered acceptable for subsequent evaluation. Cultures were maintained in Wedco cell culture incubators at 37°C, 5% CO_2 and 100% relative humidity. On day 1 (24 hours later) an upper layer consisting of 1 ml standard culture media (0.3% agarose) and drug at 2x concentration was added to appropriate cultures. All cell lines formed sufficient numbers of detectable colonies (> 60 µ) following 7-12 days' incubation. Analysis of colony formation was assessed as described for primary human tumor cell cultures.

Primary Cultures of Malignant Human Cells. Tumor specimens were dispersed enzymatically according to the method of Pavelic et al [12] and cells cultured in soft-agar bilayers according to a modification of the original Hamburger-Salmon protocol [13]. Tumor cultures evaluated in this study included 2 melanomas, 1 osteosarcoma, and carcinomas of the lung (2), breast (3), pancreas (1), ovary (1), uterus (2), vulva (1), kidney (3), and prostate (1).

Assessment of Tumor Colony Forming Units. Following 7-21 days' incubation, cultures were examined with the aid of an inverted, phase-light microscope (Leitz Diavert equipped with reticule, Ernst Leitz Co.) and scored with a computerized image analyzer, the FAS-II (Omnicon Feature Analysis System, Model II, Bausch and Lomb, Inc.). The evaluable region of each culture dish (35 contiguous fields [each 4.44 x 3.22 mm^2] equivalent to 500 mm^2) was assessed through use of the Omnicon 36-position stem cell program (Bausch and Lomb, Inc.). The mean colony count and standard error of the mean for each group of cultures (6 dishes/control group and 3 dishes/drug-treated group) were computed and tabulated by the FAS-II. Following completion of conventional analysis, culture dishes were treated with INT, re-examined and recounted.

Results

Increased Efficiency of Colony Detection. During preliminary assessments of nonstained cultures, the number of colonies registered by the image analyzer was observed to be only a fraction (approximately 10-60%) of the number of images appearing on the FAS-II display screen. The inefficiency of detecting such nonstained colonies was more prominent in cultures of transparent cells (e.g., A204) than in cultures of more optically dense cell (e.g., A101D and A1663). Despite lowering the detection threshold below that commonly utilized, the efficiency of counting colonies was still variable between cell lines and not satisfactorily reproducible for any of the cultured cell lines examined.

The use of INT in cell line cultures was observed to improve greatly FAS-II detection efficiency. Images of INT-stained colonies were readily detectable at midrange grey level detection thresholds due to the increased optical density of individual cells afforded by INT staining. Computer-scored colony counts for each of five human cancer cell lines prior to and after INT-staining are shown in Table 1. In each case, cultures treated with INT exhibit substantially higher values with ratios (nonstained to stained) ranging from .17 to .65. Similar ratios were observed on multiple occasions.

TABLE I

Relative Detection Efficiencies of Conventional Image Analysis and Image Analysis Following INT Treatment*

Cell Line	Colonies/500 mm^2		Ratio of Colony Counts (Mode A/Mode B)
	Conventional Image Analysis (Mode A)	Image Analysis p INT (Mode B)	
A101D	221 ± 15.2 (3)	341 ± 12.5 (3)	.648
A204	145 ± 9.98 (3)	850 ± 41.3 (3)	.171
A549	56.3 ± 3.85 (3)	107 ± 11.0 (3)	.526
A1663	556 ± 20.7 (3)	1105 ± 58.3 (3)	.503
CaKi-1	27.3 ± 1.50 (3)	78.7 ± 8.37 (3)	.347

*Table entries are the mean colony count (>60 μ diameter) ± 1 SEM following 7 to 10 days' incubation. The same cultures evaluated by conventional image analysis (mode A) were re-evaluated following treatment with INT (mode B).

Microscopic examination of cultures revealed that more than 80% of the cell groups exceeding a diameter of 60 μ contained moderate to large amounts of red stain while the remaining 60 μ cell groups contained little or no formazan deposition. Non-stained groups of cells appeared intact yet took up trypan blue readily and exhibited prominent orange nuclear fluorescence, suggesting that a substantial proportion of colonies appearing otherwise indistinguishable from the INT-stained colonies had died spontaneously following a limited number of cell divisions.

Compatibility of INT Staining with In Vitro Assessment of Drugs. To investigate whether INT treatment is compatible with in vitro assessment of drug sensitivities, five human cancer cell lines (A101D, A204, A549, A1663, and CaKi-1) exposed to each of 4 cytotoxic agents (doxorubicin, mitomycin C, cisplatin, and sodium azide) were analyzed prior to and after INT treatment. In all cases the dose range of a given drug producing 50% inhibition of colony formation was the same irrespective of INT treatment as shown in Figure 1. Similar steep dose-response curves were observed for all cell lines cultured in the presence of doxorubicin, mitomycin C and sodium azide. Cultures of A204 and CaKi-1 cells exhibited comparable resistance to cisplatin as that depicted for A549 cells (Fig. 1). On the other hand, cultures of A101D and A1663 cells were much more sensitive to these concentrations of cisplatin, with >98% inhibition of colony formation at 1500 ng cisplatin/ml.

In a subsequent experiment, moderately cisplatin-resistant cell lines were cultured in the presence of higher drug concentrations. Of interest was the fact complete inhibition (< 1% survival) in each cell line could be obtained only through analysis of INT-stained cultures. In A549 cultures containing cisplatin (1.5 μg/ml and greater), 5.75 ± 5.29 colonies/500 mm^2 (equivalent to 10.2 ± 9.4% survival) were detected by conventional image analysis, whereas only 0.42 ± 1.00 colonies/500 mm^2 (equivalent to 0.39 ± 0.93% survival) were detected following INT treatment (Fig. 2A). While the residual colony counts of nonstained, drug-treated cultures may appear to be a trivial fraction of that in control cultures of A549 cells, the presence of comparable counts in drug-treated CaKi-1 cultures (Fig. 2B) represented a more sizeable fraction: While INT-treated cultures exposed to cisplatin at concentrations of 1.5 μg/ml and greater exhibited 0.33 ± 0.78 colonies/500 mm^2 (equivalent to 0.42 ± 0.99 % survival), the same cultures analyzed prior to INT stain exhibited 9.33 ± 4.60 colonies/500 mm^2 (equivalent to 34.0 ± 16.8 % survival). The fact that "colonies" were detected in nonstained cultures containing a cytotoxic agent present at 1-, 3-, 10-, and 30-times the concentration employed for in vitro drug screening but not in the same cultures following INT treatment suggest that counts in nonstained cultures arise from the detection of nonvalid images within the soft-agar matrix.

Microscopic examination of drug-treated cultures revealed that many cells within colonies which had died (trypan blue uptake and orange nuclear fluorescence) during the course of incubation appeared to remain intact for days. If lysis occurred within a given colony, intracellular constituents dispersed only poorly into the surrounding soft-agar matrix, yielding an image easily detected by conventional analysis. Thus, INT staining appears to provide an appropriate index of colony survival because it permits discrimination against those colonies which have undergone spontaneous or drug-induced death.

To assess whether the use of INT is compatible with the presence of other drugs in soft-agar culture, INT treatment and image analysis were performed after brief, continuous exposure to each of the following agents (concentration in μg/ml): actinomycin D (0.010), amethopterin (1.0), m-AMSA

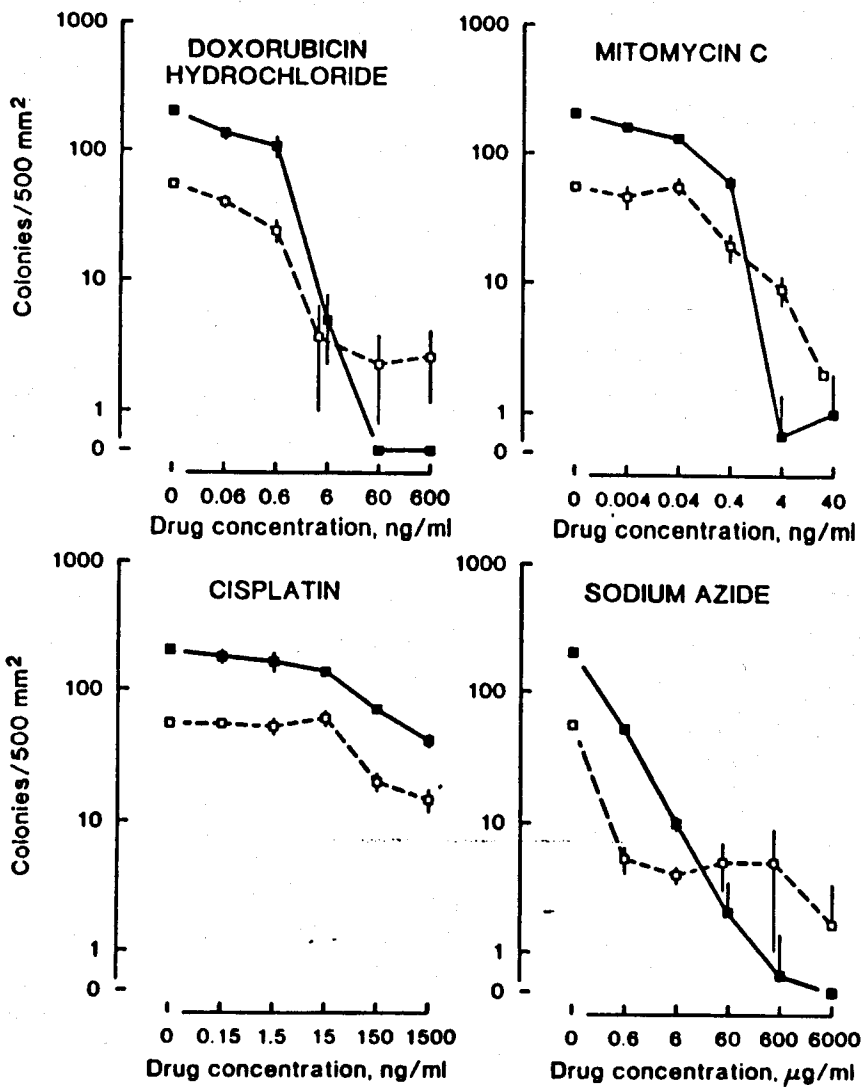

Fig. 1.

Soft agar colony formation by A-549 cells cultured in the presence of cytotoxic agents. The mean colony count (> 60 μ diameter) ± 1 SEM (6 dishes/control group and 3 dishes/drug-treatment group) are shown for conventional image analysis (- □ -) and image analysis following INT treatment (—■—)

(1.0), bleomycin (2.0), doxorubicin (0.60), etoposide (10.0), 5-fluorouracil (10.0), mitomycin C (0.040), sodium azide (6,000) and vinblastine (0.050). After incubation for 7 days, groups of A549 cell cultures (6 dishes/control, 3 dishes/drug) were pre-incubated with each agent for 6 hours prior to INT treatment. Image analyses revealed no significant alteration in colony counts for any agent.

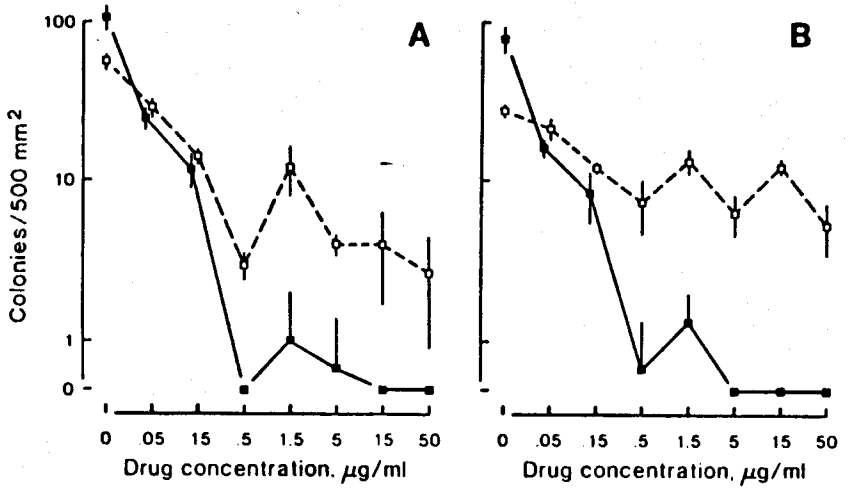

Fig. 2.

Soft-agar colony formation by A549 (A) and CaKi-1 (B) cells cultured in the presence of cisplatin. The mean colony count (> 60 μ diameter) ± 1 SEM (6 dishes/control group and 3 dishes/drug treatment group) are shown for conventional image analysis (- □ -) and image analysis following INT treatment (—■—).

Improved Detection of Drug Cytotoxicity in Primary Cultures of Human Tumor Cells. Since disaggregation of solid tumors for culture often yields substantial debris and non-viable cells and since the number of cells initially plated in such primary cultures is 500 times that employed in the present cell line experiments, it occurred to us that image analysis of nonstained primary cultures may give rise to a similar, if not greater, incidence of artifactual colony counts than cell line cultures. To examine whether the use of INT might improve the detection of growth and drug activity in primary cultures, image analyses were performed before and after INT treatment on a series of cultures derived from 17 different patients. While similar results were observed for 3 tumor specimens, improved detection of drug effects was observed following INT staining in 10 of 17 tumor specimens. Markedly nonconcordant levels of drug activity were noted for 4 tumor cell cultures. A typical example of improvement in the detection of drug effects following INT treatment is shown for one such tumor in Table II. The mean number of images detected in control cultures by conventional analysis was approximately 10% greater than that detected following INT treatment. The presence of such non-stained groups of cells in control as well as drug-treated cultures (confirmed by microscopy) no doubt contributed to the apparent lack of drug sensitivity noted with conventional image analysis. While significant reductions in colony count (< 30% survival) were observed for 8 agents by conventional analysis, following INT treatment significant reductions were observed for 12 agents, 5 of which imposed more than 90% kill. In other primary cultures, the number of colonies detected in INT-stained control plates was often less than 50% that registered by conventional image analysis, indicating the occurrence of even greater artifactual colony counts.

TABLE II

A Comparison of Drug Sensitivity Profiles of Human Tumor-Derived Cells[1] Assessed by Two Methods of Analysis

Treatment[2]	Conventional Image Analysis		Image Analysis Following INT Incubation	
	Colony Count[3]	% Survival[4]	Colony Count[3]	% Survival[4]
Control	106 ± 26 (6)	100	96.7 ± 19.9 (6)	100
L-Alanosine (50.0)	25 ± 4.6 (3)	24 *	10.7 ± 5.51 (3)	11 *
AZQ (1.0)	146 ± 44 (3)	137	223 ± 35.5 (3)	231
Galactitol (2.0)	19 ± 5.6 (3)	18 *	5.00 ± 3.00 (3)	5 **
Mitomycin C (0.040)	12 ± 2.0 (3)	11 *	15.7 ± 8.96 (3)	16 *
Bisantrene (0.50)	56 ± 11 (3)	53	26.0 ± 5.57 (3)	27 *
Mitoguazone (5.0)	80 ± 4.6 (3)	76	24.0 ± 5.00 (3)	25 *
Razoxane (0.38)	162 ± 97 (3)	153	357 ± 23.3 (2)	369
Dacarbazine (5.0)	319 ± 14 (3)	301	314 ± 18.2 (3)	325
Vinblastine (0.050)	12 ± 1.5 (3)	11 *	1.0 ± 1.73 (3)	1 **
Doxorubicin (0.60)	50 ± 4.0 (3)	47	21.0 ± 3.46 (3)	22 *
PALA (10.0)	266 ± 162 (3)	251	109 ± 30.5 (3)	113
Cisplatin (1.50)	88 ± 25 (3)	83	54.3 ± 30.0 (3)	56
Procarbazine (5.0)	242 ± 53 (3)	229	318 ± 18.0 (3)	329
Carmustine (2.0)	299 ± 52 (2)	282	281 ± 36.1 (2)	291
Etoposide (10.0)	38 ± 2.1 (3)	36	12.3 ± 2.52 (3)	13 *
Hydroxyurea (60.0)	17 ± 3.2 (3)	16 *	2.00 ± 2.00 (3)	2**
Triazinate (40.0)	265 ± 13 (3)	250	33.0 ± 3.46 (3)	34
Bleomycin (2.0)	23 ± 2.6 (3)	22 *	14.7 ± 2.08 (3)	15 *
Semustine (1.50)	156 ± 39 (3)	147	336 ± 72.7 (3)	348
Teniposide (10.0)	15 ± 1.5 (3)	14 *	0.00 ± 0.00 (3)	0 **
Actinomycin D (0.010)	25 ± 27 (2)	24 *	1.00 ± 1.41 (2)	1**

[1]Tumor Type: Grade 3 renal cell carcinoma (#1934)
[2]Continuous drug exposure (μg/ml)
[3]Table entries are the mean ± 1 S.D. and (number of dishes) for each group.
[4]Colony counts of drug-treated cultures less than 30% (*) or less than 10% (**) that of respective control cultures indicate drug sensitivity according to our drug screening protocol.

Discussion

Computerized image analysis has advanced greatly the rate and precision with which in vitro cell proliferation can be quantitated. Reports in the recent literature have acknowledged multi-faceted applications of this relatively new technique to biology and medicine (e.g., 9, 14-21). As demonstrated in the present work, the FAS-II is potentially well-suited for the detection of drug effects in soft-agar cultures of human cancer cell lines as well as tumor-derived cells. However, computerized image analysis dependent upon the size, shape, and optical density of soft-agar colonies does not discriminate between viable and non-viable groups of cells. The present findings suggest that detection of drug-induced cytotoxicity in non-stained soft-agar cultures may be inadequate. Non-viable colonies in cultured human cell lines as well as primary tumor cultures under conditions

employed in our assays often gave rise to variable, and at times significant, non-valid colony counts. On the other hand, use of INT in conjunction with image analysis was observed to provide a means to distinguish viable groups of cells (valid images) from non-viable cellular aggregates (non-valid images). In the context of in vitro quantification of drug-induced cytotoxicity, such a methodology appears to provide an appropriate index of drug effect. By definition, cytotoxic agents when present in "therapeutic" concentrations should impart nearly-complete if not total killing of drug-sensitive cells and such an endpoint should be clearly identifiable.

While a trained observer using a microscope may distinguish colonies from cellular aggregates and debris in soft-agar cultures without stains (22), an image analyzer whose detection is based primarily upon optical density may incorrectly score non-viable groups of cells or debris as valid images. The observation that dead cells disperse poorly in soft-agar culture and the fact that inhibition of colony formation appears to be blunted when assessed by conventional methodology, yet spans the entire range of 100 to 0% survival when analyzed following INT treatment, suggests that the former methodology may not accurately detect the true impact of cytotoxic agents. While results are preliminary and a much more extensive assessment of INT staining is underway, it is our judgement that the use of INT and other vital stains in conjunction with image analysis may permit more accurate quantitation of the chemosensitivity of human tumor cells.

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