

# Morphometric and Colorimetric Analyses of Human Tumor Cell Line Growth and Drug Sensitivity in Soft Agar Culture<sup>1</sup>

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## ABSTRACT

Previous studies have demonstrated the suitability of image analysis of tetrazolium-stained colonies to assess growth and drug sensitivity of human tumor cells cultivated in soft agar culture. In the present study, the potential utility of colorimetric analysis to expedite experimental drug evaluations using human tumor cell lines was investigated. The same culture dishes were assessed by image analysis and by formazan colorimetry for purposes of comparing multiple methods of measuring growth as well as growth inhibition. Replicate cultures treated with 2-(*p*-iodonitrophenyl)-3-*p*-nitrophenyl-5-phenyltetrazolium chloride or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide exhibited nearly identical colony count and volume indices as well as excellent correlation in colorimetric end points. Colony-forming unit volume analysis *versus* colorimetric assessment of the same cultures following dimethyl sulfoxide extraction of protamine sulfate-rinsed, dried soft agar cultures exhibited excellent linear correlation for both growth (Pearson *r* ranging from 0.95 to 1.00) and drug sensitivity (Pearson *r* ranging from 0.90 to 0.99, and Spearman *r* ranging from 0.82 to 0.97) and similar drug sensitivity profiles. Results of the current investigation indicate that end points of soft agar culture remain stable for a period of at least 2 weeks following assay termination. In addition, a colorimetric detection range of 1.3–2.2 log units permits determinations of survival levels ranging from 100 to 5% of respective control levels. Colorimetric analysis is anticipated to expedite soft agar colony formation assay evaluations (a) by reducing the need to use the more rigorous and time-consuming image analysis procedures to measure activity in preliminary drug sensitivity assays and (b) by permitting the determination of effective concentration ranges of new experimental agents for subsequent, more detailed investigations.

## INTRODUCTION

Soft agar colony formation assays have been utilized widely to evaluate growth, radiation sensitivity, and drug sensitivity of mammalian stem cells as well as fresh human tumor material (e.g., Refs. 1–16). Assays of drug sensitivity have been based largely upon scoring the number of colonies (which exceed an arbitrary size, often 60  $\mu$ m in diameter) in drug-treated cultures relative to that in vehicle-treated control cultures. More recently, metabolizable vital dyes (e.g., INT<sup>3</sup>) have been utilized in conjunction with soft agar culture to facilitate CFU enumeration as well as to more accurately distinguish viable from nonviable cell groups in cultures exposed to drug (17–20). Recent studies have also demonstrated the utility of tetrazolium

colorimetry in a variety of growth/growth inhibition assays in fluid culture microplate systems (e.g., Refs. 21–26). In the present study we have investigated the suitability of colorimetric analysis of tetrazolium-stained soft agar cultures. The objectives of the current study were to determine (a) whether tetrazolium-stained soft agar cultures are stable for analysis at time intervals distant from the time of assay, and (b) whether colorimetric analysis provides a reasonable approximation of growth and growth inhibition. Findings indicate that morphometric and colorimetric end points are stable following culture termination and that colorimetric analysis is well suited for defining effective drug concentration ranges in human tumor cell lines.

## MATERIALS AND METHODS

**Cell Cultivation.** Cell lines utilized in the current study were derived from multiple forms of human cancer: A-204 rhabdomyosarcoma (27); A-549 lung carcinoma (27); HT-29 colon adenocarcinoma (28); K-562 chronic myelogenous leukemia (29); HL-60 promyelocytic leukemia (30); and NCI-H460 large cell lung carcinoma (31). Each cell line was cultivated in a standard culture medium: RPMI 1640 (Quality Biologicals, Inc., Gaithersburg, MD) supplemented with 10% defined fetal bovine serum (HyClone Laboratories, Inc., Logan, UT) and 2 mM L-glutamine (Quality Biologicals) without antibiotics. Each cell line was subjected to quality assurance tests and was cryopreserved at multiple passage levels as previously described (25). Maintenance cultures as well as soft agar culture assays were incubated under conventional conditions (37°C, 5% CO<sub>2</sub>, 95% air, and 100% relative humidity). Adherent monolayer cultures were passaged weekly with the DeLarco formulation of trypsin/EDTA (Quality Biologicals); leukemia cell lines were passaged weekly by dilution. Each cell line was cultivated for a minimum of 2 passages following thaw prior to experimentation and was maintained in exponential growth for a maximum of 20 serial passages from a specific cryopreserved reference cell stock (working seed stock). Cultures were replenished with fresh culture medium 24 h prior to harvesting for assays.

**Chemical Reagents and Solutions.** Tetrazolium and formazan reagents were purchased from Sigma Chemical Co. (St. Louis, MO): MTT (M2128); MTT formazan (M2003); INT (I8377); and INT formazan (I7375). DMSO was purchased from American Burdick and Jackson Laboratories (Muskegon, MI); spectrophotometric grade product 081 and stored in unopened bottles at room temperature in the dark or in 50-ml sterile plastic tubes at –20°C in the dark. Protamine sulfate (salmine, P4380) was purchased from Sigma. Phosphate-buffered saline and normal saline were purchased from Quality Biologicals.

All chemotherapeutic agents were obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute. Crystalline stock materials were stored at –20°C. Solvent-reconstituted chemotherapeutic agents were prepared at high concentration, partitioned into multiple aliquots, and stored at –70°C. Just prior to culture application the contents of frozen vials were thawed and mixed. Measured aliquots (20–150  $\mu$ l) were transferred by micropipet (Gilson Pipetman; Model P-200) equipped with polypropylene tips to standard culture medium within polypropylene tubes (Nunc cryotubes), serially diluted in culture medium containing appropriate concentration of vehicle, and applied within 10 min to appropriate culture dishes.

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<sup>3</sup> The abbreviations used are: INT, 2-(*p*-iodonitrophenyl)-3-*p*-nitrophenyl-5-phenyltetrazolium chloride; CFU, colony-forming unit; DMSO, dimethyl sulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; P/S, protamine sulfate; T/C, percentage of control, treatment/control  $\times$  100%; IC<sub>50</sub>, drug concentration imposing 50% growth inhibition.

**MTT Stock Solution.** The MTT stock solution (5 mg/ml) was prepared as follows: 1 g MTT/200 ml phosphate-buffered saline was sterile filtered (0.22- $\mu$ m filter units; Corning 25932) and stored at 4°C for a maximum of 1 month. MTT working solution (1 mg/ml) was prepared just prior to culture application by diluting MTT stock solution 1:5 (v/v) in prewarmed standard culture medium. INT solution (1 mg/ml) was prepared as follows: 300 mg INT in 300 ml prewarmed deionized distilled H<sub>2</sub>O were heated to 100°C in a microwave oven, allowed to cool, sterile filtered, stored at 4°C for a maximum of 1 month, and utilized in culture without further dilution. P/S buffer (2.5%, m/v) was prepared as follows: 12.5 g P/S were added slowly to 500 ml normal saline with stirring, allowed to dissolve, sterile filtered, and stored at room temperature for a maximum of 6 weeks.

**In Vitro Growth/Growth Inhibition Assays.** The soft agar colony formation assay outlined below represents a modification of the procedures previously described (20). Each 35-mm culture dish (No. 3001; Falcon Plastics, Oxnard, CA) was coated with a 1-ml base layer containing 0.7% agarose (Seaplaque; FMC Corp., Rockville, ME). On day 0, cells in maintenance culture were dissociated as described above, washed once in growth medium, filtered through 30- $\mu$ m polyester mesh, and subcultured by layering 0.625 to 20  $\times$  10<sup>3</sup> viable cells in 0.5 ml culture medium containing 0.3% agarose over replicate base culture layers. Culture dishes were then transferred to a refrigerator (4°C) for 15 min, to room temperature for 10 min, and then to culture incubators. For growth assays an upper layer of 0.5 ml fluid culture medium was applied to each culture on day 0. For drug sensitivity assays, an upper layer of 0.5 ml culture medium plus 0.1 ml culture medium containing drug ( $n = 3$ /each concentration) and/or drug vehicle ( $n = 6$ ) was applied to cultures on day 1. Cultures were stained with one of two tetrazolium reagents, 1 ml of INT solution (16-h incubation), or 1 ml of MTT working solution (4-h incubation) at multiple time intervals (usually days 1, 4, and 7).

**Protamine Sulfate Buffer Rinses.** Following incubation with tetrazolium, fluid medium on the surface of each culture was removed by aspiration and replaced with 1.5 ml P/S buffer. Plates were maintained at 4°C for 16–24 h. This rinse procedure was repeated a second time prior to final fluid aspiration and image analysis of formazan-stained colonies. Use of P/S buffer rinses was observed to reduce the optical density of soft agar matrix by image analysis and to permit better discrimination between viable cells and agar matrix due to a reduction in culture blank mean absorbance from 0.094 to 0.036 unit for MTT and from 0.061 to 0.042 unit for INT. Evaluation of replicate cultures stained with INT or MTT by image analysis and by colorimetry revealed that each end point was stable over the course of 16 days following termination of culture.

**Image Analysis.** Cumulative counts of colonies (diameters,  $\geq 60 \mu$ m) and cumulative volumes of CFU (diameters,  $\geq 20 \mu$ m) were determined with an Omnicon FAS-II (Bausch & Lomb, Rochester, NY) following calibration with an Omnicon test plate 3 and polystyrene microspheres embedded in soft agar matrix as described previously (20). The evaluable region of each culture dish (35 contiguous fields equivalent to 51% of the cell layer culture volume) was analyzed, using an Omnicon program in which measurements of CFU diameters and calculations of CFU volumes assume spherical geometry. Selective scoring of viable cell groups was achieved by adjustment of the instrument detection threshold (minimum optical density) to exclude images of nonstained cell material and debris. Data were collected directly from the FAS-II with an IBM PC AT computer equipped with Telios communication software and analyzed through use of Lotus Symphony software. Subsequent to image analysis, cultures were permitted to dry overnight in the dark in a vented containment hood and then extracted for formazan colorimetry.

**Formazan Colorimetry.** Complete formazan extraction was accomplished over the course of 4 h following the addition and occasional agitation of 1 ml DMSO to each dried culture. Aliquots (150  $\mu$ l) of DMSO extract were transferred to individual microculture plate wells by micropipet (Gilson) and were analyzed with a plate colorimeter (Dynatech MR 600) as described previously (25).

Culture dishes containing formazan reagents were utilized for determinations of culture extraction efficiency and preparation of standard

curves in the following manner. For MTT formazan stock, 40 mg of the reagent were allowed to dissolve in 100% ethanol for 1 h with occasional mixing. For direct measurement in microculture plates, stock was diluted in DMSO initially at 1:20, followed by 7 serial dilutions of 2 (150  $\mu$ l of each added to replicate wells). For extraction efficiency determinations and standard curve generation stock was diluted in 100% ethanol; aliquots (1 ml) over a range of concentrations (7.5–0.0293  $\mu$ g/ml) were applied to each of 6 empty culture dishes and air dried. For INT formazan stock, 20 mg reagent were allowed to dissolve in 20 ml acetone for 1 h with occasional mixing. Just prior to application, stock solution was diluted serially 1:2. Aliquots (125  $\mu$ l) of each concentration were applied to each of 3 glass coverslips, allowed to air dry, and transferred to empty culture dishes.

**Statistical Methods.** The statistical analysis of agreement among the three methods of soft agar colony formation assay analysis (CFU volume, colony count, and colorimetry) was conducted in several steps. The results of the 2 standard morphometric methods were correlated with each other as well as with those of the new colorimetric procedure in each of 6 cloning assays and 53 drug sensitivity assays.

For cloning experiments, statistical analyses are based upon 6 inoculation densities (625–20,000 cells/dish, 3 dishes/density). MTT was used in each of the 6 cell line-cloning experiments; INT was used in replicate culture sets in 3 of these experiments. Results obtained from each of the two morphometric methods were compared with each other and with the colorimetric results by means of the Pearson correlation coefficient.

For the drug sensitivity experiments, the percentage of vehicle control (T/C) values were calculated by each of the three methods for each assay. The respective values obtained from the different methods were compared by means of the Pearson correlation coefficient ( $r$ ). Separate sets of correlations, one set stratified on cell line and one set stratified on drug, were also calculated. Since a single 5–6-log concentration range was chosen for each drug to span the effective concentration ranges of all cell lines, we were concerned about the possibility that the observed drug sensitivity profiles might be highly bimodal, with most of the T/C values falling in the 90–100% range or the 0–10% range, and that we might thereby obtain very high Pearson correlation coefficients, even though the analysis methods agreed only in the categorization of high versus low sensitivities. To guard against this, we calculated the Spearman correlation coefficients ( $r$ ) as well, since the Spearman coefficient is the correlation of the ranks of the data and the ranks are uniformly distributed and cannot, therefore, be bimodal.

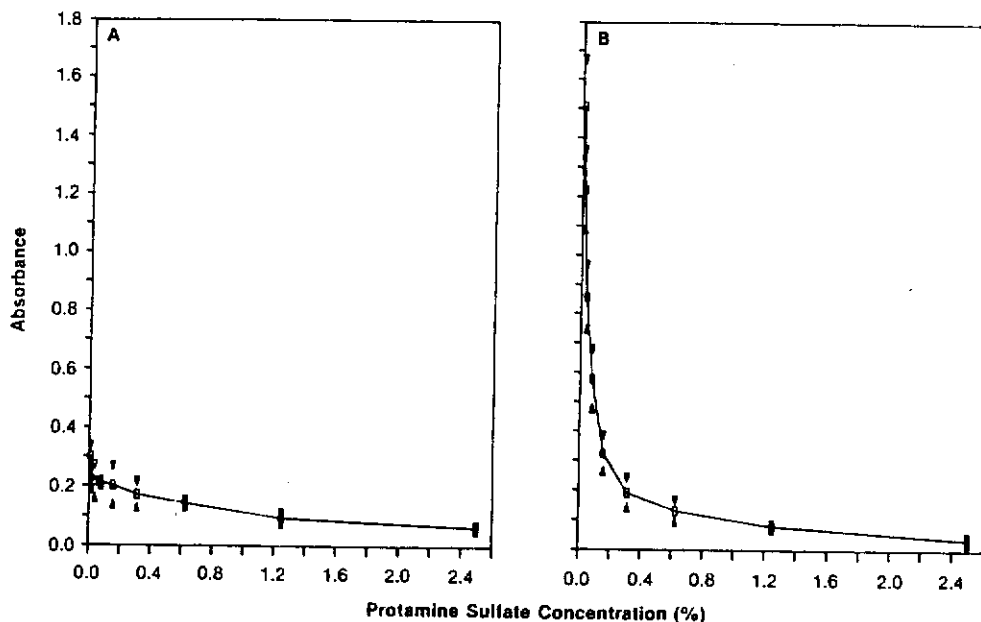
A separate test of the agreement among the analysis methods was also carried out on differences in T/C values observed between the three end points in each experiment. For each morphometric method, T/C values were subtracted from T/C values obtained by colorimetry. The same analyses were performed to compare the two morphometric methods, in which T/C values for colony count were subtracted from T/C values for CFU volume. Analyses were performed on data sets overall as well as repeated on data sets stratified by cell line.

## RESULTS

**Colorimetry of Formazan Reagents in Soft Agar Matrix.** Development of a colorimetric procedure to measure overall growth/growth inhibition in soft agar culture was conducted in two steps: (a) development of a plate washing, drying, and extraction procedure appropriate for soft agar cultures coupled with (b) adaptation of the procedure for formazan colorimetry of fluid microplate cultures to that of soft agar cultures. It is well known that fluid as well as soft agar matrix cultures containing INT or MTT (but lacking cells) exhibit significant "background" formazan production during incubation at 37°C. These background levels continue to increase gradually with time despite extensive rinsing with standard buffers (e.g., phosphate-buffered saline, normal saline, or Hanks' balanced salt solution) or refrigeration of the cultures.

When MTT-treated cultures are allowed to air dry at room temperature, such background increases markedly and to the

Fig. 1. "Background" formazan extracted from soft agar culture "blanks" following two rinses with P/S buffer. Replicate culture "blanks" were set up, rinsed, dried, and extracted as described in "Materials and Methods." Mean absorbances  $\pm$  SD ( $n = 3$ ) for each of 8 P/S concentrations are depicted for INT-treated (A) and MTT-treated (B) blanks.



point that viable colonies are obscured from view. On the other hand, terminating the tetrazolium incubation by the addition of P/S buffer eliminates subsequent background formazan generation. As shown in Fig. 1, background absorbance of dishes rinsed with normal saline are somewhat elevated for INT-treated soft agar cultures ( $0.303 \pm 0.036$  unit) and are very high for MTT-treated soft agar cultures ( $1.51 \pm 0.16$  units). Following two rinses with P/S buffer, absorbances were significantly reduced, with minimum levels ( $0.063 \pm 0.007$  and  $0.033 \pm 0.012$  unit for INT and MTT, respectively) observed following two rinses with 2.5% P/S.

While image analysis can discriminate between viable and nonviable cell groups in cultures stained with INT, the detection range for colorimetric analysis is much improved following two rinses with P/S buffer. Likewise, cultures stained with MTT are suitable for image analysis if the plates are simply refrigerated following tetrazolium incubation. However, colonies in cultures stained with MTT which are permitted to dry without previous P/S buffer treatment are obscured from view and cannot be measured colorimetrically. It is important to note that INT and MTT formazan extraction by DMSO requires that plates be dried in advance of solvent application, since hydrated agar matrix relinquishes formazan too slowly to be practically useful.

Fig. 2 illustrates the linearity of MTT formazan reagent colorimetry. MTT formazan in DMSO solvent dispensed directly into 96-well plates (Fig. 2A) exhibits linear regression ( $r = 0.998$ ,  $n = 48$ ). Likewise, as shown in Fig. 2B extraction of MTT formazan (previously applied to the surface of 35-mm dishes) results in reasonable recovery (75%) and excellent linear regression ( $r = 0.998$ ,  $n = 54$ ).

**Image Analysis versus Colorimetry of Formazan Extracted from Cloning Assays.** Results of experiments described above were obtained utilizing formazan reagents in soft agar culture matrix. In the remaining experiments, image analysis and colorimetry were performed, utilizing soft agar cell cultures stained with tetrazolium salts. Overall growth and cloning efficiency of a given cell line in soft agar culture is dependent upon multiple parameters. Colony count and cumulative volume generally exhibit a linear or near-linear relationship with cell inoculation over a range of densities with growth at lower

levels dependent upon critical cell mass and growth at upper levels limited by availability of culture medium nutrients. Plots of CFU count versus inoculation density and especially cumulative CFU volume versus inoculation density typically deviate significantly from linearity at these "lower" and "upper" limits of inoculation and generally exhibit optimal growth at densities

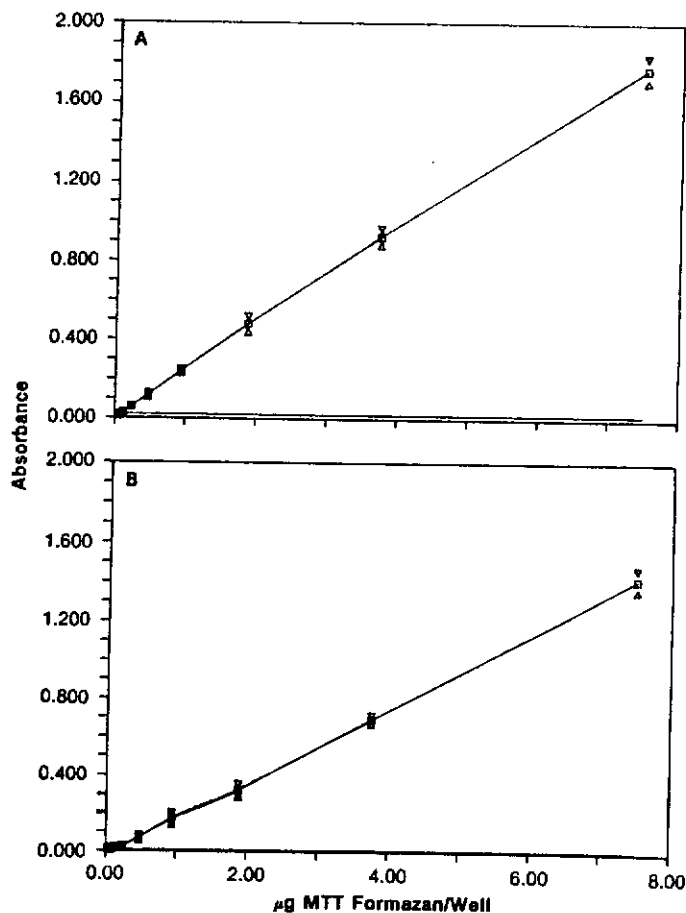


Fig. 2. MTT formazan colorimetry. A, reagent added directly to analysis wells; B, MTT formazan applied to the surface of replicate dishes, covered with culture medium containing agarose, rinsed with P/S, dried, and extracted with DMSO.

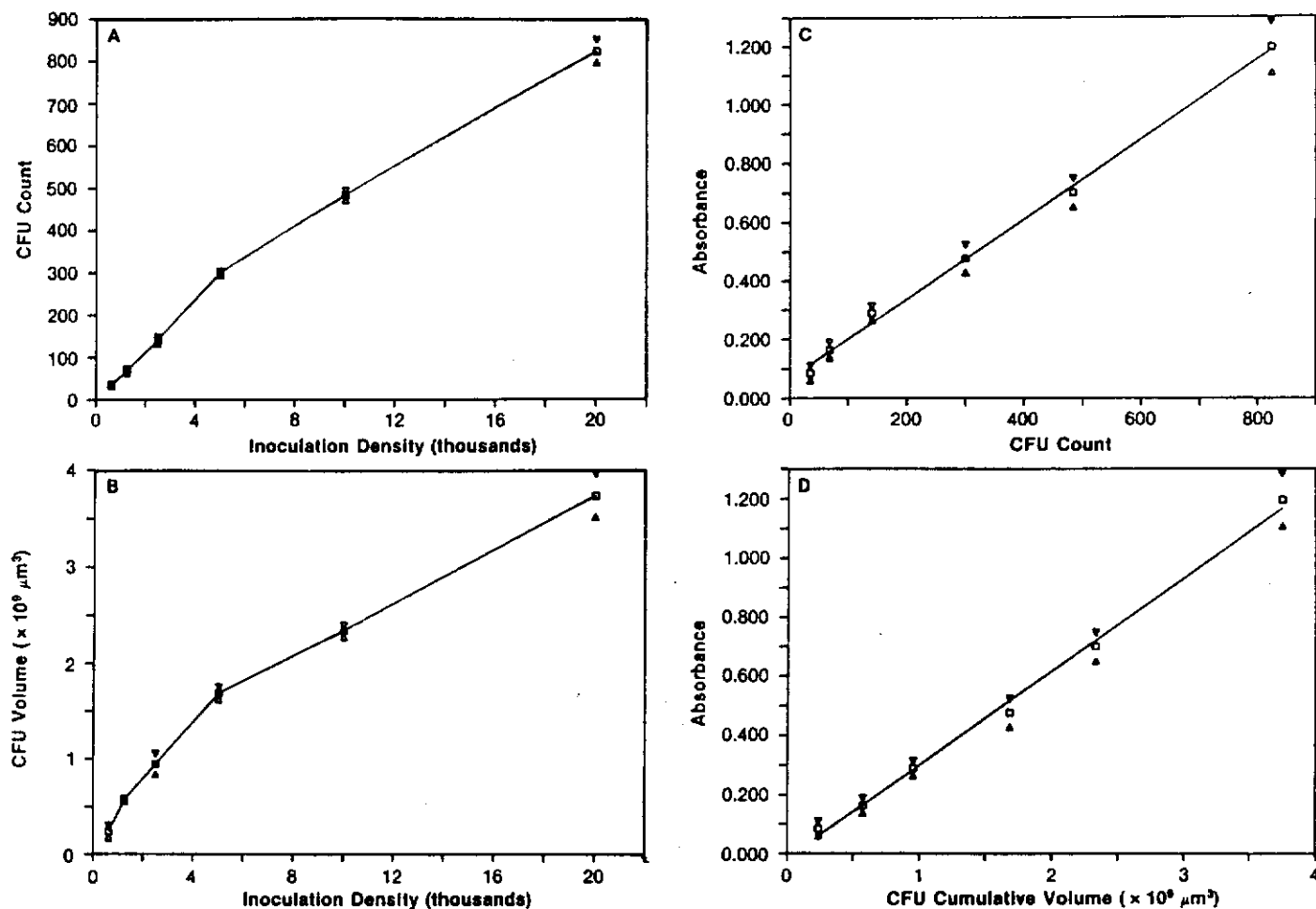


Fig. 3. Soft agar colony formation by NCI-H460 cells. Inoculation on day 0 (625–20,000 cells/2 ml/35-mm dish); MTT incubation (4 h) on day 7; A, CFU count and cloning efficiency. B, computer-assisted volume analysis; C, linear regression analysis of CFU count versus extracted formazan; D, linear regression analysis of cumulative CFU volume versus extracted formazan. The means  $\pm$  SD ( $n = 3$ ) are depicted in each group.

intermediate in value (e.g., Fig. 3, A and B). Nevertheless, formazan colorimetry of such cloning assays correlates with both CFU count and volume in a linear manner (e.g., as shown in Fig. 3, C and D). For 6 cell line cloning assays treated with MTT,  $r$  was observed to range from 0.95 to 1.00 with 16 of the 18 coefficients  $\geq 0.98$ . For three cell line cloning assays treated with INT,  $r$  was observed to range from 0.98 to 1.00 with 8 of the 9 coefficients  $\geq 0.99$ . In addition, replicate cultures treated with either INT (16-h incubation) or MTT (4-h incubation) exhibited nearly identical CFU count and volume indices as well as corresponding colorimetric end points (Fig. 4).

**Stability of P/S-rinsed Soft Agar Cultures.** The stability of P/S-rinsed soft agar cultures was assessed by using replicate dishes of HT-29 cells cultivated for 7 days, stained with MTT or INT, rinsed twice with P/S buffer, and then stored under refrigeration for 0, 2, 9, and 16 days prior to image analysis and formazan colorimetry. Fig. 5A illustrates the results of linear regression analysis for cumulative CFU volume versus inoculation density of MTT-treated cultures ( $0.994 < r < 0.996$ ). Slope factors for image analysis ranged from 0.6358 to  $0.6593 \times 10^6$  (3.7% difference), and all values fell within a 95% confidence interval of the day 0 slope factor ( $0.6358 \pm 0.0364$ ). Likewise, intercept values for image analysis ranged from  $-64.7 \times 10^6$  to  $51.0 \times 10^6$  (overall deviation of 7.46% from the average cumulative CFU volume) and all values fell within a 95% confidence interval of the day 0 measurement ( $-64.7 \times 10^6 \pm 240.2 \times 10^6$ ). Fig. 5B illustrates results of linear regression

analysis for absorbance versus inoculation density of MTT-treated cultures ( $0.979 < r < 0.991$ ). Although the slope factor for colorimetric analysis on day 2 (0.000206) slightly exceeded the 95% confidence interval of the day 0 slope factor ( $0.000179 \pm 0.000024$ ), the remaining measurements of days 9 and 16 (0.000191 and 0.000199) and all intercept values fell within the 95% confidence interval for the day 0 group ( $-0.00346 \pm 0.0955$ ). Similar results were observed for image analysis and formazan colorimetry of INT-treated cultures (data not shown). In addition, microscopic inspection of formazan-stained colonies over the course of 2 weeks following culture termination indicated that P/S did not remove formazan once deposition had occurred. Even the fine, needle-like formazan crystals which extended beyond some colony margins remained intact following extended periods of refrigeration following P/S rinses (16 days).

**Image Analysis versus Colorimetry of Formazan Extracted from Drug-treated Cultures.** Formazan colorimetry also correlated well with morphometric indices in drug-treated cultures. A given cell line was exposed to 5 to 6 concentrations of each of 10 selected chemotherapeutic agents (see Table 1). In all experiments, drug sensitivity profiles for each method of analysis were observed to span the entire concentration-effect range (yielding T/C values from 100 to approximately 0%). Unfortunately, data from the testing of five agents (doxorubicin HCl, mitomycin C, 5-fluorouracil, cisplatin, and carmustine were evaluated concurrently) in one cell line (A-204) had to be

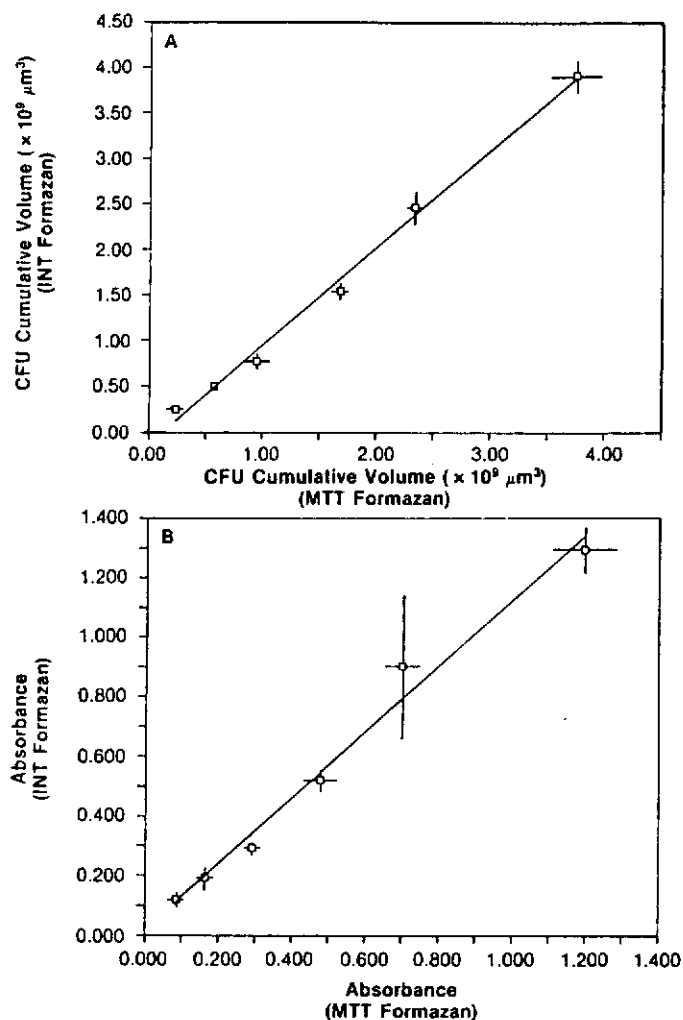


Fig. 4. Linearity and correlation of computer-assisted volume analysis and tetrazolium colorimetry (same experiment as described for Fig. 3). Correspondence in CFU volume (A) and colorimetry (B) between INT and MTT treated cultures (each point represents the mean  $\pm$  SD,  $n = 3$  replicate cultures for each tetrazolium).

excluded from statistical analysis due to a technical problem in the processing of cultures for colorimetry. An inadequate number of P/S rinses resulted in prominent background absorbances and a large proportion of T/C values which fell well within the negative range. Statistical analyses of data from the remaining 53 of the 58 drug sensitivity assays are summarized in Tables 1 and 2. In these analyses, colorimetry T/C values which fell below 3% were all taken as 0% since 3% generally approximates the detection limit for colorimetric analysis of soft agar cultures.

In general, the three methods of culture analysis correlated well. Overall, correlation coefficients ranged from 0.860 to 0.964. CFU volume *versus* formazan colorimetry exhibited the highest correlations overall as well as for data stratified by cell line and by drug. Pearson coefficients ranged from 0.901 to 0.988 and Spearman coefficients ranged from 0.820 to 0.969. CFU volume *versus* colony count analyses exhibited a similar range of correlations, 0.879 to 0.996 for Pearson and 0.838 to 0.985 for Spearman. Colony count *versus* colorimetric analyses exhibited somewhat less correlation with coefficients of 0.766 to 0.965.

A tendency for the distribution of T/C values in a given experiment to be bimodal (*i.e.*, values concentrated in the 90–100% range or in the 0–10% range; *e.g.*, as shown in Fig. 6) raised concerns that this bimodal distribution might yield high

Pearson correlation coefficients due to the presence of only high and low T/C values. However, the results of Spearman analysis (on T/C value ranks) generally confirmed the correlations between the three end points exhibited by the Pearson analyses. The correlation coefficients for vinblastine (which demonstrated a particularly extreme bimodal distribution of T/C values with only a few intermediate drug sensitivity values across cell lines) are a notable exception. Nevertheless, in such cases the effective concentration range (*i.e.*, 90–10% of vehicle control values) for all culture analysis end points fall within the same 1-log drug concentration range. Such results demonstrate the utility of using colorimetric analysis to determine an upper limit to the effective drug concentration range and to select an appropriate serial dilution factor to measure intermediate levels of drug activity in a subsequent more detailed morphometric analysis of drug sensitivity.

One additional correlation analysis was performed as a means to avoid possible problems associated with interpretation of bimodal T/C value distributions. For each drug, analysis was restricted to the highest concentration level yielding mean T/C values (across the cell lines) which, for all three culture analysis methods, fell between 10 and 90% (referred to as the "midrange concentration" in Table 2). We then calculated the Pearson correlation coefficients. With the exception of chlorambucil, correlation coefficients for CFU volume *versus* colorimetry exceeded 0.884. Correlations involving colony count were significantly less in most cases (possibly for reasons suggested

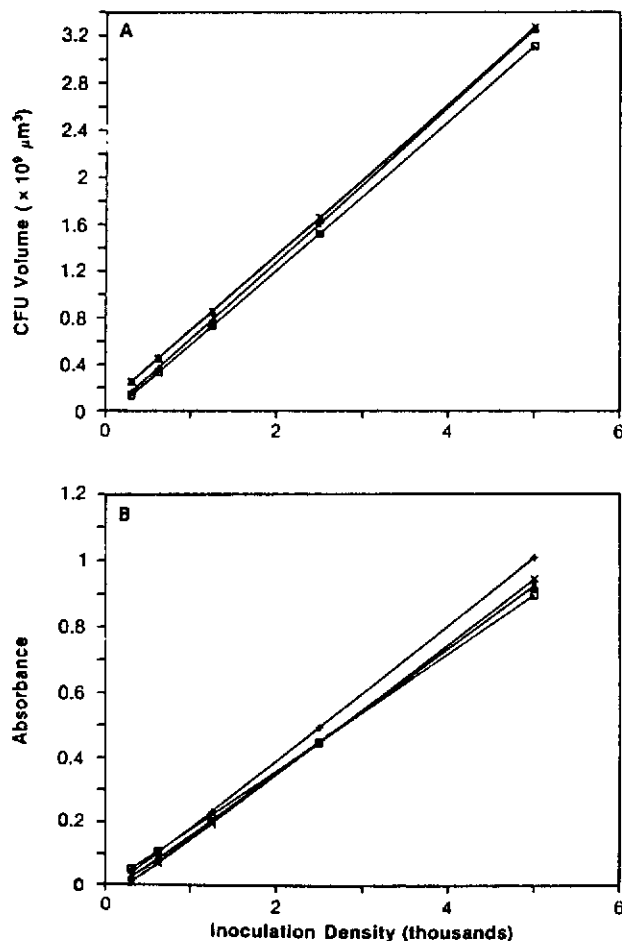


Fig. 5. Stability of image analysis and formazan colorimetry following P/S buffer rinses. Soft agar cultures (3 replicate cultures/each of 5 inoculation densities) were stored under refrigeration for 0 ( $\square$ ), 2 ( $+$ ), 9 ( $\Delta$ ), and 16 ( $\times$ ) days prior to analysis. Cumulative CFU volume (A) and formazan colorimetry (B).

Table 1 Correlations of morphometric versus colorimetric assessments (T/C values) of drug sensitivity

	N <sup>a</sup>	Formazan colorimetry vs. CFU volume <sup>b</sup>	Formazan colorimetry vs. colony count <sup>b</sup>	CFU volume vs. colony count <sup>b</sup>
Over-all	312	0.964 (0.942)	0.901 (0.860)	0.919 (0.918)
By cell line				
A-204	24	0.959 (0.935)	0.959 (0.935)	0.996 (0.985)
A-549	49	0.955 (0.928)	0.965 (0.876)	0.992 (0.956)
HL-60	60	0.972 (0.910)	0.831 (0.810)	0.879 (0.842)
HT-29	60	0.988 (0.938)	0.980 (0.929)	0.976 (0.957)
K-562	60	0.969 (0.963)	0.930 (0.827)	0.893 (0.838)
NCI-H460	59	0.961 (0.927)	0.901 (0.892)	0.883 (0.947)
By drug				
Doxorubicin	29	0.969 (0.903)	0.924 (0.801)	0.950 (0.876)
Mitomycin C	29	0.973 (0.959)	0.889 (0.824)	0.922 (0.890)
5-Fluorouracil	29	0.972 (0.918)	0.869 (0.793)	0.895 (0.863)
Cisplatin	29	0.988 (0.962)	0.956 (0.939)	0.944 (0.920)
Carmustine (BCNU) <sup>d</sup>	29	0.971 (0.963)	0.939 (0.866)	0.945 (0.920)
Actinomycin D	35	0.957 (0.912)	0.906 (0.853)	0.922 (0.948)
Bleomycin	35	0.966 (0.969)	0.866 (0.853)	0.921 (0.914)
Chlorambucil	24	0.901 (0.886)	0.837 (0.811)	0.900 (0.898)
Mercuric chloride	36	0.983 (0.956)	0.908 (0.871)	0.927 (0.913)
Vinblastine	36	0.972 (0.824)	0.950 (0.766)	0.975 (0.932)
By drug (for mid-range <sup>c</sup> concentration)				
Doxorubicin	5	0.939	0.849	0.864
Mitomycin C	5	0.884	0.576	0.859
5-Fluorouracil	5	0.959	0.614	0.810
Cisplatin	5	0.978	0.878	0.899
Carmustine (BCNU)	5	0.987	0.975	0.934
Actinomycin D	6	0.906	0.621	0.680
Bleomycin	6	0.992	0.853	0.893
Chlorambucil	4	0.705	0.534	0.640
Mercuric chloride	6	0.990	0.856	0.845

<sup>a</sup> N, number of pairs of T/C values. Each T/C value is derived from concurrent testing in an experiment with 6 replicate cultures for vehicle control and 3 replicate dishes for each drug concentration. There were 5-6 serial dilutions of 10 (except in a few cases for carmustine (BCNU) where 6 serial dilutions of 4 were used) with highest concentrations ( $\mu\text{g/ml}$ ) as indicated: Doxorubicin HCl (6.0); mitomycin C (4.0); 5-fluorouracil (715); cisplatin (150); carmustine (BCNU, 200); actinomycin D (1.0); bleomycin sulfate (200); vinblastine sulfate (5.0); mercuric chloride (100); and chlorambucil (100). Chlorambucil was not tested in the A-204 or A-549 cell lines.

<sup>b</sup> r values, with the Pearson correlation coefficient followed by the corresponding Spearman correlation coefficient in parentheses.

<sup>c</sup> The midrange concentration is defined as the largest concentration yielding T/C values between 10% and 90% for all methods.

<sup>d</sup> BCNU, bischloroethylnitrosourea.

below). Results for vinblastine do not appear in these analyses because grouped data did not satisfy our definition of midrange concentration.

Despite overall agreement among the three modes of analysis, two types of disparity were sometimes observed between methodology end points. First, colony count data tended to exhibit "shoulders" in the 70-100% of control range, whereas sensitivity measured in the same dishes by volume analysis and colorimetry tended to be more "rounded" (e.g., doxorubicin, mitomycin C, 5-fluorouracil, and mercuric chloride; Fig. 6). Such shoulders were especially noticeable for cell lines in which mean colony diameters of control cultures significantly exceed 60  $\mu\text{m}$ . In the drug sensitivity experiments performed in this study (2 experiment sets/cell line) the following mean colony diameters ( $\mu\text{m}$ ) were observed: A-204, 61; A-549, 76; HL-60, 145; HT-29, 108; K-562, 149; and NCI-H460, 193. It is noteworthy that cell lines exhibiting mean colony diameters much greater than 60  $\mu\text{m}$  (HL-60, K-562, and NCI-H460) are associated with diminished correlation coefficients for colony count versus CFU volume and colony count versus colorimetry (see Table 1). These results are not unexpected since by definition colonies with nominal diameters of 60, 120, and 240 would each be scored as 1 in a counting scheme but would differ in mass (and

Table 2 Mean differences in T/C values derived from morphometric versus colorimetric assessments of drug sensitivity

	N <sup>a</sup>	Colorimetry minus CFU volume <sup>b</sup>	Colorimetry minus colony count <sup>b</sup>	CFU volume minus colony count <sup>b</sup>
Over-all	312	0.084 (60+) -0.106 (40-)	0.105 (49+) -0.193 (51-)	0.073 (45+) -0.203 (43-)
By cell line				
A-204	24	0.093 (58+) -0.183 (42-)	0.122 (67+) -0.110 (33-)	0.076 (88+) -0.000 (4-)
A-549	49	0.132 (59+) -0.140 (41-)	0.140 (57+) -0.090 (43-)	0.049 (65+) -0.055 (20-)
HL-60	60	0.073 (47+) -0.079 (53-)	0.161 (43+) -0.241 (57-)	0.129 (38+) -0.240 (45-)
HT-29	60	0.063 (60+) -0.060 (40-)	0.056 (45+) -0.100 (55-)	0.031 (40+) -0.112 (50-)
K-562	60	0.049 (65+) -0.147 (35-)	0.028 (37+) -0.316 (63-)	0.038 (28+) -0.265 (68-)
NCI-H460	59	0.108 (68+) -0.084 (32-)	0.112 (59+) -0.176 (41-)	0.116 (40+) -0.242 (41-)

<sup>a</sup> See Table 1, Footnote a. Tables 1 and 2 are based on the same pharmacological data set.

<sup>b</sup> Respective means of the positive and negative T/C value differences. Numbers in parentheses, percentages for positive and negative differences.

presumably in formazan deposition) by factors of 1, 8, and 64, respectively.

To examine this disparity more closely, the mean positive and mean negative differences in T/C values were calculated for each pair of end points (Table 2). The overall analysis yielded modest mean positive and negative differences of approximately 10% for the colorimetry T/C value minus the CFU volume T/C value. When the colony count T/C value was subtracted from the T/C value for either of the alternate methods, the mean negative difference was approximately 20% overall, a reflection of the shoulders exhibited by the colony count drug sensitivity profiles. When the mean difference analysis is stratified by cell line, it can be seen that this phenomenon is restricted to the cell lines with greater mean colony diameters (HL-60, K-562, and NCI-H460).

While colorimetric data and volume analysis showed good agreement in the upper portion of concentration-effect curves, colorimetric analyses sometimes exhibited greater values and a more gradual decrease in the 0-10% survival region than volume analysis and count analysis (e.g., actinomycin D, 5-fluorouracil; Fig. 6). This disparity is probably due to the fact that an image analyzer at low-power magnification cannot detect viable single cells or cell groups <20  $\mu\text{m}$  in diameter, whereas colorimetric analysis detects the cumulative mass of all viable cells in culture irrespective of CFU size. Whether a given agent imposes cytotoxic activity can be determined by enumerating viable and nonviable cells in control versus drug-treated cultures in representative fields at higher magnification.

Despite shoulders in the 70-100% range and differences in slope in the 0-10% range, the three methods of culture analysis exhibited similar profiles for a given agent and IC<sub>50</sub> values which generally fell within relatively narrow ranges. With the exception of actinomycin D and mercuric chloride, where IC<sub>50</sub> values for volume and count exhibited ranges of 3.3- and 8.8-fold, respectively (IC<sub>50</sub> values for volume analysis versus colorimetry, 1.6- and 1.5-fold, respectively), IC<sub>50</sub> values for eight other agents range by factors of 1.1- to 2.2-fold: actinomycin D, 0.61-2.1 ng/ml; carmustine, 4.4-5.3  $\mu\text{g/ml}$ ; bleomycin sulfate, 0.45-0.98  $\mu\text{g/ml}$ ; cisplatin, 0.31-0.48  $\mu\text{g/ml}$ ; chlorambucil, 2.7-3.2  $\mu\text{g/ml}$ ; doxorubicin hydrochloride, 16-22 ng/ml; 5-fluorouracil, 0.12-0.23  $\mu\text{g/ml}$ ; mitomycin C, 6.4-12 ng/ml; vinblastine sulfate, 1.4-1.5 ng/ml; and mercuric chloride, 38-340 ng/ml.

Differences in detection range associated with the respective

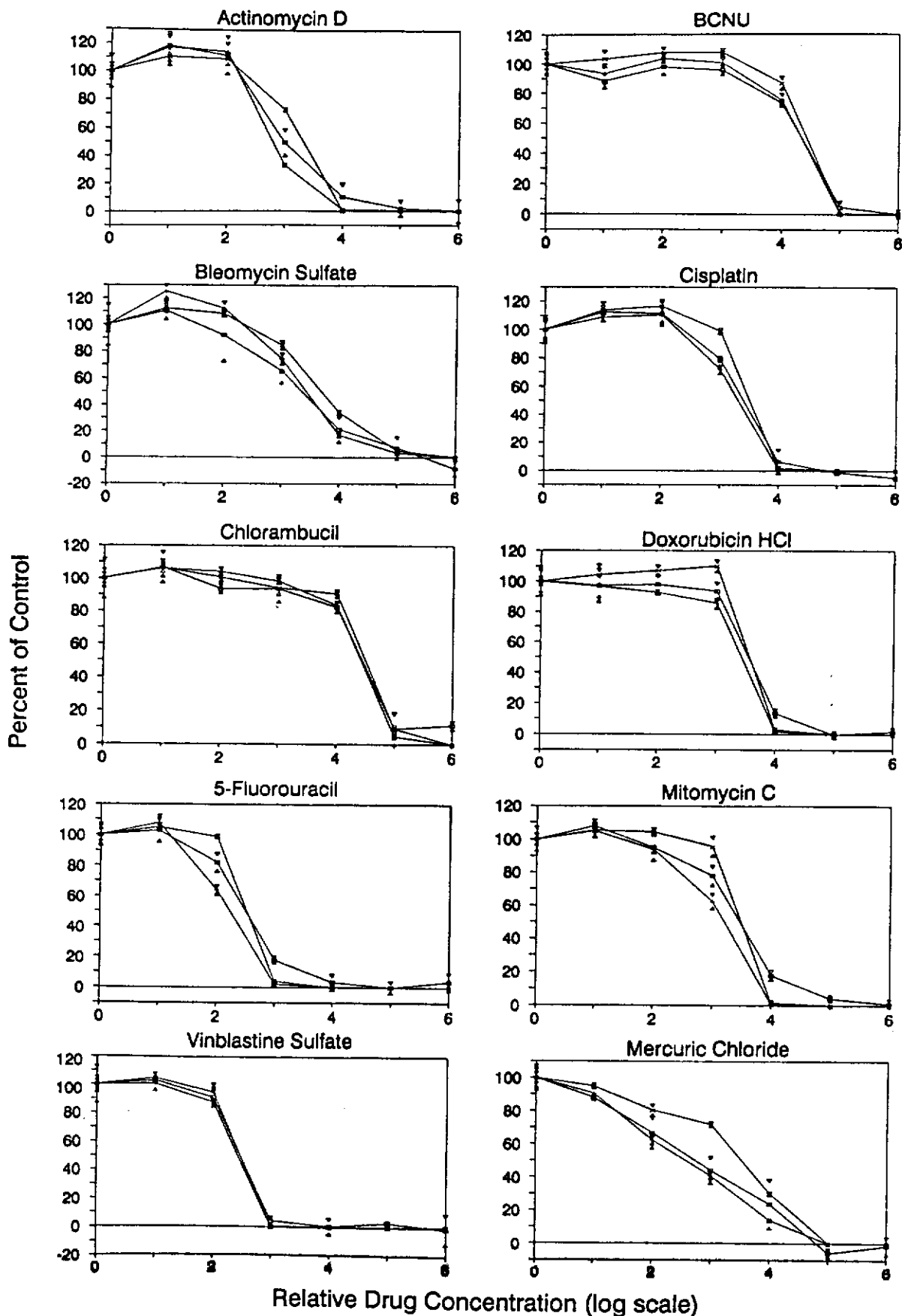


Fig. 6. Drug sensitivity profiles of a human colon adenocarcinoma cell line (HT-29) measured by CFU volume analysis (+), colony count analysis (x), and colorimetry (□). "Continuous" drug exposure to a range of six concentrations (see description in Table 1). Data represent the mean  $\pm$  SD ( $n = 6$  for vehicle control-treated cultures and  $n = 3$  for cultures treated with each drug concentration).

Table 3. Detection ranges for colorimetric and morphometric analyses of selected human tumor cell lines in soft agar culture<sup>a</sup>

Cell line	Experiment set	MTT formazan colorimetry <sup>b</sup>			Colony count <sup>c</sup>			Cumulative volume analysis <sup>d</sup>		
		$\mu\text{g}$ formazan/control culture	Control culture: background ratio	$\text{Log}_{10}$ (C/B)	Colony count/control culture	Control culture: detection limit ratio	$\text{Log}_{10}$ (C/DL)	CFU volume/control culture ( $\times 10^6 \mu\text{m}^3$ )	Control culture: detection limit ratio	$\text{Log}_{10}$ (C/DL)
A-204	1	8.88	22.09	1.344	672	2,018	3.305	255.8	56,720	4.754
	2	11.02	27.41	1.438	912	2,739	3.429	344.4	76,360	4.883
A-549	1	12.85	31.97	1.505	1,031	3,096	3.491	393.6	87,270	4.941
	2	19.45	48.38	1.685	1,185	3,559	3.551	522.8	115,900	5.064
HT-29	1	42.53	105.8	2.025	1,987	5,967	3.776	1,664	369,000	5.567
	2	41.62	103.5	2.015	2,384	7,159	3.855	1,965	435,700	5.639
HL-60	1	47.63	118.5	2.074	1,699	5,102	3.708	3,681	816,200	5.912
	2	40.31	100.3	2.001	1,174	3,526	3.547	4,228	937,500	5.972
K-562	1	30.19	75.10	1.876	2,016	6,054	3.782	3,771	836,100	5.922
	2	33.33	82.91	1.919	2,013	6,045	3.781	3,776	837,300	5.923
NCI-H460	1	57.38	142.7	2.155	1,067	3,204	3.506	3,271	725,300	5.861
	2	68.01	169.2	2.228	824	2,497	3.397	3,744	830,200	5.919

<sup>a</sup> Table values are the means of 3–6 dishes/cell line on each of two separate occasions.

<sup>b</sup> Formazan levels represent the total amount generated per control culture. Table values are the means of 3–6 dishes/cell line. The background level of MTT formazan detected in soft agar culture blanks is  $0.402 \pm 0.062$  (SD)  $\mu\text{g}/\text{dish}$ .

<sup>c</sup> Colony counts (CFU diameters  $\geq 60 \mu\text{m}$ ) were determined for the evaluable region of each culture dish (mean of 3–6 dishes/cell line) as described in "Materials and Methods." The colony count detection limit is 1 colony/3 culture dishes (0.333 colony/dish).

<sup>d</sup> Cumulative volumes of CFU (diameters  $\geq 20.4 \mu\text{m}$ ) were determined for the evaluable region of each culture dish (mean of 3–6 dishes/cell line). The volume analysis detection limit is  $0.00451 \times 10^6 \mu\text{m}^3/\text{dish}$  (equivalent to a single  $20.4\text{-}\mu\text{m}$ -diameter CFU, 14 contiguous picture points).

analysis methodologies are summarized in Table 3. Seven days following inoculation of 5,000 or 10,000 cells/dish MTT formazan production ranged from 8.88 to 57.4  $\mu\text{g}/2\text{-ml}$  culture, resulting in detection ranges for the colorimetric end point of 1.34 to 2.23 log units. Image analysis of these same growth control cultures resulted in colony counts ( $>60 \mu\text{m}$  diameter) ranging from 672 to 2384 and cumulative CFU volumes ranging from 256 to  $4228 \times 10^6 \mu\text{m}^3$  (51% of culture region evaluated). Thus, detection ranges (observed for the same experiments) were 3.31–3.86 log units for colony count and 4.75–5.97 log units for cumulative CFU volume analysis.

It is important to acknowledge the potential impact of the "static" cell population in drug sensitivity measurements. Not all CFU achieve "colony" status in a 7-day soft agar colony formation assay. While mean colony counts ( $\geq 60 \mu\text{m}$  in diameter CFU) range from 672 to 2384 for the six cell lines (as shown in Table 2), mean CFU counts ( $\geq 20.4 \mu\text{m}$  in diameter) were as follows: A-204, 2263, 2748; A-549, 1930, 2270; HT-29, 3014, 2921; HL-60, 1806, 1206; K-562, 2156, 2162; and NCI-H460, 1216, 1000. One can define the "proliferating" cell fraction of a given culture to be equal to the  $\geq 20\text{-}\mu\text{m}$  CFU count/5,100 (the number of cells within the evaluable region of cultures inoculated with 10,000 cells/dish) and the "nonproliferating" or static cell fraction to be  $1 - [\text{CFU}(\geq 20 \mu\text{m})/5100]$ . From the mean cell volume of each cell line (based upon area measurements and volume calculations for  $>100$  cells/cell line) and the cumulative CFU volume, one can approximate the number of cells in the proliferating fraction, their doubling time, and the number of cells in the static cell fraction. By calculation, the static cell populations (percentage of total, day 7) for cell lines used in these experiments are on the order of 6% for A-204, 5% for A-549, 0.6% for HT-29, 1.2% for HL-60, 1.5% for K-562, and 1.7% for NCI-H460.

These calculations suggest that the nonproliferating cell population represents a significant fraction of the total cell population in slower growing cell lines (e.g., A-204 and A-549). However, other observations would indicate that they contribute less in terms of colorimetric signal. For each cell line used in this study, the majority of nonproliferating cells do not survive 7 days of incubation. This phenomenon has been investigated extensively on a previous occasion (e.g., Ref. 19) and as part of this study. Microscopic inspection of replicate cultures treated with MTT or acridine orange/ethidium bromide dem-

onstrate that on day 7 the majority of (what would appear to be) single cells lack intact nuclei and only a fraction (at most 30%) of these cells exhibit formazan deposition. It is important to acknowledge the fact that cell lines with slower growth rate or lower cloning efficiencies in soft agar culture would have a more substantial contribution to the colorimetric end point *versus* morphometric end point from the static cell population. Nevertheless, for any cell line it is possible to estimate the number and mass of viable, nonproliferating cells/culture through the combined use of vital stains and microscopic evaluation as described above.

## DISCUSSION

The objective of the current study was to assess potential means to expedite experimental drug evaluations subjected to the soft agar colony formation assay. Such an assay may represent an important adjunct to a first stage *in vitro* drug screen by providing a means to determine whether new agents exhibit differential activity in an alternate culture format and, if so, what fraction and/or mass of cells within a given cell line population is effectively inhibited by a given drug concentration. Results demonstrate that colorimetric analysis is well suited to assays exhibiting mild to moderate growth inhibition and that colony count and cumulative volume analysis can be reserved for subsequent, more detailed evaluations of drug activity. The protocol described in the study (a) applies to cultures stained with either INT or MTT, (b) improves optical clarity of culture preparations for microscopy and for image analysis, and permits either form of analysis to be selected and scheduled at time intervals distant from assay termination). Results indicate that when the soft agar culture format is utilized, colorimetric analysis provides a simple alternative method to measure overall growth inhibition and to identify effective drug concentrations which can reduce the need for more rigorous and time-consuming efforts of counting and/or measuring colonies with a microscope or image analyzer in preliminary drug sensitivity assays. Colorimetric analysis is anticipated to be particularly useful in defining effective concentrations of new experimental agents for a given cell line; i.e., the concentration range as well as which serial dilution factor (10, 4, 2, or  $\sqrt{2}$ ) is best suited for subsequent detailed concentration-effect testing. In addition, the facts that cultures can be stored, inspected macroscopically



and/or microscopically, and scheduled for analysis are anticipated to improve the efficiency of experimental drug evaluations in soft agar culture.

MTT incubation for 4 h or INT incubation for 16 h generally resulted in appropriate and comparable levels of colony staining (formazan deposition) within the soft agar culture system described above (*i.e.*, Fig. 4). Tetrazolium incubation coupled with P/S rinses resulted in clearer visualization of colonies by microscope, improved resolution of formazan-stained colonies from background matrix by image analysis, and a wider detection range in the colorimetric measurement of DMSO-extracted formazans. Under optimal assay conditions, MTT generally exhibited higher cellular absorbance and lower backgrounds than that of INT-stained cultures.

The mechanism(s) by which protamine sulfate inhibits tetrazolium adsorption and formazan generation is not entirely clear. Protamines, which are low molecular weight cationic proteins containing a high proportion of arginine residues (32, 33), have been used in a variety of applications, including: heparin antagonism; insulin complexation (34); culture surface attachment matrix (35-37); inhibition of tumor angiogenesis (38, 39); modulation of phagocytosis, chemotaxis, and mitogenic activity (40-42); and precipitation of cytosolic receptor proteins (43, 44). Previously, Altman (45, 46) used P/S ostensibly to block adsorption of tetrazolium salts to protein in an effort to limit subsequent "nonspecific" formazan deposition within tissue sections. Our observations indicate that P/S (*a*) interferes with the reduction of a "nonsubstantive" tetrazolium salt (*i.e.*, MTT) as well as reduction of a "substantive" tetrazolium salt (*i.e.*, INT) and (*b*) permitted cultures to be stored for extended periods of time (up to 16 days was evaluated) under refrigeration without appreciable loss or gain in formazan-based morphometric or colorimetric end points. These results indicate that P/S effectively stabilizes culture-associated tetrazolium/formazan and thereby permits end point analyses to be conveniently scheduled rather than to be completed within a limited time interval following the termination of tetrazolium incubation.

Results of image analysis, formazan colorimetry, and microscopic examinations indicated that "background" formazan levels noted in cultures treated with 2.5% P/S most likely were generated during the step of tetrazolium incubation at 37°C (4 h for MTT and 16 h for INT) rather than during the steps of plate rinsing and drying. The greater magnitude in cell-mediated formazan generation for MTT over INT per unit time most likely reflects the fact that MTT has a lower redox potential than INT. The greater background formazan levels noted for INT *versus* MTT-treated cultures were probably due to a combination of (*a*) greater adsorption of INT by culture matrix and (*b*) the longer incubation time required for INT reduction. While P/S buffer "stabilized" tetrazolium/formazan in hydrated cultures for 16 days, the best assay results (lowest background levels) were obtained when colorimetry was performed within 8 h of the plate-drying step. Dried cell culture dishes (and culture blanks) maintained at room temperature over the course of 2-5 days turned darker. Such plate darkening was accentuated in cultures containing certain standard agents at elevated concentrations (*i.e.*, 5-fluorouracil  $\geq 715$   $\mu\text{g/ml}$ , mitomycin C  $\geq 4$   $\mu\text{g/ml}$ , and chlorambucil  $\geq 100$   $\mu\text{g/ml}$ ).

Even though formazan colorimetry offers a simple quantitative alternative to the more cumbersome colony count procedures in terms of ease of measurement and use of complex instrumentation, it is important to identify limitations of colorimetric analysis. First, the determination of optimal culture

parameters for a given cell line (*e.g.*, inoculation density, culture duration, cloning efficiency) requires scoring and interpretation of cell line growth characteristics by conventional microscopic examination or image analysis prior to the conduct of drug sensitivity assays. Second, colorimetric analysis has a limited range of detection due to "background" formazan generation by culture matrix. Thus, while data indicate that colorimetric analysis permits the approximation of drug sensitivity profiles in soft agar culture, it is important to emphasize the point that colorimetric analysis is not considered to be a replacement for conventional analyses. Instead, colorimetric analysis is viewed as an efficient means to identify appropriate assay parameters for subsequent colony formation assays, where, *e.g.*, pharmacological indices, morphology, and other attributes of individual colonies surviving drug exposure can be evaluated in greater detail.

Although culture medium lacking cells exhibited only a marginal capacity to reduce tetrazolium salts under conventional culture conditions, the same culture matrix had a significant capacity to reduce tetrazolium salts during the process of drying. Of the 1000  $\mu\text{g}$  applied to culture dishes initially, 150  $\mu\text{g}$  tetrazolium/formazan remained per culture dish following fluid aspiration and two normal saline rinses (assuming no adsorption by the components of soft agar matrix). Approximately 15  $\mu\text{g}$  INT and 130  $\mu\text{g}$  MTT (by measurement) were recovered as formazans following the process of drying from cultures not rinsed with P/S buffer. In contrast, rinsing cultures twice with 2.5% P/S buffer reduced formazan production appreciably: "background" levels of 1.6  $\mu\text{g}$  INT formazan or 0.402  $\mu\text{g}$  MTT formazan represented small fractions of the tetrazolium salt present in dishes following two rinses as well as the amounts of cell-generated formazan (9-68  $\mu\text{g}$  MTT formazan and 8-132  $\mu\text{g}$  INT formazan). As shown in Table 1 detection ranges for six selected cell lines were from 1.3 to 2.2 log units (equivalent to control:background ratios of 22.1 to 169). Alternatively, a background level of 0.402  $\mu\text{g}$  represented 0.24 to 4.5% of the respective control culture formazan level for these six selected cell lines. Such collective data indicated that colorimetric analysis can detect growth inhibition up to, and for many cell lines exceeding, 95%. By contrast, colony count and volume analysis of the same culture dishes exhibited detection ranges of 3.3 to 3.8 and 4.7 to 5.9 log units, respectively, which corresponded to 0.014 to 0.049 and 0.00033 to 0.0055% survivals, respectively.

The soft agar colony formation assay represents an important technique for experimental drug testing. Soft agar cultures provide a culture format alternate to that of fluid cultures which are often used in *in vitro* screening assays. Macroscopic/microscopic inspection permits the observer (*a*) to determine whether drug activity is mild, moderate, or prominent and (*b*) whether the agent affects all cells or subsets in a heterogeneous cell line population. In addition, since soft agar cultures are amenable to colorimetric as well as morphometric modes of analysis, the observer can determine which end point is most appropriate for a given application. Moreover, replicate culture dishes are amenable to evaluation by yet other methodologies. For example, colonies cultivated under specific conditions are amenable to histological/cytological stains and electron microscopy (47, 48). It is noteworthy that soft agar prevents loss of cells from culture during washing steps which can occur in fluid culture assay systems due to spontaneous release and/or drug-induced detachment from plastic surfaces. Since viable cell detachment and perturbed growth have been observed in fluid cultures containing insoluble synthetic agents as well as crude extracts

of natural products, the suitability of agar-based colorimetric assay systems (including that described above) is currently under investigation for subsequent evaluation of such agents.

In summary, observations and data described above indicate that colorimetric analysis is capable of measuring growth and approximating effective drug concentration ranges (5–100% survival) in soft agar culture. Such colorimetric analysis is anticipated to provide indices useful for comparisons with other growth formats and other colorimetric assays. In addition, such colorimetric analysis can facilitate selection of appropriate drug concentration ranges and serial dilution factors to be used in subsequent colony formation assays, where pharmacological activity can be evaluated in greater detail.

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