Cancer Chemother Pharmacol (1981) 6:205–210

Cancer Chemotherapy and Pharmacology

Articels

Methodologic Problems in Clonogenic Assays of Spontaneous Human Tumors

F. Roy MacKintosh, Thomas L. Evans, and Branimir Ivan Sikic

Department of Medicine, Oncology Division, Stanford University School of Medicine, Stanford, CA 94305, USA

Summary. *Colony formation in soft agar was used to investigate growth properties and drug sensitivity in 102 tumor specimens from 91 patients. Sufficient colony growth for sensitivity testing with various drugs was obtained in 36 of 67 specimens (54%) with adequate cell yield and pathologically documented malignancy. Room temperature (20–24° C) is superior to both 4° C and 37° C for 12–36 h storage and transport of malignant effusions. By contrast, fine mincing in sterile saline or balanced salt solution, and refrigerated storage (4° C) appear optimal in experiments with three solid tumors. The use of buffered NH_4Cl to lyse red blood cells markedly reduced plating efficiencies, and also reduced the percentage of tumors in which drug sensitivities could be tested from 64% to 38%. Several combinations of potential growth factors and culture media have been tested. Insulin enhanced plating efficiency (PE) in all six adenocarcinomas tested. Drug sensitivity of tumors was not affected by varying plating efficiency up to five-fold in two tumors. In eleven cases tumor cells were exposed to combinations of two or more drugs, and results assessed for evidence of drug interactions. In almost all cases, these two-drug combinations produced additive cell killing rather than either antagonistic or greater-than-additive effects.*

Introduction

This report describes our initial experience in studying the human tumor clonogenic assay. We have received 102 specimens from 91 patients. Our efforts have been directed toward methodologic problems and questions relating to validation of the clonogenic assay. These include optimal methods of cell disaggregation, effects of red blood cell lysis, storage conditions prior to assay, media, and effects of growth factors. In addition, because of the clinical importance of combination chemotherapy, we have begun to approach the problem of extending the assay to the evaluation of combinations of chemotherapeutic agents.

In addition to clinical applications, the clonogenic assay may be very useful as a marker of drug sensitivity in studies of the cellular pharmacology of spontaneous human tumors, particularly mechanisms of resistance to anticancer agents and interactions of these drugs. Along with spontaneous human tumors, we have used the murine solid tumors, B16 mouse melanoma and Lewis lung carcinoma, and human ovarian carcinoma cell lines as model systems in these investigations.

Reprint requests should be addressed to B. I. Sikic, Stanford University Medical Center, Department of Medicine (Oncology), Room MO11, Stanford, CA 94305, USA

Materials and Methods

Chemotherapeutic agents were obtained either from standard commercial sources (5-FU, DTIC, doxorubicin) or from the Investigational Drugs Branch, National Cancer Institute, Bethesda, MD (courtesy of Ruth Davis, Toxicology Branch, and Dr. John Douros, Developmental Therapeutics Program). All culture media and heat-inactivated sera were obtained from Gibco. Cefoxitin and streptomycin (100 μg/ml) were routinely added to culture media and balanced salt solutions.

Solid tumor specimens were finely minced with a single-edge razor blade mounted in a modified portable jig saw (Black and Decker Model 7580). The minced tumor specimen and effusions which contained clusters of tumor cells were then treated for 30 min at 37° C with an enzyme cocktail (J. Martin Brown, poersonal communication) consisting of pronase 0.5 mg/ml, collagenase type I, 0.2 mg/ml, and DNase I, 0.2 mg/ml, all from Sigma, in Hank's Balanced Salt Solution (HBSS) (without Ca^{2+} and Mg^{2+}), and filtered through sterile gauze and a 200 mesh stainless steel screen. Cell counts were obtained by hemocytometer and viability by trypan blue exclusion. Cells were exposed to chemotherapeutic agents in concentrations ranging from 0.001 to 10 μg/ml, at 37° C in HBSS/10% newborn calf serum (NBCS) for

0344-5704/81/0006/0205/$ 01.20

1 h, washed twice with HBSS/10% NBCS, and plated at 5×10^5 cells/ml. Cells were cultured in multiwell plates with 16.6-mm diameter wells. We presently use an underlayer of 0.5 ml McCoy's: Waymouth's media (1 : 1) with 15% NBCS in 0.5% noble agar, and an overlayer of 0.5 ml cell suspension plated in McCoy's : Waymouth's (1 : 1) media/15% NBCS + 5 µg/ml porcine insulin with 0.3% agar.

Colony counts at 14–28 days were performed with an inverted microscope at 13× or 40× magnification. Aggregates of 30 cells or more were considered colonies. Sensitivity to drugs was assessed according to the methods of Salmon et al. [6] and the most recent analyses [2] of data from the Tucson group.

Lysis of red blood cells (rbc) was performed when an excess of rbc was present (rbc:mononuclear cell ratio > 100 : 1), according to a modification of the procedure of Roos and Loos [4]. The packed single cell pellet was resuspended in three volumes of cold (4° C) buffered NH_4Cl, mixed by inversion for 3 min, and allowed to stand at room temperature for 7 min. The cells were then centrifuged for 8 min at 450 *g* at 40° C and the supernatant containing rbc lysate and cell membranes discarded.

Histologic confirmation of the malignant nature of colonies was performed by preparing fixed and stained specimens from soft agar layers [5]. In addition, slides of single cell suspensions are prepared routinely for staining with hematoxylin and eosin, Wright-Giemsa, and Papanicolaou stains. These are examined with a cytopathologist and differential counting of malignant and normal cells is performed.

Excess tumor cells have been stored in liquid nitrogen, at a concentration of 10^7 cells/ml Waymouth's medium plus 15% NBCS plus 10% DMSO. The cells were frozen slowly by being placed in an insulated container in a −90° C freezer prior to transfer to liquid N_2. Thawing was performed rapidly by immersion in a 37° C water bath.

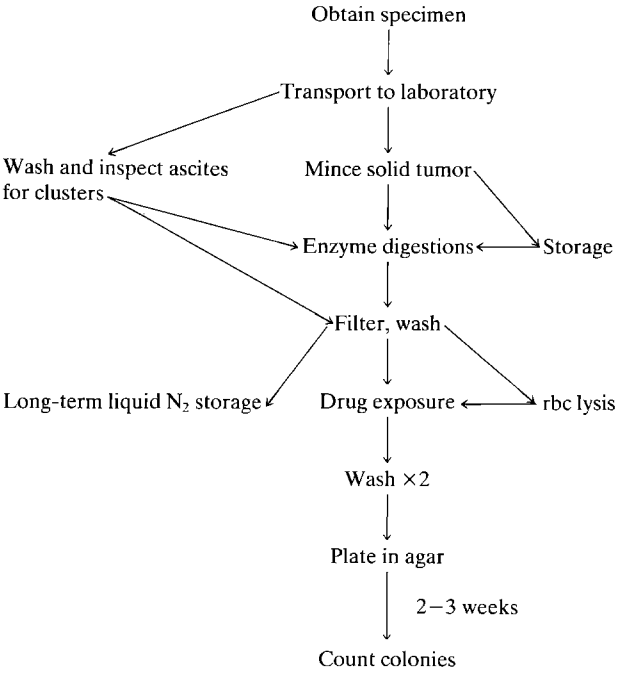

Fig. 1. Steps of a clonogenic assay for spontaneous human tumors

Results

We have concentrated on ovarian adenocarcinomas and have processed 60 specimens from 51 patients. Plating efficiency adequate for drug sensitivity testing ($\geq 0.01\%$) was achieved in 26 of the 41 specimens (63%), with adequate cell yields and pathologic documentation of ovarian malignancy. From 20 of these ovarian cases, 96 drug dose-colony survival curves were obtained. Sensitivity to the drug was indicated in 28 tests, and resistance in 68. Retrospective sensitivity correlation (based on clinical failure of chemotherapy prior to performance of clonogenic assay) showed that resistance both in vivo and in vitro was present in 22 of 24 (92%) drug tests in nine patients. In several cases in vitro sensitivity was detected for drugs not usually considered clinically useful for ovarian carcinomas, including vinblastine in 8/15 cases and bleomycin in 3/13 cases. In three prospective trials of vinblastine therapy for patients judged sensitive to vinblastine by the assay there has been one minimal clinical response of short duration.

In nonovarian malignancies, adequate colony growth has been obtained in 10/26 specimens from a variety of tumor types. In one patient, a complete clinical response has been obtained to mitomycin and actinomycin combination therapy for a sarcoma, after determination of in vitro sensitivity to these agents.

Exploration of the methodologic variables associated with the steps of the assay outlined in Fig. 1 has included both spontaneous human tumors and the model systems indicated.

Mechanical vs Enzymatic Cell Disaggregation

Our standard method of disaggregation of solid tumors involves fine mincing and enzymatic digestion with a mixture of pronase, collagenase, and DNAse, based on work within our laboratory with Lewis lung carcinoma. The enzyme mixture yielded 93% viability by trypan blue exclusion, as against 43% for 0.05% trypsin, and 10% for the purely mechanical method of Salmon and co-workers [6]. Similar superiority of enzymatic disaggregation for viable cell yield from solid tumors has been noted by others [J. M. Brown and M. Rosenblum, personal communication; 3]. Studies are in progress to compare the effects of these disaggregation methods on drug sensitivities of tumor cells.

Table 1. Effects of storage conditions on cell viability and yield from three solid tumor specimens stored for 24 h

	Conditions	Percent viability	Viable cell yield/gram	Percent PE	Colonies/gram of tumor
1. Sarcoma, SA-1	Minced, 4° C, 0.9% NaCl	93	5.2×10^6	0.58	3.0×10^4
	Solid, 4° C, 0.9% NaCl	59	3.9×10^6	0.66	2.6×10^4
	Minced, 22° C, 0.9% NaCl	43	2.4×10^6	0.58	1.4×10^4
	Solid, 22° C, 0.9% NaCl	11	1.8×10^6	0.65	1.2×10^4
2. Ovarian carcinoma, OV-35	Minced, 4° C, 0.9% NaCl	88	11.0×10^6	0.017	1.9×10^3
	Minced, 4° C, Waymonth's/15% NBCS	94	9.0×10^6	0.009	0.8×10^3
	Minced, 22° C, Waymonth's/15% NBCS	79	9.7×10^6	0.016	1.5×10^3
	Minced, 22° C, 0.9% NaCl	65	9.9×10^6	0.004	0.4×10^3
3. Ovarian carcinoma, OV-40	Minced, 4° C, medium	69	1.5×10^6	0.010	1.5×10^2
	Minced, 4° C, HBSS	48	2.1×10^6	0.012	2.5×10^2
	Minced, 22° C, HBSS	44	9.6×10^5	0.003	0.3×10^2
	No storage	53	2.6×10^6	0.029	7.5×10^2

Table 2. Effects of insulin on clonogenic PE of human adenocarcinomas in soft agar

Tumor	Control PE (%) without insulin	PE (%) with insulin, 5μg/ml	Insulin effects, % of control PE
Ovarian	0.004	0.010	250%
Ovarian	0.22	0.64	291%
Ovarian	0.12	0.28	233%
Ovarian	0.14	0.34	243%
Colon	0.034	0.095	279%
Endometrial	0.0005	0.0016	319%

Short-term Storage and Transport Conditions

We have recommended that samples of malignant effusions be transported at room temperature (20–24° C), with the addition of 10 units heparin/ml or one ampule (3 g) of EDTA (Endrate®) per liter. Under such conditions of 12- to 36-h storage and transport, cell viability has usually been over 90%, with viability and PE superior to those obtained with storage at either 4° C or 37° C. Similar results have been reported for a large number of malignant effusions [8].

Viability of cells within solid tumors appears to be better maintained with refrigerated storage than at room temperature or 37° C. Experiments with three large solid tumors are illustrated in Table 1. In a sarcoma, fine mincing increased viability on storage compared with no mincing, and 4° C was superior to 22° C in percent viability and viable cell yield per gram of tumor. Refrigeration was also superior to storage at room temperature for two ovarian solid tumors (Table 1). Storage in sterile saline or balanced salt solution was at least equivalent to storage in complete medium for these tumors.

Removal of Red Blood Cells

We have treated 20 single cell suspensions of ovarian carcinomas with buffered NH_4Cl to lyse excess rbc. Subsequent clonal growth with PE of at least 0.01% was achieved in 6/16 of these specimens (38%), compared with successful growth in 18/28 (64%) of ovarian carcinomas not treated with NH_4Cl ($P < 0.05$). Direct comparison of the effect of NH_4Cl lysis on PE in four ovarian tumors has revealed markedly greater PEs without the use of NH_4Cl in three out of four cases (0.57% vs 0.04%, 1.6 vs < 0.001; 0.04 vs 0.03, and 0.07 vs 0.01).

Effects of Insulin

Table 2 summarizes our data concerning the effect of insulin on PE. Insulin, 5 μg/ml, increased PE in each of six tumors tested, to a mean of 270% of control. Higher concentrations of insulin did not enhance this effect in two cases.

Combination Chemotherapy in vitro

Simultaneous exposure to two or more drugs has been tested in eleven tumors. In most cases, the colony survival after two-drug treatment has been similar to the calculated product of the survivals after exposure to each drug separately (Fig. 2). 'Synergy', or significantly greater than expected cell killing, was observed in one tumor for the combination of bleomycin and vinblastine, with an expected survival fraction of 0.49 and an observed survival of 0.05 on two-drug exposure (Fig. 2). Such an interaction did not occur in several other tumors with this same pair

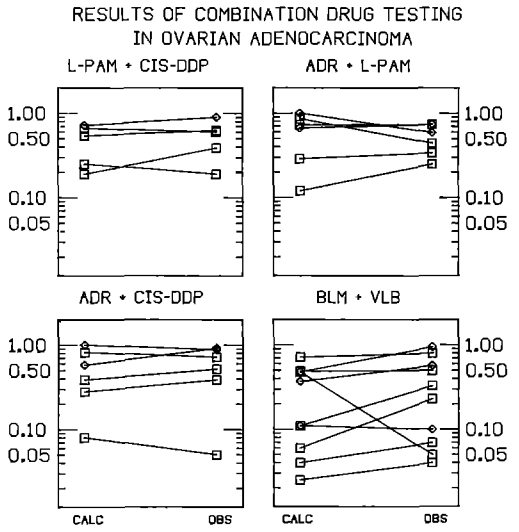

Fig. 2. Fractional colony survival after exposure to combinations (CBS) is compared with that calculated by multiplying the separate fractional colony survivals for the individual drug exposures indicated. Dosage was 1 h at 0.02 ng/ml (◇) or 0.1 ng/ml (□)

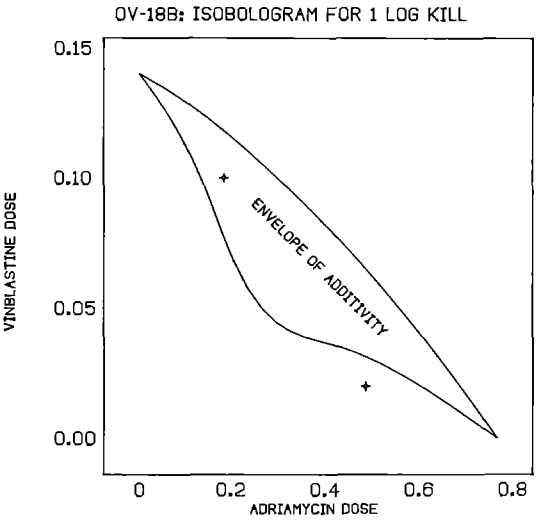

Fig. 3. An isobologram plot for the effect and interaction of adriamycin and vinblastine in a primary human tumor specimen. Drug doses are in micrograms per milliliter for 1 h

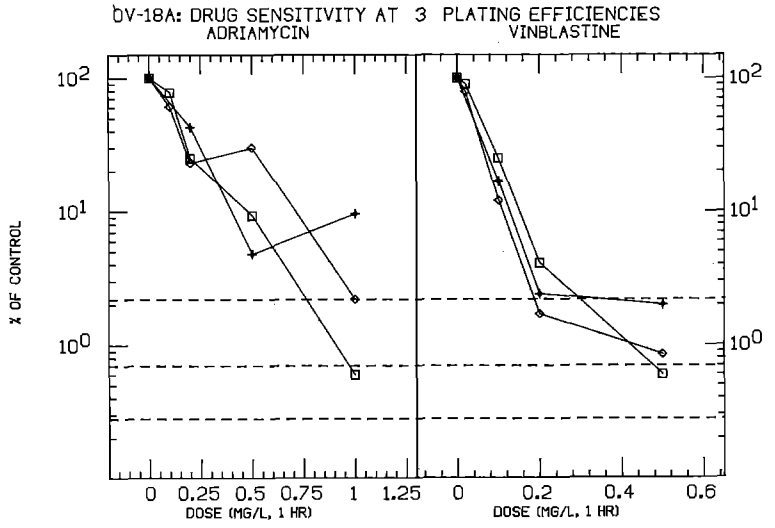

Fig. 4. Drug dose-colony survival data are displayed for an ovarian tumor specimen plated under three different growth conditions. Each *point* represents the average of counts in two wells. The *horizontal dashed lines* represent the lower limits of survival detectable (one colony per two wells) at each of the three PEs. (◇) P.E. – 0.07%; (□) P.E. – 0.029%; (⊕) P.E. – 0.019%

of drugs, however. Similarly, we observed simple additive effects for the combinations of melphalan with adriamycin, melphalan with cisplatin, and adriamycin with cisplatin. Significant antagonistic effects between two drugs were not observed in these experiments.

In one human tumor the combination of adriamycin and vinblastine was tested at several concentrations. When survival curves were analyzed by the isobologram technique described by Steele and Peckham [7] it again appeared that the results of two-drug treatment were additive (Fig. 3).

Plating Efficiency and Drug Sensitivity

In two ovarian carcinomas, colony survival curves have been obtained with cells from fresh human tumors at more than one control PE, by varying storage conditions or growth medium. A five-fold change in plating efficiency of the controls for OV-9 did not alter the conclusion of sensitivity to adriamycin or resistance to cisplatin. With OV-18, plating under three different growth conditions altered PE from 0.04% to 0.08% without significant change in

the sensitivity profile to adriamycin, vinblastine (Fig. 4) or cisplatin.

Long-term Storage in Liquid Nitrogen

We have attempted serial studies of clonogenicity and drug sensitivity in cells after storage in liquid nitrogen. Viability after thawing such cells has ranged from 10%–70%, with a marked decrease in PE.

Discussion

It is clear that clonogenic assay of human tumors is an evolving technique and will require continued refinement and development of methodology. Individualization of media and growth factors for specific tumor types is being reported and will hopefully increase both the plating efficiency of specimens and the percentage of specimens which can be successfully assayed [1].

The evidence from other laboratories, as well as our own, indicates that combined mechanical and enzymatic disaggregation is superior to mechanical disaggregation of solid tumors. Whether drug sensitivities are different for enzyme treatment than for purely mechanical dissaggregation is not yet known.

Our current recommendations for transport and storage of specimens are as follows: Ascites and other malignant effusions should be shipped at room temperature, with the addition of 10 units preservative-free heparin/ml or one ampule (3 g) of EDTA (Endrate®)/liter if the effusion is visibly bloody. Solid tumors should be minced finely and transported under refrigeration, suspended in 0.9% sterile NaCl. The necessity for removal of rbc prior to drug exposure and plating is open to question, especially in light of the marked decrease in PE observed after exposure of tumor cells to buffered NH_4Cl as an rbc-lysing reagent. We are currently testing the use of osmotic shock with sterile distilled water as a method for selective removal of rbc.

A major criticism of human tumor clonogenic assays has been the low PEs, leading to the argument that those few cells which are able to give rise to colonies under stringent in vitro conditions may not be representative of the population of tumor stem cells which are of clinical importance. While our experience has been similar to others in that PEs even as high as 1% are unusual, we are encouraged by our experience in two human tumors and in experiments with model systems, in which variation in PE did not alter the shape of the drug dose-colony curves or the conclusions about drug sensitivity.

The assessment of drug sensitivity in combination chemotherapy will require considerably more experience. In idealized cases, interactions in which the colony survival from exposure to a pair of drugs is substantially less than the product of the separate fractional survivals represent synergy, or greater-than-additive effects. The assumption in such single-dose comparisons is that cell survival is a logarithmic function of drug dose [7]. In most cases in the clonogenic assay we found the product of the separate survivals to be similar to the actual survival on exposure to the drug pair. This would represent simple additivity.

A more definitive method of analyzing drug interactions is by isobolograms, as proposed by Steele and Peckham [7]. However, this requires detailed individual drug survival curves and data for numerous concentrations of the drug pairs in question. Therefore, it will seldom be possible to use this approach with fresh human tumor specimens. It can prove useful when previous drug sensitivities are known and frozen or sequential specimens are available, or when it is known in advance that only two drugs are being actively considered for use in a particular clinical setting. However, substantially more clinical correlation will be needed to assess the usefulness of either method of testing drug pairs. We are currently studying specimens from ovarian carcinoma patients with single- and multiple-drug exposures in vitro, and will be accumulating prospective data on the correlation of in vitro drug sensitivity and possible drug interactions with treatment outcomes after combination chemotherapy.

We are screening alternative measures of drug sensitivity along with the clonogenic assay in selected cases and in experiments with model systems. However, short-term biochemical measures of drug response are influenced by the presence of non-malignant cells in the primary specimen, and the degree to which this will affect results remains problematic.

Acknowledgements. This work was supported by National Institutes of Health grants 1 R01 CA 27478, CA-5 P30, CA 19408-05, and BRSG RR-5353, and by American Cancer Society grant IN-32U.

References

1. Laboisse CL, Augeron C, Potet F (1981) Growth and differentiation of human gastrointestinal adenocarcinoma stem cells in soft agarose. Cancer Res 41:310–315
2. Moon TE (1980) Quantitative and statistical analysis of the association between in vitro and in vivo studies. In: Salmon SE

(ed) Cloning of human tumor stem cells. Alan Liss, New York
3. Pavelic ZP, Slocum HK, Rustum YM, Creaven PJ, Karakousis C, Takita H (1980) Colony growth in soft agar of human melanoma, sarcoma, and lung carcinoma cells disaggregated by mechanical and enzymatic methods. Cancer Res 40: 2160–2164
4. Roos D, Loos JA (1970) Changes in the carbohydrate metabolism of mitogenically stimulated human peripheral lymphocytes. Biochim Biophys Acta 222: 505–582
5. Salmon SE, Buick RN (1979) Preparation of permanent slides of intact soft-agar colony cultures of hematopoietic and tumor stem cells. Cancer Res 39: 1133–1136
6. Salmon SE, Hamburger AW, Soehnlen B, Durie BGM, Alberts DS, Moon TE (1978) Quantitation of differential sensitivity of human-tumor stem cells to anticancer drugs. N Engl J Med 298: 1321–1327
7. Steel GG, Peckham MJ (1979) Exploitable mechanisms in combined radiotherapy-chemotherapy: the concept of additivity. Int J Radiat Oncol Biol Phys 5: 85–91
8. Von Hoff DD, Cowan J, Harris G, Reisdorf G (1981) Human tumor cloning: feasibility and clinical correlations. Cancer Chemother Pharmacol 6: 265–271

Received November 1980