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Chemosensitivity of Human Neoplasms with in vitro Clone Formation

Experience at the University of Southern California – Los Angeles County Medical Center

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Summary. *We analyze experience with 600 specimens for in vitro chemosensitivity assessment of human neoplasms utilizing a soft agar colony-forming technique. Good test reproducibility is demonstrated. Disaggregation with collagenase enhances yield and does not alter chemosensitivity profiles. Therapeutic exposure to chemotherapy prior to biopsy reduces in vitro sensitivity to the specific agents used in vitro. The cyclophosphamide derivatives 4-hydroperoxycyclophosphamide (4-HC) and phosphoramide mustard are active in vitro, and produce comparable rank order sensitivities among tested tumors. There is marked reduction of in vitro 4-HC sensitivity in patients with prior therapeutic cyclophosphamide exposure, supporting the use of this derivative in test systems. Rank order of test results among specimens is compared at 0.1 μg and 10 μg drug/ml. Substantial differences in rank order at these two dose levels are demonstrated, indicating that the in vitro test dose selected is an important variable.*

Introduction

Chemosensitivity assessment of human malignancies by means of colony formation in soft agar [2, 4, 5] is a developing technique, which offers the promise of significant predictive power for guiding the treatment of cancer [1, 6]. Major areas for development include improvement in test efficiency, optimization of the in vitro end-point, and refinement of correlative data. We report here our initial experience at the University of Southern California and within the Los Angeles basin on a spectrum of human malignancies. Methodologic studies have included exploration of the use of enzymatic disaggregation, estimates of test reproducibility, and the in vitro use of 4-hydroperoxycyclophosphamide (4-HC) and phosphoramide mustard (PM) as active derivatives of cyclophosphamide [3, 7, 8]. The impact of specific prior chemotherapy upon in vitro test score is documented. In vitro test results are compared at 0.1 μg and 10 μg drug/ml to assess the influence of the in vitro test dose.

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Materials and Methods

Specimens were contributed by physicians at the University of Southern California-Los Angeles County Medical Center and affiliated hospitals, and physicians within the Los Angeles basin. Received were primary tumors, lymph nodes, solid metastases, heparinized malignant effusions, and lavage specimens from bladder. The culture medium was McCoy's 5A (K-C Biologicals, Lenexa, Kansas) with 50 units penicillin/ml, 50 μg streptomycin/ml, 20 m*M* HEPES (Sigma) and 24 m*M* $NaHCO_3$, pH 7.4, in air atmosphere, with additions as specified below.

Specimens were transported and processed at ambient temperature until the time of incubation with drug.

Upon being received, solid tumors were minced with sharp instruments, and collagenase (Worthington, type III) was added to a final concentration of 0.2 mg/ml. Depending on ease of dissociation, specimens were left for from 1 h–24 h in collagenase at ambient temperature prior to being sequentially forced with teflon pestles through 20 mesh and 200 mesh (80 μm) stainless steel screens. Cells were collected by centrifugation at 900 *g* for 2 min and resuspended in medium with 6% heat-inactivated calf serum (0.5 h at 56°) at a concentration of 10^6 nucleated cells/ml.

Drugs used were standard patient treatment formulations except for 4-HC and PM, which were gifts of Dr M. Colvin. Drugs were diluted with sterile water or dissolved in the diluent provided by the manufacturer and then diluted with sterile water to a concentration of 100 μg/ml. Then 1 *M* HCl, 1 *M* NaOH, or ethanol was added as required to dissolve HMM, procarbazine and melphalan. Leukocyte interferon (Israel Institute for Biological Research) was diluted to 3000 units/ml with sterile water. Test doses of drugs were dispensed and stored at −20° C.

Drug exposure of 1-ml aliquots of cells was at 0.1 μg/ml and 10 μg/ml for 1 h at 37° C unless otherwise stated. Following incuba-

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tion, cells were collected at 900 *g* and plated in duplicate. Control tubes were incubated without drug. Each 24-mm well received 0.5 ml each of an agar underlayer and a cell containing soft agar overlayer. Each milliliter of agar underlayer contained 6 mg TSB, 180 μg sodium pyruvate, 30 μg serine, 460 μg glutamine, 40 μg ascorbic acid, 1.6 units insulin, 80 μg asparagine, 30 μg DEAE dextran (Pharmacia), 12% horse serum, 8% heat-inactivated calf serum, and 5 mg (0.5%) agar (Difco, Bacto-Agar).

The cell overlayer (0.5 ml per well) contained in each milliliter of medium: 0.45×10^6 nucleated cells, 220 μg sodium pyruvate, 40 μg serine, 58 μg glutamine, 50 μg ascorbic acid, 2 units insulin, 100 μg asparagine, 38 μg DEAE dextran, 6.25×10^{-2} μ*M* 2-mercaptoethanol, 15% (V/V) horse serum, 10% heat-inactivated calf serum, and 3 mg (0.3%) agar.

Cultures testing interferon and methylprednisolone had the agents added to the cell layer at 300 units/ml and 10 μg/ml, respectively. Cultures were examined and photographed by phase microscopy immediately after plating, and were usually counted at 9 days. Clones are identified as compact groups of cells no less than 50 μm in diameter, which develop during incubation. Routinely, clones from ten 200x fields were counted and data expressed as percent survival comparing drug to control plates.

To facilitate comparison of drug sensitivities for each drug concentration, percent survival scores have been transformed to a percentile distribution. The percentile is computed as the cumulative frequency for each drug concentration of a stated or lower percent survival score. The data base used is made up of the scores from all patients not treated with the specific agent.

Stained microscopic mounts (to be reported elsewhere) have been prepared of all cell suspensions and of all agar cultures to allow review of initial as well as day 9 appearance.

Results

1. Scope of Experience

Six hundred specimens were evaluated in a 12-month period. Seventy-six percent of the specimens grew successfully and allowed the testing of three or more drugs. Seven percent of specimens were not attempted because fewer than 1 million cells were

Table 1. Scope of experience: number of specimens of each tumor type reported between April 1980 and March 1981 ($n = 420$)

Carcinoma			357
Gastrointestinal		58	
Stomach	14		
Colorectal	28		
Pancreas	9		
Liver	2		
Esophagus	2		
Gallbladder	2		
Intestine	1		
Genitourinary		119	
Kidney	31		
Urothelium	33		
Prostate	6		
Ovary	42		
Uterus	7		
Respiratory		42	
Lung	41		
Adenocarcinoma	12		
Large cell	5		
Oat cell	12		
Mesothelioma	3		
Squamous	6		
Undifferentiated	3		
Bronchogenic	1		
Breast		103	
Head and Neck		5	
Mouth	1		
Pyriform sinus	1		
Thyroid	3		
Skin		6	
Squamous	4		
Basal cell	1		
Mycosis fungoides	1		
Unknown primary		24	
Adenocarcinoma	24		
Germinal neoplasms			27
Testis		26	
Embryonal	15		
Teratocarcinoma	4		
Seminoma	4		
Mixed neoplasms	3		
Ovary		1	
Dysgerminoma	1		
Sarcoma			12
Osteogenic	1		
Leiomyosarcoma	3		
Malignant fibrous histiocytoma	1		
Rhabdomyosarcoma	3		
Embryonal	2		
NOS	4		
Myeloma			6
Melanoma			10
Mixed Müllerian			1
Uterus		1	
Wilm's Tumor			1
CLL			2
Lymphoma			3
Thymoma			1

recovered. Six percent were pathologically negative for malignancy and did not grow. The remaining 11% did not grow or fewer than three drugs were tested.

Table 1 shows the number of each tumor type reported. Sensitivity information was provided for an average of 12 drugs per specimen (range 1–21). Our experience encompasses most solid neoplasms.

2. *Cloning Efficiency*

Cloning efficiency is computed as the number of clones counted per 10^2 cells plated and is presented in Table 2. There is a large coefficient of variance. Cells from primary tumor and from lymph node appear to be more efficient than those from solid metastases or effusions.

Table 2. Cloning efficiency[a]

	$\bar{X}$	SD	SE
T	0.67	1.70	0.26
N	0.70	0.98	0.21
M solid	0.34	1.09	0.18
M fluid	0.26	0.36	0.06

[a] Computed as number of clones per 10^2 cells plated. $\bar{X}$, average; SD, standard deviation; SE, standard error; T, tumor; N, lymph node; M solid, solid metastases; M fluid, effusions

3. *Collagenase*

Pretreatment of solid specimens with collagenase resulted in a highly variable but usually substantial increase in the number of liberated cells. Occasional specimens which disaggregate easily show no benefit. In other tumors, almost always for example in breast carcinoma, cell yields are augmented. We have examined Collagenase III (Worthington) for effects on viability and drug sensitivity, and have found none of significance. The experiments were performed on effusions to evaluate potential effects of enzyme on cells separately from its influence on cell recovery by tissue disaggregation. Cells were collected, suspended in medium at 5×10^5 cells/ml, and the sample divided. Half the specimen was exposed to collagenase (0.2 mg/ml for 24 h at room temperature) and the other half remained in medium with no collagenase present. The cells were then collected, exposed to drug and plated in agar.

Results of two experiments are presented in Fig. 1a and b. There are no apparent effects of collagenase pretreatment on clone viability, either in control or drug-treated dishes.

4. *Reproducibility*

Reproducibility has been assessed by determinations of chemosensitivity on serial specimens. We have obtained lavage, biopsy, and surgical specimens from

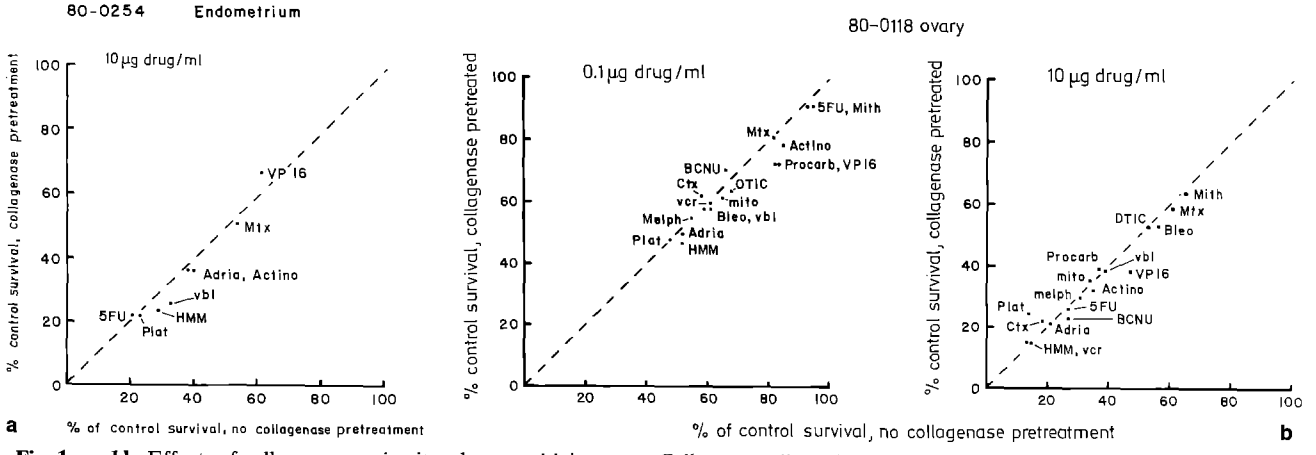

Fig. 1a and b. Effects of collagenase on in vitro drug sensitivity score. Cells were collected from ascitic fluid from patients with endometrial carcinoma (*a*) and ovarian carcinoma (*b*). Collagenase was added to a concentration of 0.2 mg/ml to half of each specimen, and the divided aliquots were incubated at ambient temperature for 24 h prior to chemosensitivity assessment. The percent control colony-forming units (CFU) after collagenase exposure is shown on the *vertical axis* and the percent control CFU without collagenase pretreatment is shown on the *horizontal axis*. Each *point* represents the observed survivals after treatment with the indicated drug. The number of CFU per 10 HPF is shown for control (no drug) plates in the presence and absence of collagenase pretreatment: (**a**) Collagenase 36, no Collagenase 35; (**b**) Collagenase 33, no Collagenase 31

Table 3. Reproducibility: Comparison of drug response among multiple specimens from three patients

Specimen source	No. of control clones[a]	Percent control survival[a]								
		Adria	Inter	Melph	Plat	VP16	5-FU	Mtx	4-HC	PM
1. Cytoscopy	116	117	45	54	74	42				
Lavage	108	102		62	78					
Urine	80	100								
Cystectomy	664	110	49	48	72	50				
2. Lavage	153	50								
Cystectomy	41	36								
3. Ascites d_1	21	69		38	45		29	45	29	62
Ascites d_2	18	81		39	53		33	33	36	72

[a] Percent control survival for each drug and number of clones in control plates for each specimen are given

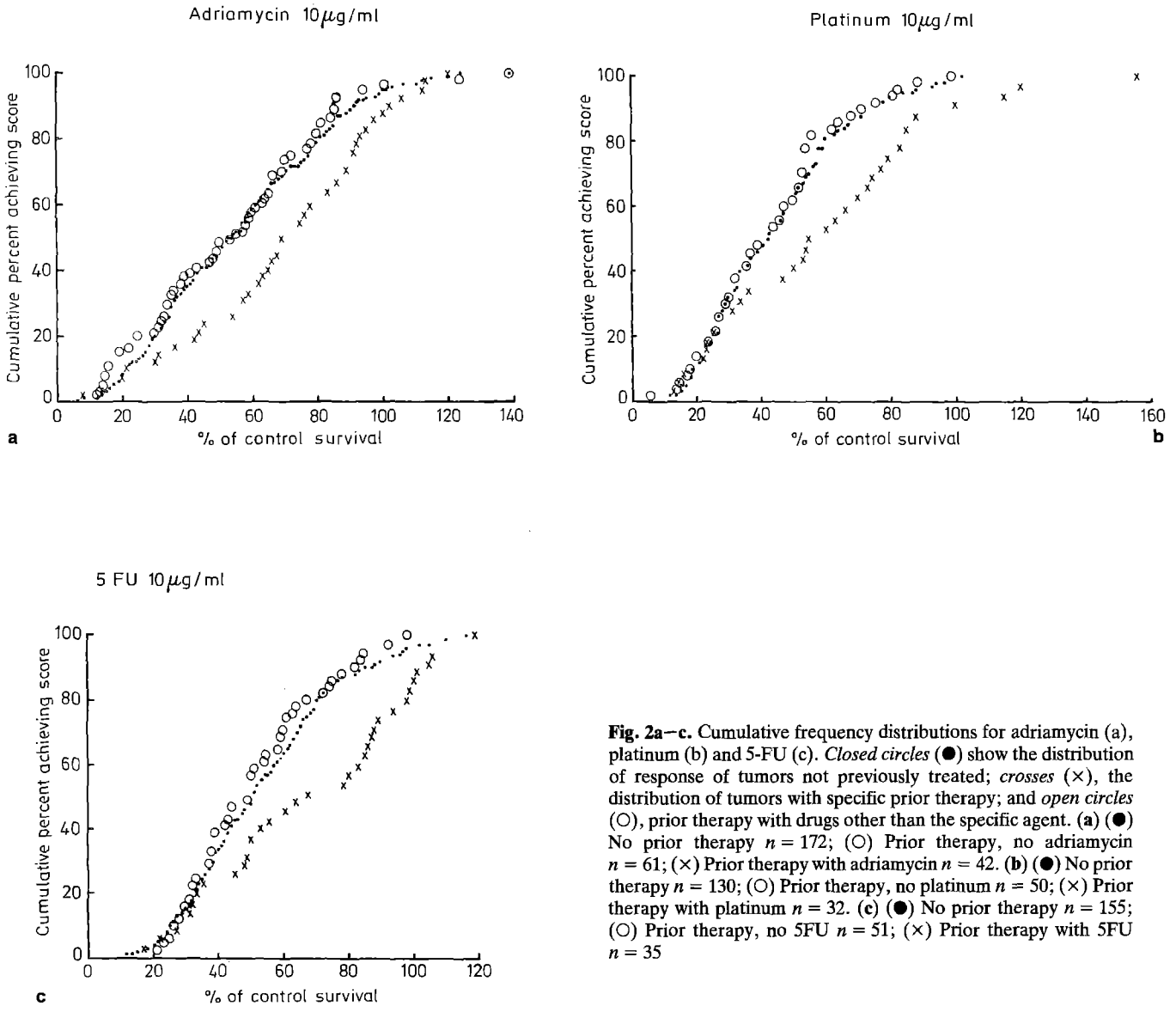

Fig. 2a–c. Cumulative frequency distributions for adriamycin (a), platinum (b) and 5-FU (c). *Closed circles* (●) show the distribution of response of tumors not previously treated; *crosses* (×), the distribution of tumors with specific prior therapy; and *open circles* (○), prior therapy with drugs other than the specific agent. (**a**) (●) No prior therapy $n = 172$; (○) Prior therapy, no adriamycin $n = 61$; (×) Prior therapy with adriamycin $n = 42$. (**b**) (●) No prior therapy $n = 130$; (○) Prior therapy, no platinum $n = 50$; (×) Prior therapy with platinum $n = 32$. (**c**) (●) No prior therapy $n = 155$; (○) Prior therapy, no 5FU $n = 51$; (×) Prior therapy with 5FU $n = 35$

bladder and repeated paracentesis specimens in ovarian cancer. These sequential determinations are illustrated for three neoplasms in Table 3 and generally show close agreement.

5. *Effect of Prior Therapy*

The effect on percent control survival of patient exposure to the tested drug prior to biopsy is shown in Fig. 2a–c. Mean percent survival is increased following specific prior drug exposure to adriamycin, platinum, and 5-fluorouracil, but not methotrexate (not shown). The cumulative frequency distributions demonstrate that the influence of specific prior drug exposure extends the entire range of observed sensitivities. Occasional tumor specimens retain in vitro sensitivity despite prior exposure.

6. *Cyclophosphamide: Active Derivatives*

Thirty tumor specimens were exposed in vitro to both 4-HC and PM. Both agents showed in vitro effects at 10 μg/ml. The average clone survival following 4-HC was 44.9% and that following PM was 70.3%. Percentile transformation for 4-HC effect and for PM effect was carried out and the paired data are plotted in Fig. 3. Linear regression analysis of the percentile transforms of 4-HC and PM indicates a slope of 0.82 and a correlation coefficient of 0.82. This result is consistent with similarity or identity of involved molecular pathways to achieve cell kill for the two derivatives.

The cumulative frequency distribution of percent survival scores following 4-HC exposure are presented in Fig. 4 for previously untreated patients ($n = 71$), for patients treated with drugs other than cyclophosphamide ($n = 16$), and for patients treated with chemotherapy including cyclophosphamide ($n = 21$). There is a major shift in 4-HC sensitivity associated with prior cyclophosphamide exposure.

7. *Comparative Evaluation of in vitro Test Dose by Percentile Ranking*

To evaluate the effect of in vitro drug concentration on apparent relative sensitivity we separately ranked the in vitro scores obtained at 0.1 and 10 μg drug/ml for all patients tested at the two dose levels. Rank order for each drug has been normalized by a

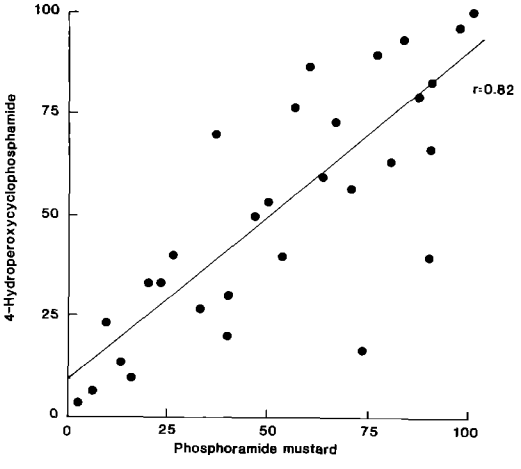

Fig. 3. Percentile distribution scores for 30 tumors tested against both cyclophosphamide derivatives, 4-HC and PM. The linear regression of the paired scores resulted in y-intercept = 9.1, slope = 0.82, and $r = 0.82$, suggesting dependence on similar or identical cell properties for clone inhibition

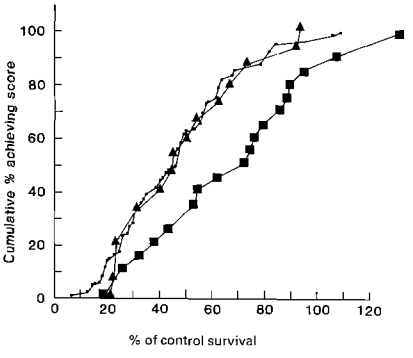

Fig. 4. Response of individual specimens to 4-HC in vitro. The distribution of fractional survival following 4-HC exposure is shown as the cumulative percent of individual specimens which achieve the indicated or lower percent survival score. *Closed circles* (●) represent specimens from patients with no prior chemotherapy; *triangles* (▲), patients with prior chemotherapy not including cyclophosphamide; *boxes* (■), patients with prior chemotherapy including cyclophosphamide. Note that specimens from patients previously treated with cyclophosphamide show greater in vitro resistance to 4-HC than do specimens from patients with no prior chemotherapy or chemotherapy other than cyclophosphamide. (●) No prior chemotherapy ($n = 71$) (▲) Prior chemotherapy, no cyclophosphamide ($n = 16$) (■) Prior chemotherapy, with cyclophosphamide ($n = 21$)

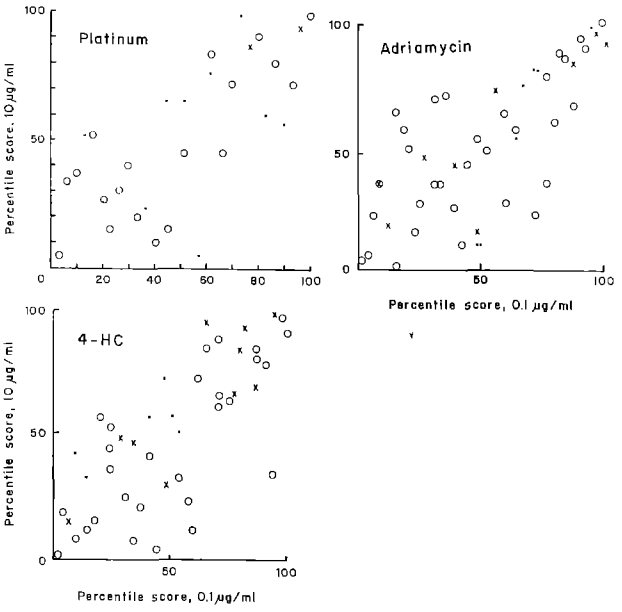

Fig. 5. Comparative evaluation of in vitro test dose by percentile ranking. In vitro scores obtained at 0.1 and 10 µg drug/ml were ranked and normalized by a percentile transform. Shown are data for platinum, adriamycin, and 4-HC. The *vertical axis* shows percentile score for 10 µg/ml and the *horizontal axis*, 0.1 µg/ml. *Open circles* (○) represent patients with no prior chemotherapy; *closed circles* (●), patients with prior chemotherapy not including the specific drug; and *crosses* (×), patients with prior therapy including the specific drug

percentile transform such that a lower in vitro score (large drug effect) is assigned a lower percentile. Data for platinum, adriamycin, and 4-HC are presented in Fig. 5. There is considerable deviation from the lines of identity, which becomes greater at the lower percentile scores (greater drug effect). This reflects non-parallel in vitro dose response curves between patients and demonstrates that the rank order of sensitivity among patients is strongly dependent upon the selected in vitro test dose. There was no clear influence of prior drug therapy upon the comparative effect.

Discussion

We have documented that in vitro chemosensitivity assessment may be performed on most specimens submitted to our laboratory for that purpose. The high success rate reflects close surgical cooperation and control of specimen transport under ambient temperature conditions (data not presented). Chemosensitivity results are reproducible and are not altered by collagenase pretreatment. Routine use of this enzymatic facilitation of disaggregation will enhance test usefulness for most specimens.

The cyclophosphamide derivatives 4-HC and PM are active in vitro, 4-HC being more potent. The close correlation obtained from the paired percentile transformation supports the view that activities of both drugs depend on similar cellular properties and that the percentile transformation technique normalizes data effectively across a range of sensitivities. The demonstration of effect of specific prior drug exposure to cyclophosphamide on the distribution of in vitro 4-HC response supports the validity of utilizing this derivative as an activated cyclophosphamide surrogate. Similar arguments have been developed in animal tumor systems [9].

We demonstrate that specimens from patients who have received prior treatment with a specific agent tend to be less sensitive in vitro to that drug; but for 4-HC, adriamycin, platinum, and 5-FU previous exposure to other drugs does not shift the observed distribution of sensitivities from untreated cohorts. Our analysis pooled prior experience with all other agents. Specific instances of collateral sensitivity or resistance are likely but will require more detailed examination.

Test validation requires clinical correlation of test results with subsequent in vivo response. It is a complex task to establish the appropriate test dose range and the criterion for significant in vitro response for each drug. Published correlations have utilized 10% of concentration × time drug levels to select the in vitro test dose [6]. We are exploring the potential usefulness of ranking each test score within our overall patient experience using a percentile transform. Comparison over a two-log drug concentration range demonstrates different patient rank order at the two test doses reflecting non-parallel individual dose response curves. Thus it is clear that selection of the appropriate test dose for each drug will be important for optimal clinical validity.

References

1. Alberts DS, Chen HG, Salmon SE et al. (1980) In vitro clonogenic assay for predicting response of ovarian cancer to chemotherapy. Lancet 1:340–342
2. Bradley TR, Metcalf D (1966) The growth of mouse bone marrow cells in vitro. Aust J Exp Biol Med Sci 44:287
3. Colvin M, Padgett CA, Fenselau C (1973) A biologically active metabolite of cyclophosphamide. Cancer Res 33:915–918
4. Hamburger AW, Salmon SE (1977) Primary bioassay of human tumor stem cells. Science 197:461

5. Puck TT, Marcus PI, Ciecura SJ (1955) Clonal growth of mammalian cells in vitro. J Exp Med 103: 273
6. Salmon SE, Hamburger AW et al. (1978) Quantitation of differential sensitivity of human tumor stem cells to anticancer drugs. N Engl J Med 294: 1321
7. Takaniejawa A, Matsumoto S, Iwata T, Katagiri K, Tochino Y, Yamaguchi K (1973) Studies on cyclophosphamide metabolites and their related compounds. II. Preparation of an active species of cyclophosphamide and some related compounds. J Am Chem Soc 95: 985–986
8. Van der Steen J, Timmer EC, Westrau JG, Benckhuysen C (1973) 4-Hydroperoxidation in the Fenton oxcidation of the antitumor agent cyclophosphamide. J Am Chem Soc 95: 7535–7536
9. Volm M, Mattern J, Wayss K (1977) Detection of induced tumour-resistance to cyclophosphamide by the in vitro short-term test. Arch Gynaekol 223: 249–257

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