Jpn. J. Cancer Res. 87, 91–97, January 1996

Evaluation of the Relative Cytotoxic Effects of Anticancer Agents in Serum-supplemented versus Serum-free Media Using a Tetrazolium Colorimetric Assay

Chun-Ming Tsai,[1,2,5] Reury-Perng Perng,[1] Kuo-Ting Chang,[1] David Venzon[3] and Adi F. Gazdar[4]

[1]Section of Thoracic Oncology, Chest Department, Veterans General Hospital-Taipei, Taipei, Taiwan (ROC) 11217, [2]Department of Medicine, Medical School, National Yang-Ming University, Taiwan (ROC) 11217, [3]Biostatistics and Data Management Section, Clinical Oncology Program, National Cancer Institute, Bethesda, Maryland 20892, USA and [4]Simmons Cancer Center and Department of Pathology, University of Texas Southwestern Medical Center, Dallas, Texas, USA

Most cell culture and *in vitro* drug sensitivity assays utilize serum-supplemented media (SSM). However, fully defined serum-free media (SFM) offer several advantages and are being used increasingly for initiation and maintenance of cell cultures. Because serum inhibits the *in vitro* cytotoxicity of certain antineoplastic agents, we investigated the inter-relationships between medium type, cell proliferation and cytotoxic effect. Twenty-four human lung cancer cell lines were tested with nine anticancer agents in both media types. A semi-automated tetrazolium (MTT) colorimetric assay was used for assaying cell survival. Cell lines initiated and maintained in SFM preferentially proliferated in that medium type or proliferated equally well in both media types. In contrast, cell lines established in SSM varied considerably in their medium of preference. There appeared to be a direct correlation or trend between cell proliferative rate and cytotoxicity of all drugs with the possible exceptions of methotrexate and carmustine. In general, the cell lines were more sensitive to anticancer agents when they were exposed in the culture medium in which they preferentially proliferated. Therefore, to determine the influence of culture media on cytotoxicity, we analyzed the data only from lines that replicated equally efficiently in both media. After correction for cell proliferative rate, SSM had a negative effect on the cytotoxic action of some drugs (especially methotrexate, 5-fluorouracil and, to a lesser extent, mitomycin-C). Our results demonstrate that fully defined SFM may be suitable for initiating cell lines and for use in *in vitro* cytotoxicity assays for selection of individualized therapy or for screening of new anti-neoplastic agents, and thus may increase the number of antineoplastic agents that can be tested satisfactorily.

Key words: Serum-free medium — Drug testing — Anticancer agent — Lung cancer

A number of *in vitro* assays have been used for predicting clinical response and for the screening of new therapeutic agents.[1–3] While the range of assays that may be used for testing cell lines is large, only a limited number can be utilized for testing fresh tumor specimens, because tumors consist of varying proportions of tumor cells admixed with stromal cells. Culture of tumors in routine serum-supplemented medium (SSM) frequently results in overgrowth or persistence of stromal cells. Only assays capable of discriminating between the responses of tumor and stromal cells may be utilized. Unfortunately, most assays currently utilized for clinical testing, including clonogenic, dye exclusion and differential adhesive assays are laborious, technically difficult, or subjective.

The tetrazolium (MTT) dye colorimetric assay is a semi-automated, rapid, objective, and highly reproducible bioassay. It has been used in a variety of cell culture systems, including human lung cancers, to evaluate *in vitro* chemosensitivity, radiosensitivity and growth stimulation.[4–13] The major drawback of this assay is its inability to distinguish tumor from stromal cells. Thus, it can only be applied to cell lines or other populations of "pure" tumor cells. Currently, we are attempting to modify the assay for clinical testing.

Serum-free media (SFM) have been developed for the selective growth of most lung cancers. These media include HITES medium for small cell lung cancer (SCLC)[14,15] and ACL-4 medium for non-SCLC (NSCLC) cancers, especially large cell and adenocarcinomas.[16–18] These SFM have two major roles in drug sensitivity testing (DST). Their use permits the selective growth of tumor cells. Thus, assays that do not discriminate between tumor and stromal cells can be applied within a relatively short time after obtaining a tumor sample. In addition, as the *in vitro* cytotoxicity of some drugs is partially inhibited in SSM, the use of SFM may increase the range of cytotoxic agents that can be tested *in vitro*. We have utilized the MTT assay and a panel of human lung cancer cell lines grown in SSM and SFM to investigate the inter-relationships between medium type,

[5] To whom correspondence should be addressed, at the Section of Thoracic Oncology, Chest Department, Veterans General Hospital-Taipei.

cell growth and the cytotoxic effects of a variety of anticancer drugs.

MATERIALS AND METHODS

Cell lines, media and culture methods Twenty-four lung cancer cell lines, 8 SCLC and 16 NSCLC (8 adenocarcinomas, 1 squamous cell carcinoma, 3 adenosquamous cell carcinomas, and 4 large cell carcinomas) were tested. All of the cell lines were established and characterized in the NCI-Navy Medical Oncology Branch, as described previously.[19,20] In all instances, the initial medium was RPMI-1640 either supplemented with 10% heat-inactivated fetal bovine serum (R10 medium) or a fully defined medium for the selective growth of either SCLC (HITES medium)[15] or NSCLC (ACL-4).[18] While SCLC lines can be initiated in fully defined HITES medium, the success rate is higher in HITES medium supplemented with modest amounts of serum (2–5%) (A. F. Gazdar, unpublished data). Initial cell culture methodology usually included both media types, with maintenance of the culture in the medium type that supported proliferation preferentially. All of the lines were maintained in the medium in which they had been established, either R10, HITES, ACL-4 or HITES supplemented with 5% serum (HITES-5). Most SCLC lines lack substrate adherence and grow as floating cell clusters in SSM and SFM.[19–21] In contrast, NSCLC lines usually demonstrate substrate adherence and grow as monolayer cultures in SSM. However, most SFM, including ACL-4, lack attachment factors present in serum, and NSCLC cells usually float when cultured in SFM (Table I).

DST DST was performed by a modification of the method of Mossman.[4] All of the lines were tested in both SSM (R10) and the appropriate SFM. The SFM used for testing SCLC lines was HITES medium supplemented with 0.2% bovine albumin (RIA grade, Sigma Chemical Co., St. Louis, MO) (HITES-A medium). Preliminary experiments indicated that SCLC growth in microtiter wells was superior in HITES-A medium to that in HITES. A single batch of serum and single stock solutions of growth factors were used for all of the studies.

To obtain single cell suspensions, cells were washed twice with phosphate-buffered saline, and disaggregated with trypsin-EDTA. They were then resuspended in the tested growth media (SSM and SFM) and seeded into 96-well microtiter plates in a volume of 180 μl. The optimal seeding density of individual cell lines was predetermined as described previously,[6] substituting a 5-day cell incubation. Cell lines were tested simultaneously in

Table I. Cell Line Characteristics

Cell line	Cell type	Initial growth medium	Substrate adherence	Prior chemotherapy
SCLC				
NCI-H735	SCLC	HITES	No	Yes
NCI-H792	SCLC	HITES	No	Yes
NCI-H738	SCLC	HITES-5	No	No
NCI-H774	SCLC	HITES-5	No	No
NCI-H1105	SCLC	HITES-5	No	No
NCI-H1304	SCLC	HITES-5	No	Yes
NCI-H841	SCLC	R10	Partial	Yes
NCI-H889	SCLC	R10	No	No
NSCLC				
NCI-H676	Adenocarcinoma	ACL-4	No	No
NCI-H820	Adenocarcinoma	ACL-4	No	No
NCI-H1355	Adenocarcinoma	ACL-4	No	No
NCI-H1435	Adenocarcinoma	ACL-4	No	No
NCI-H23	Adenocarcinoma	R10	Yes	No
NCI-H358	Adenocarcinoma	R10	Yes	No
NCI-H838	Adenocarcinoma	R10	Yes	No
NCI-H1437	Adenocarcinoma	R10	Yes	No
NCI-H125	Adeno-squamous ca	R10	Yes	No
NCI-H322	Adeno-squamous ca	R10	Yes	No
NCI-H647	Adeno-squamous ca	R10	Yes	No
NCI-H226	Squamous cell ca	R10	Yes	No
NCI-H460	Large cell ca	R10	Yes	No
NCI-H661	Large cell ca	R10	Yes	Yes
NCI-H1299	Large cell ca	R10	Yes	No
NCI-H1334	Large cell ca	R10	Yes	No

SSM and SFM. After incubation for approximately 16 h (to allow cells to recover from trypsinization), 20 μl of drug or saline was added to test and control wells, respectively. After exposure to drugs for 4 days, the remaining steps of the MTT assay were performed as previously described.[6] Data points represent the mean of 8 wells. Cell number per microtiter well was proportional to the absorbance of the solubilized formazan at 540 nm.[6, 10–13, 22] Thus, the surviving cell fraction after drug exposure was considered to be the mean absorbance of the test wells/mean absorbance of control wells. The results, reported as IC_{50} values, which were defined as the drug concentration required to inhibit cell growth (reduce absorbance) by 50%, are the means of three independently performed assays.

Chemotherapeutic agents Nine drugs used in the therapy of lung cancer, namely doxorubicin, carmustine, cisplatin, 5-fluorouracil, mitomycin-C, methotrexate, nitrogen mustard, vincristine, and etoposide were tested. Nitrogen mustard was used as a substitute for cyclophosphamide, as the latter is inactive *in vitro* unless "activated."[23] The drugs were formulated as for clinical use, except for carmustine, which was obtained from Sigma Chemical Co. We elected to test carmustine instead of lomustine because of the relative insolubility of the latter. All drugs were prepared fresh for each experiment and were dissolved in saline or water except carmustine which was dissolved in 50% ethanol. All drugs were subsequently diluted in saline. Each drug was tested over a 3–7 log range, using 10-fold dilutions, to cover the entire dose-response curves (as determined by preliminary testing), whenever possible.

Data analysis We used the mean values of the proliferation index (PI) in three independently performed assays to compare the relative activities of a cell line in SFM and SSM. The PI was defined as follows:

$$PI = \ln\left(\frac{\text{absorbance/cell number plated per well in SFM}}{\text{absorbance/cell number plated per well in SSM}}\right)$$

In the formula, ln represents the natural log. Cell lines with positive PI values proliferated preferentially in SFM, those with negative PI values proliferated preferentially in SSM, and those whose PI values were close to zero proliferated equally well in both media.

The survival fractions at the test concentrations in SSM and SFM were correlated by Pearson product-moment correlation[24] and plotted. Examples are displayed in Fig. 1. The area under the regression line was defined as the sensitivity index (SI). The mean SI value of the three independently performed assays was used to reflect the relative sensitivity in the two types of test media. Thus, drugs having mean SI values for a particular cell line of less than 0.5 were more cytotoxic in SFM and those with mean SI values of greater than 0.5 were more cytotoxic in SSM. Drugs having mean SI values of 0.5 were equally cytotoxic in both media. We did not utilize IC_{50} values for calculating the SI values because the IC_{50} values for some drugs could not always be determined in both media (see below). However, for those drugs for which the IC_{50} values could always be generated, there was an excellent correlation between SI and the natural log of the ratios of the IC_{50} values (data not shown).

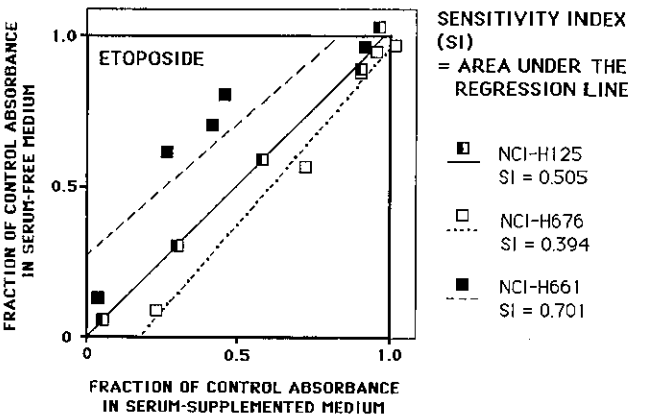

Fig. 1. Examples demonstrating determination of the sensitivity index (SI). The correlations between cell survivals of the cell lines exposed to etoposide in SFM and SSM are displayed. Each point represents the cell survival (the mean value of three independent assays) at a given drug concentration. The area under the regression line was defined as the sensitivity index of a particular cell line for the drug tested. The SI value represents the relative chemosensitivity of the line in the two media types. Thus, cell line NCI-H125 (SI=0.505) is equisensitive to etoposide in both media, while line NCI-H676 (SI=0.39) is relatively more sensitive in SFM, and cell line NCI-H661 (SI=0.7) is relatively more sensitive in SSM.

In this study, mean PI value was correlated with mean SI value in order to evaluate the relationship between proliferation and chemosensitivity in different media by means of the Spearman rank correlation.[24] We found a trend between cell proliferation and cytotoxicity (see below). Therefore, to evaluate the effect of serum on cytotoxicity, only those cell lines whose proliferation was approximately equal in both media were utilized. After an analysis of the data, cell lines with PI values between 0.3 to −0.3 were judged to have met this criterion, and the dose-response curves of these cell lines were plotted. Drug concentrations resulting in almost complete or almost no cytotoxicity were deemed to be of no interest. The drug concentrations chosen for analysis for all drugs (except for vincristine) were those resulting in 20% to 80% cytotoxicity. Because the drug response curves for vincristine were relatively "flat," the drug concentrations

Table II. Relationship between Initial Growth Medium and Proliferation Index

Cell line	Cell type	Initial growth medium	Proliferation index (PI)[a]
Preferential growth in SSM			
NCI-H1299	NSCLC	R10	−2.526±0.094
NCI-H661	NSCLC	R10	−1.897±0.115
NCI-H841	SCLC	R10	−0.810±0.047
NCI-H1437	NSCLC	R10	−0.533±0.038
NCI-H322	NSCLC	R10	−0.386±0.013
NCI-H23	NSCLC	R10	−0.334±0.021
Equal growth in both media			
NCI-H647	NSCLC	R10	−0.216±0.018
NCI-H460	NSCLC	R10	−0.164±0.009
NCI-H820	NSCLC	ACL-4	−0.087±0.010
NCI-H792	SCLC	HITES	−0.060±0.009
NCI-H838	NSCLC	R10	−0.057±0.001
NCI-H125	NSCLC	R10	−0.046±0.003
NCI-H226	NSCLC	R10	0.032±0.002
NCI-H1334	NSCLC	R10	0.082±0.007
NCI-H774	SCLC	HITES-5	0.195±0.019
NCI-H358	NSCLC	R10	0.230±0.006
NCI-H1304	SCLC	HITES-5	0.261±0.018
NCI-H1435	NSCLC	ACL-4	0.262±0.020
NCI-H1105	SCLC	HITES-5	0.280±0.019
NCI-H738	SCLC	HITES-5	0.294±0.015
Preferential growth in SFM			
NCI-H889	SCLC	R10	0.302±0.023
NCI-H735	SCLC	HITES	0.339±0.028
NCI-H676	NSCLC	ACL-4	0.436±0.031
NCI-H1355	NSCLC	ACL-4	0.703±0.054

a) Mean±SE of three independent experiments.

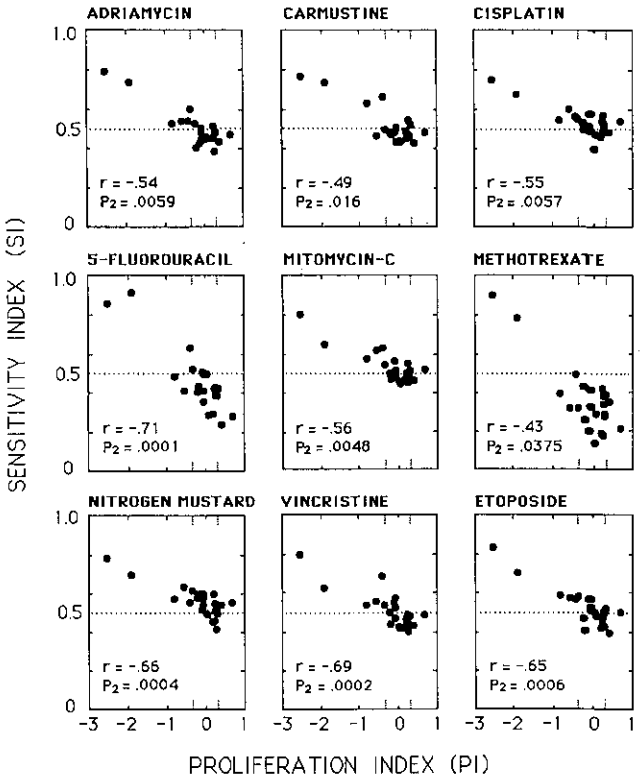

Fig. 2. Relationship of cell proliferation to chemosensitivity. The proliferation and sensitivity indices were correlated for all 24 lines for the nine drugs tested. In general, cell lines that proliferated preferentially in a particular medium type were more chemosensitive in that medium. The Pearson correlation coefficients (r values) and two-sided P values are reported.

chosen were those that resulted in cytotoxicity of 40% to 80%. The differences for given cell lines at several concentrations of a given drug were analyzed by using Hotelling's T^2 test (for a single concentration this is equivalent to the paired Student's t test).[25] Because nine tests were carried out simultaneously, only P_2 values <0.0056 were considered to be statistically significant, using the Bonferroni procedure for testing multiple hypotheses.[26]

RESULTS

Characteristics of cell lines The characteristics of the cell lines used in this study are presented in Table I. They included 8 SCLC lines, as well as 16 NSCLC lines of various histological subtypes. Five lines were from patients who had received prior chemotherapy. Six lines were established and maintained in completely defined media, while the remaining lines were initiated in SSM. All of the SCLC lines lacked substrate adherence (one line demonstrated partial adherence). Adherence of NSCLC lines was dependent on the type of culture medium. The 12 NSCLC lines initiated in SSM (which contains attachment factors) demonstrated substrate attachment. In contrast, the 4 NSCLC lines initiated in ACL-4 medium (which lacks attachment factors) lacked substrate attachment.

Effect of culture media on cell proliferation The influence of the initial culture medium on cell proliferation in both media types is presented in Table II. Most of the cell lines (18/24), including all of those initiated in SFM, proliferated preferentially in SFM, or equally well in both media types. In contrast, all of the 6 cell lines that proliferated preferentially in SSM were initiated in the medium. We devised an SI to compare the relative cytotoxicity of drugs in the different media types. Examples of cell lines tested for sensitivity to etoposide are displayed in Fig. 1. Cell line NCI-H125 has SI 0.5, indicating that it is equisensitive to the drug in both SFM and SSM media. Cell line NCI-H661 with SI 0.7, is relatively more sensitive to the drug in SSM.

We devised the SI instead of using the ratio of the IC_{50} values in the two media types because the latter could not always be determined for some drugs in SSM (5-fluorouracil, methotrexate, and vincristine). For the other six drugs, for which the IC_{50} values could always be determined in both media, there was an excellent correlation between the SI values and the natural log values of the ratios of IC_{50} values. By Pearson product-moment correlation[24] the correlation coefficients for these drugs were between 0.9 and 1.0, and the P_2 values were less than 0.0001.

Relationship between cell proliferation and chemosensitivity To determine whether there was a relationship between cell proliferation and chemosensitivity, we correlated the proliferative and sensitivity indices (Fig. 2). There was a significant correlation of the two indices for all drugs with the exceptions of methotrexate and carmustine. These results indicate that cell lines tend to be more sensitive to drugs when they are tested in the particular medium type in which they preferentially proliferate.

Effect of culture media on chemosensitivity For evaluating the effect of SSM on drug cytotoxicity, only data for the 14 cell lines (5 SCLC and 9 NSCLC) which proliferated approximately equally well in both media types were utilized. The mean dose-response curves of these lines in the two media types are displayed in Fig. 3. The

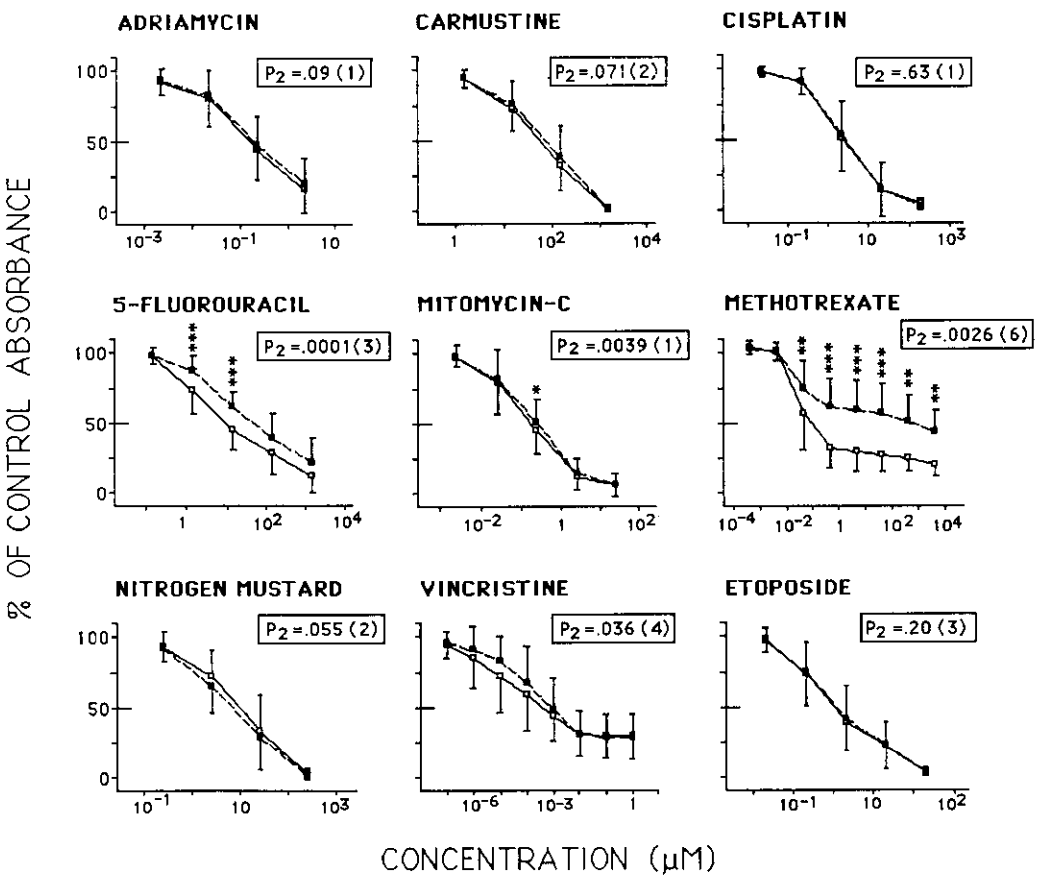

Fig. 3. Mean dose-response curves of the 14 cell lines which proliferated equally well in both media types. The mean surviving fractions when tested with the nine cytotoxic agents in SFM (open squares with solid lines) and SSM (closed squares with dashes) are shown with one standard deviation (vertical dash marks). The boxes contain the P_2 values for comparison of the two curves, as mentioned in "Materials and Methods." The numbers enclosed in parentheses after the P_2 values represent the number of drug concentrations used for statistical analyses (see "Materials and Methods"). In addition, individual drug concentrations at which statistically significant differences were noted between the two media types are asterisked: * $P_2 <$ 0.0056, ** $P_2 < 0.0011$, *** $P_2 < 0.00011$. The highly significant result in the case of mitomycin-C is explained by the very high correlation between the results across experiments. The difference in the surviving fractions between SSM and SFM tended to be strongly positive in this and other cases. Unfortunately, only one concentration of mitomycin-C was available for analysis using the criteria established prior to analysis (see "Materials and Methods").

surviving fractions were similar for all drugs except for methotrexate, 5-fluorouracil and mitomycin-C.

DISCUSSION

We have previously demonstrated that our cell lines provide useful models to study the chemo- and radiosensitivity patterns of lung cancers.[6,7,9] These lines are currently being used in clinical trials based on selection of individualized combinations of new therapeutic agents. For both purposes, it is essential to select the test conditions that yield optimal results.

While SSM from the basis of most cell culture systems, fully defined SFM offer many advantages, and are increasingly being used for the initiation and maintenance of tumor cell lines including human lung cancers.[15,17] Among the disadvantages of SSM is the inhibition of the *in vitro* cytotoxicity of some antineoplastic agents. For these reasons, we investigated whether SFM offer advantages for *in vitro* drug testing. Before the relative cytotoxicity of drugs in these two media types could be compared, the effects of the different media on cell proliferation, and the effects of cell proliferation on the cytotoxicity of drugs had to be investigated.

Our results demonstrate that lung cancer lines initiated and maintained in fully defined SFM preferentially proliferate in that medium type, or in both media types. In contrast, cell lines established in SSM varied considerably in their medium of preference, with some lines preferentially replicating in SFM (Table II).

There was a definite correlation or trend between the proliferation and sensitivity indices for all of the drugs with the possible exceptions of methotrexate and carmustine. These results indicated that the *in vitro* cytotoxicity of most chemotherapeutic agents is influenced by the rate of cellular proliferation. Therefore, to determine the influence of culture media on cytotoxicity, we analyzed the data only from lines that replicated approximately equally efficiently in both media.

Serum-supplemented media had a negative effect on the cytotoxic action of some drugs (especially methotrexate, 5-fluorouracil and, to a lesser extent, mitomycin-C), independent of the effect on cell proliferation. The cellular protective effects of serum on methotrexate and 5-fluorouracil are mediated via hypoxanthine and thymidine contained in undialyzed serum.[27-30] The final concentrations of hypoxanthine and thymidine in the R10 medium we used (5–12 μM and 0.85–0.93 μM, respectively) were 2–12 times higher than the levels normally present in the circulation.[30,31] Cisplatin and vincristine bind avidly to serum proteins,[32,33] and thus, theoretically, may demonstrate reduced cytotoxicity in SSM. However, under our assay conditions, we failed to detect any significant differences in the cytotoxicity of these drugs in the two media types. The reasons for the modest effects of serum on the cytotoxicity of mitomycin-C are not apparent.

In the present study, our results demonstrate that almost all of the cell lines which did not grow equally well in both media types proliferated preferentially in the particular medium in which they were grown (9/10, the only exception being H889) (Table II). Therefore, for purposes other than selection of individualized therapy and screening of new antineoplastic agents, the medium in which the cell lines are grown will be the medium of choice for *in vitro* drug testing. However, fully defined SFM may be suitable for initiating cell lines and for use in *in vitro* cytotoxicity assays for selection of individualized therapy or for screening of new anti-neoplastic agents, and thus may increase the number of anti-neoplastic agents that can be tested satisfactorily.

ACKNOWLEDGMENTS

This work was supported in part by grant NSC78-0412-B075-35 from the National Science Council, Taiwan (ROC).

(Received July 26, 1995/Accepted October 20, 1995)

REFERENCES

1) Hamburger, A. W. and Salmon, S. E. Primary bioassay of tumor stem cells. *Science,* **197,** 461–463 (1977).
2) Weisenthal, L. M. and Lippman, M. E. Clonogenic and nonclonogenic *in vitro* chemosensitivity assays. *Cancer Treat. Rep.,* **69,** 615–632 (1985).
3) Baker, F. L., Spitzer, G., Ajani, J. A., Brock, W. A., Lukeman, J., Pathak, S., Tomasovic, B., Thielboldt, D., Williams, M., Vines, C. and Tofilon, P. Drug and radiation sensitivity measurements of successful primary monolayer culturing of human tumor cells using cell adhesive matrix and supplemented medium. *Cancer Res.,* **46,** 1263–1274 (1986).
4) Mossman, T. Rapid colorimetric assay for cellular growth survival: application to proliferation and cytotoxic assays. *J. Immunol. Methods,* **65,** 55–63 (1983).
5) Denziot, F. and Lang, R. Rapid colorimetric assay for cell growth and survival: modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Methods,* **89,** 271–277 (1986).
6) Carmichael, J., Degraff, W. G., Gazdar, A. F., Minna, J. D. and Mitchell, J. B. Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemo-

sensitivity testing. *Cancer Res.*, **47**, 936–942 (1987).
7) Carmichael, J., Degraff, W. G., Gazdar, A. F., Minna, J. D. and Mitchell, J. B. Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of radiosensitivity. *Cancer Res.*, **47**, 943–946 (1987).
8) Nakanishi, Y., Mulshine, J. L., Kasprzyk, P. G., Natale, R. B., Maneckjee, R., Avis, I., Treston, A. M., Gazdar, A. F., Minna, J. D. and Cuttitta, F. Insulin-like growth factor-1 can mediate autocrine proliferation of human small cell lung cancer cell lines. *J. Clin. Invest.*, **82**, 354–359 (1988).
9) Carmichael, J., Mitchell, J. B., Degraff, W. G., Gamson, J., Gazdar, A. F., Johnson, B. E., Glatstein, E. and Minna, J. D. Chemosensitivity testing of human lung cancer cell lines using the MTT assay. *Br. J. Cancer*, **57**, 540–547 (1988).
10) Tsai, C.-M., Gazdar, A. F., Venzon, D. J., Steinberg, S. M., Dedrick, R. L., Mulshine, J. L. and Kramar, B. S. Lack of *in vitro* synergy between etoposide and *cis*-diamminedichloroplatinum(II). *Cancer Res.*, **49**, 2390–2397 (1989).
11) Tsai, C.-M., Gazdar, A. F., Allegra, C., Perng, R.-P. and Kramer, B. S. Enhancement of fluorinated pyrimidine-induced cytotoxicity be leucovorin in human lung cancer cell lines. *Int. J. Cancer*, **46**, 101–105 (1990).
12) Tsai, C.-M., Gazdar, A. F., Perng, R.-P. and Kramer, B. S. Schedule dependent *in vitro* combination effects of methotrexate and 5-fluorouracil in human tumor cell lines. *Int. J. Cancer*, **47**, 401–407 (1991).
13) Tsai, C.-M., Shiao, S.-H., Frey, C.-M., Chang, K.-T., Perng, R.-P., Gazdar, A. F. and Kramer, B. S. Combination cytotoxic effects of *cis*-diamminedichloroplatinum(II) and 5-fluorouracil with and without leucovorin against human non-small cell lung cancer cell lines. *Cancer Res.*, **53**, 1079–1084 (1993).
14) Simms, E., Gazdar, A. F., Abrams, P. G. and Minna, J. D. Growth of human small cell (oat cell) carcinoma of the lung in serum-free growth factor supplemented medium. *Cancer Res.*, **40**, 4356–4363 (1980).
15) Carney, D. N., Bunn, P. A., Gazdar, A. F., Pagan, J. F. and Minna, J. D. Selective growth in serum-free hormone supplemented medium of tumor cells obtained by biopsy from patients with small cell carcinoma of the lung. *Proc. Natl. Acad. Sci. USA*, **78**, 3185–3189 (1981).
16) Brower, M., Carney, D. N., Oie, H. K., Gazdar A. F. and Minna, J. D. Growth of cell lines and clinical specimens of human non-small cell lung cancer in a serum-free defined medium. *Cancer Res.*, **46**, 798–806 (1986).
17) Gazdar, A. F. and Oie, H. K. Growth of cell lines and clinical specimens of human non-small cell lung cancer in a serum-free defined medium. *Cancer Res.*, **46**, 6011–6012 (1986).
18) Gazdar, A. F. and Oie, H. K. Cell culture methods for human lung. *Cancer Genet. Cytogent.*, **19**, 5–10 (1986).
19) Carney, D. N., Gazdar, A. F., Bepler, G., Guccion, J., Marangos, P. J., Moody, T. W., Zweig, M. H. and Minna, J. D. Establishment and identification of small cell lung cancer cell lines having classic and variant features. *Cancer Res.*, **45**, 2913–2923 (1985).
20) Ruckdeschel, J. C., Oie, H. K. and Gazdar, A. F. *In vitro* characterization of non-small cell lung cancer. *In* "Lung Cancer. Basic and Clinical Aspects," ed. H. H. Hansen, pp. 49–59 (1986). Martinus Nijhoff, Boston.
21) Gazdar, A. F., Carney, D. N., Russell, E. K., Sims, H. L., Baylin, S. B., Bunn, P. A., Guccion, J. G. and Minna, J. D. Establishment of continuous clonable cultures of small-cell carcinoma of the lung which have amine precursor uptake and decarboxylation cell properties. *Cancer Res.*, **40**, 3502–3507 (1980).
22) Gazdar, A. F., Tsai, C.-M., Park, J.-G., Ihde, D. C., Mulshine, J., Mitchell, J. B. and Minna, J. D. *In vitro* assays for predicting clinical response in human lung cancer. *In* "Prediction of Tumor Treatment Response," ed. J. D. Chapman, L. J. Peters and H. R. Withers, pp. 175–186 (1989). Pergamon Press, New York.
23) Friedman, O. M., Myles, A. and Colvin, M. Cyclophosphamide and related phosphamide mustards: current status and future prospects. *Adv. Cancer Chemother.*, **1**, 159–164 (1979).
24) Rermington, R. D. and Schork, M. A. "Statistics with Applications to the Biological and Health Sciences," 2nd Ed. (1985). Prentice Hall, New Jersey.
25) Afifi, A. A. and Clark, V. "Computer-aided Multivariate Analysis" (1984). Wadworth Inc., Belmont.
26) Fleiss, J. L. "The Design and Analysis of Clinical Experiments" (1986). John Wiley and Sons, New York.
27) Yoshida, M., Hoshi, A. and Kuretani, K. Prevention of antitumor effect of 5-fluorouracil by hypoxanthine. *Biochem. Pharmacol.*, **27**, 2979–2982 (1978).
28) Jackson, R. C. Modulation of methotrexate toxicity by thymidine: sequence-dependent biochemical effects. *Mol. Pharmacol.*, **18**, 281–286 (1980).
29) Martin, D. S., Stolfi, R. L., Sawyer, R. C., Nayak, R., Spiegelman, S., Young, C. W. and Woodcock, T. An overview of thymidine. *Cancer*, **45**, 1117–1128 (1980).
30) Sobrero, A. F. and Bertino, J. R. Endogenous thymidine and hypoxanthine are a source of error in evaluating methotrexate cytotoxicity by clonogenic assays using undialyzed fetal bovine serum. *Int. J. Cell Cloning*, **4**, 51–62 (1986).
31) Holden, L., Hoffbrand, A. V. and Tattersall, M. H. Thymidine concentrations in human sera: variations in patients with leukemia and megaloblastic anemia. *Eur. J. Cancer Clin. Oncol.*, **16**, 115–121 (1980).
32) Wilson, A. P. Cytotoxicity and viability assays. *In* "Animal Cell Culture: A Practical Approach," ed. R. I. Freshney, pp. 183–216 (1986). IRL Press, Oxford.
33) Donigian, D. W. and Owellen, R. J. Interaction of vinblastine, vincristine and colchicine with serum proteins. *Biochem. Pharmacol.*, **22**, 2113–2119 (1973).