Normalization of in Vitro Sensitivity Testing of Human Tumor Clonogenic Cells

Verena Hug, Howard Thames, George R. Blumenschein, Gary Spitzer, and Benjamin Drewinko

Departments of Internal Medicine [V. H., G. R. B.], Biomathematics [H. T.], Developmental Therapeutics [G. S.], and Laboratory Medicine [B. D.], M. D. Anderson Hospital and Tumor Institute, Houston, Texas 77030

ABSTRACT

The use of normal bone marrow (granulocyte-macrophage colony-forming units) as a point of reference to normalize the in vitro activities of anticancer agents has been investigated. The cytotoxic effects of four substituted anthraquinone derivatives, and of vinblastine on myeloid progenitors of different donors were reproducible up to a cell kill of approximately 60%. Equitoxic in vitro concentrations for normal bone marrows did not correlate with in vivo pharmacokinetic concentrations of these drugs. Breast tumor progenitor cells of 46 specimens were more sensitive than were bone marrow progenitors to the anthraquinone derivatives in 26 to 39% of instances, ratios which are similar to the clinically observed response rates of patients with breast carcinoma to these agents. Tumors were either sensitive or resistant to all four drugs in 68% (10 tumors were more sensitive, and 21 tumors were less sensitive than normal bone marrow); but in 32% of instances there were differences in tumor sensitivity for the four drugs, and the assay could select one to three drugs for which the tumor sensitivity was greater than that of bone marrow. Correlations of in vitro sensitivity and of clinical response to single agent treatments were determined in 21 patients, and the concordance was 71%. The value of the assay in predicting clinical response ranked best for sensitivity determinations within the normalized dose ranges, when testing within three different dose ranges was compared in a group of six patients. The concordance was higher in the small (1 or 2 metastatic sites) than in the large (≥3 metastatic sites) tumors (85 versus 50%), indicating a confounding influence of tumor load on the ability of the assay to predict efficacy of treatment. A rule of thumb is proposed for altering the in vitro sensitivity test results for large tumors that improves the overall concordance to 90%.

INTRODUCTION

It is generally assumed that a dose-response effect holds for most antitumor agents used in the treatment of solid tumors. However, dose escalation is generally limited to concomitant myelosuppressive effects. Therefore, the effectiveness of antitumor treatments should be judged in reference to normal host tissue tolerance.

Cytotoxic effects of anticancer agents on tumor progenitor cells can be measured in vitro by determining their inhibitory effects on the clonogenicity of these cells (18, 19), and drug-induced in vitro inhibition of granulocyte-macrophage colony formation has been reported to correlate with in vivo myelosuppression (14). Since both progenitor cell types can be recovered under comparable culture conditions (1, 6, 7, 9, 17), we have explored the feasibility of normalizing the in vitro activities of anticancer drugs to doses isoeffective for bone marrow toxicity, in order to measure the relative cytotoxic potential of such agents. In this way, the value of in vitro tumor sensitivity determinations to predict for clinical response can be tested in dose ranges based rationally on bone marrow tolerance of individual patients.

MATERIALS AND METHODS

Patients. Normal bone marrow cells were obtained by aspiration from the posterior iliac crest from healthy volunteers, donors of allogeneic bone marrow for transplantation to patients with leukemia, and from untreated patients with solid tumors, whose bone marrow was not involved with tumor. Bone marrow aspirates were collected in 2 ml of CMF-HBSS containing 50 units of preservative-free heparin (Fisher Scientific Co., Houston, TX). Fifty-six breast tumor samples were selected for this study among 105 specimens collected from patients with Stage III or IV disease during a 12-month period. Eight samples originated from primary and 33 from metastatic solid tumors, and 15 originated from pleural or ascitic fluids. Fourteen patients were untreated at the time of sample collection, and 42 had previously received between 1 and 5 treatment regimens. Tumor samples were collected in 10 ml of culture medium containing 15% FCS. An informed consent was obtained before specimens were acquired for experimentation.

Chemical Agents. Antitumor agents were obtained from the following manufacturers: doxorubicin (Adriamycin) and 4'-epidoxorubicin, Farmitalia Carlo Erba S.D.A., Italy; dihydroxanthracenedione (mitoxantrone; CL232-315) and 9,10-anthracenedicarboxaldehyde (bisantrene; CL216, 942), American Cyanamid Co., Pearl River, NY; and vinblastine, Eli Lilly and Co., Indianapolis, IN.

GM-CFU Assay. Mononuclear bone marrow cells were separated by Ficoll-Hypaque density centrifugation (density, 1.077) to obtain light-density cells. The cell fraction was further depleted of cells adherent to plastic and of cells that formed rosettes with sheep RBC, according to the method described by Weiner et al. (24). A semisolid double-layer agar system, modified from that originally described by Bradley and Metcalf (1) and Pluznik and Sachs (17) was used for cultures (23). Briefly, underlayers were prepared by combining equal volumes of 2.4 x α-MEM (K. C. Biological, Kansas City, KS), to which 30% FCS and 10% human placental conditioned medium as a source of colony-stimulating factor had been added, and of 1.0% agar (Bacto-Agar; Difco Laboratories, Detroit, MI). One-ml volumes of this mixture were poured into 35-mm Corning Petri dishes and, after solidification, were overlayered with 1-ml volumes of the upper layer mixture, which was composed of α-MEM, 15% FCS, and 0.3% agar, and to which 106 mononuclear cells admixed.

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with graded drug concentrations had been added. Triplicate cultures were obtained for each concentration and were incubated for 8 days in a humidified atmosphere of 7% CO₂ at 37°C.

**BT-CFU Assay.** Solid tumor tissue samples were debrided and diced into 1-mm cubes with scalpels, and single cells were teased into suspension with a 25-gauge needle. The cells were then suspended in an enzyme mixture of type III collagenase, elastase (Worthington Biochemical Corp., Freehold, NJ) and DNase (Sigma Chemical Co., St. Louis, MO) at a final strength of 1.0, 0.3, and 0.005%, respectively, for 16 hr at 37°C under continuous agitation. Cells obtained from malignant effusions were treated in the identical manner, with the exception that no elastase was used for their dissociation. After enzyme incubation, cells were washed in CMF-HBBS and set into semisolid suspension cultures, using the method described by Hamburger and Salmon (6, 7), adapted for the growth of breast tumors. These culture modifications for breast tumors are described elsewhere (9). Briefly, a 50:50 mixture of nutrient mixtures, Ham's F12 medium, and Dulbecco's modified Eagle medium (Grand Island Biological Co., Grand Island, NY) supplemented with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, 50 ng of epidermal growth factor/ml, 10 μg of bovine crystalline insulin/ml (Collaborative Research, Inc., Waltham, MA), 5 x 10⁻⁷ M 17-β-estradiol, and 25 μg of hydrocortisone/ml (Sigma Chemical Co.), was used as a plating medium for the underlayers, and α-MEM for the upper layers. Conditioned medium was derived from supernatants of 3 established human breast cancer cell lines (MDA-MB 231, MDA-MB 435, and MDA-MB 468), and was added to the underlayers; 10% horse serum was admixed to the underlayers and 15% FCS to the upper layers. FCS was heat inactivated prior to use, but horse serum was not. Cultures were incubated in a fully humidified atmosphere of 7% CO₂ in air at 37°C for 14 days. One control plate was fixed with 0.5 ml 3% glutaraldehyde and stored at 4°C to serve as reference for clump contamination.

**Drug Exposure.** All drugs were reconstituted to a 100 x stock solution with CMF-HBSS. Further dilutions were made in 2 x a-MEM. Two-ml aliquots of the 2 x final concentrations were stored at -70°C. At the time of experimentation, the aliquots were thawed, and agar and cells were added. Triplicate cultures were obtained for bone marrow progenitors, and one to 3 cultures for tumor progenitors.

**Scoring of Colonies.** GM-CFU (aggregates of 40 or more cells) were scored, using an Olympus SZ dissecting microscope. BT-CFU were scored with an Olympus IMT inverted microscope at x40. Aggregates with a uniform morphology that had ≥40 cells or a smallest diameter of ≥75 μm at x40, or both, were counted as colonies. The glacialaldehyde-fixed plates were scored using the same criteria, and the number of clumps so enumerated were subtracted from the scores of culture plates. Cultures with less than 10 colonies were not evaluated for tumor sensitivity.

**RESULTS**

The in vitro characteristics of bone marrow and tumor cultures are illustrated in Table 1. The plating efficiency for granulocyte-macrophage progenitors was higher and more predictable. The coefficients of variations of replicate plates were high, particularly within the colony-forming range, where drug sensitivity determinations are generally made. The median plating efficiency of the 56 tumors used for this study was 0.0144% (0.0100% for malignant effusions and 0.0146% for solid tumors). There was no difference in plating efficiency of tumors derived from patients who had been treated and those who had not been treated (0.0154 and 0.0144%, respectively). In spite of the use of enzymatic tissue disaggregation, pure single-cell suspensions were not obtained from all specimens. Refrigerated control plates were therefore used, as described, to eliminate artifacts in the sensitivity determination of clonogenic tumor cells, generated by aggregates of cells in the cell suspension.

The response of bone marrow cells from 4 different normal donors to mitoxantrone is illustrated in Chart 1. Surviving fractions of progenitors followed a steep log-linear relationship over an initial dose range which produced a 0 to 60% cell kill. The effect per unit dose beyond that range was less, and also more variable. These biphasic dose responses, with an initial steep and reproducible phase and a subsequent shallow and variable phase, were observed for all drugs tested.

Chart 2 illustrates the different cytotoxic effects, on a weight basis, of 4 substituted anthraquinone derivatives. As shown in Table 2, the dose ranges of comparable in vitro activity could not be predicted from pharmacokinetically derived parameters, even for structurally related compounds.

In order to test the survival of GM-CFU after exposure to any one of the five
Initially, the effects of the 4 substituted anthraquinone derivatives were measured on breast tumors derived from 46 patients. Drugs tested, e.g., bisantrene, is plotted in units of D50 of bisantrene for these cells, dose bisantrene/D50 bisantrene, a straight line results (Chart 3, dashed line). The same straight line describes the survival of GM-CFU after exposure to any of the other drugs, if the dose is normalized to the corresponding D50 of that drug. In other words, the units of the abscissa are dimensionless doses, computed by dividing the dose of a given drug by its D50.

The solid curves in Chart 3 show the response of a tumor to 3 drugs, with doses scaled to the respective D50s, as described in the previous paragraph. [Although the results of the comparisons would be independent of choice of scaling dose in the range where the log-linear survival model is valid (0 to 60% of GM-CFU kill), examination of the confidence bands in Charts 1 and 2 shows that D50 is defined with as good precision as any other choice.] In theory, log-linear survivals are also expected for tumor progenitors. However, the observed survival curves more often followed a biexponential course, as sketched. Possible explanations for this effect include small sample size (adequate for testing of either one drug at 4 concentrations in triplicates, or 4 drugs at 3 concentrations in single determinations), and contamination of the single-cell suspensions with cell clumps. Because of these variabilities observed with tumor cultures, 2 measures of sensitivity were used in the comparisons. In the first, the surviving fractions of BT- and GM-CFU after exposure to the D50 of a given drug for normal bone marrow were compared. In the second, for each drug the areas enclosed by the BT- and GM-CFU survival curves from 0 to 60% cell kill of GM-CFU, were estimated and compared to clinical response. The area is negative for tumors which are more sensitive than bone marrow ("S"), zero for tumors which are equally sensitive to bone marrow ("E"), and positive for tumors which are less sensitive than bone marrow ("R").

Initially, the effects of the 4 substituted anthraquinone derivatives were measured on breast tumors derived from 46 patients. Using the D50 method of comparison, 39% of the tumors were more sensitive than normal bone marrow to Adriamycin, 31% to 4'-epidoxorubicin, 28% to mitoxantrone, and 26% to bisantrene. These observed in vitro sensitivities of breast tumors correlate with the clinically observed responsiveness to these agents of patient populations similar to that from which the tumors were derived (3, 10, 16, 21, 25). Whereas the majority of these tumors showed an equal sensitivity pattern to Adriamycin and to analogues (23% sensitive to all 4 drugs and 45% resistant to all 4 drugs), in 32% of tumors the sensitivity to one or more analogues differed from the sensitivity to Adriamycin.

The in vitro sensitivities determined on the tumors of these 46 patients were then grouped according to (a) whether the patients had been exposed to Adriamycin; and if so, (b) whether they were clinically sensitive or resistant. The 14 patients of Group A (Chart 4) had no prior exposure to Adriamycin, and about 40 to 50% of these patients are expected to respond to treatment with Adriamycin (8, 13). The 12 patients of Group B had received Adriamycin in the past, but were considered to be still sensitive to it at the time the tumor samples were collected, while the 20 patients in Group C were clinically resistant to Adriamycin. Fifty % of tumors in Group A were more sensitive to Adriamycin than was normal bone marrow, whereas of those in Group B, 67% were more sensitive, and of those in Group C, only 10% were more sensitive than was normal bone marrow. Chart 4 demonstrates that normalized in vitro testing gave results for Adriamycin.
mycin in rough accord with what was expected clinically (3, 8, 13). Moreover, it appears that, at least in vitro, tumor sensitivity to the substituted anthraquinone derivatives was similar to the tumor sensitivity to Adriamycin. That is, tumors of patients who were clinically sensitive to Adriamycin were more sensitive than was normal bone marrow to 4'-epidoxorubicin in 75% of instances, to mitoxantrone in 70% of instances, and to bisantrene in 71% of instances. In contrast, tumors of patients that were clinically resistant to Adriamycin were more sensitive than was normal bone marrow to 4'-epidoxorubicin in 11% of instances, to mitoxantrone in no case, and to bisantrene in 29% of instances. This correlates with our limited clinical experience with these agents.

Twenty-one of the 46 patients whose tumors were assayed for drug sensitivities received subsequent treatment with single-agent chemotherapy. Table 3 illustrates the associations of normalized in vitro drug sensitivities of those tumors with the clinical response to the agent tested (results were the same with both measures of comparisons in 18 of 21 instances). Adriamycin was tested in 3 instances, 4'-epidoxorubicin in 6 instances, bisantrene in 8 instances, and vinblastine in 4 instances. The concordance of findings was 71%.

In view of the log-linear response of the BT-CFU over an initial dose range (Chart 3), drug-induced cell killing may in part be a random phenomenon. Therefore, as tumor size increases, it may be expected that BT-CFU sensitivity will become less effective as a predictor of clinical response. The findings presented in Table 4 are consistent with these remarks. Concordance decreased from 85 to 50% when in vitro sensitivity was compared to clinical response in patients with 3 or more metastatic sites. As shown in Table 5, this disadvantage could be partly offset by downgrading the in vitro classification of response. Thus, "S" and "E" in vitro are unchanged for small (one or 2 metastatic sites) tumors but can be decreased to "E" and "R," respectively, for large tumors. When this rule of thumb is applied, the overall concordance was increased to 90% (the computation of significance is not meaningful for such small patient numbers and has therefore been omitted).

In 6 of the 21 patients, sample sizes were large enough to allow testing at 3 dose levels: (a) D50 for normal bone marrow; (b) conventional pharmacokinetically derived doses (5 to 10% of peak plasma concentration); and (c) nonphysiological dose range (100 x D50 for normal bone marrow). Table 6 shows that the predictive value for clinical response of the clonogenic tumor cell assay ranked better if derived from testing at doses of comparable in vitro drug activity. The in vitro activities of pharmacokinetically derived doses was unpredictable for different drugs. Thus, the inhibition of granulocyte-macrophage colony formation for Adriamycin was 25%, and it was greater than 99% for bisantrene. Testing at high doses, which are of no clinical relevance, gave concordant results in only 1 of 6 of the cases (17%). Twenty-nine of 30 drug sensitivities determined on breast tumors of 10 patients were equally classified when related to the patients' own bone marrow and to the standard tolerance curves derived from normal donors, as described.

**DISCUSSION**

Standardization of in vitro drug activity is essential for comparative drug evaluation. Established tumor cells are not suitable for this purpose, since their altered metabolism is representative...
neither of normal tissue nor of the majority of primary tumors. In contrast, bone marrow represents the dose-limiting host tissue organ for most anticancer agents. Therefore, generation of an in vitro therapeutic index that compares inherent drug sensitivities of tumor and bone marrow progenitors has a rationale in tumor biology. Since these progenitors recover from cytotoxic insults to a degree and at a rate determined by their inherent sensitivity, it is reasonable to assume that only agents that affect tumor cells more profoundly than bone marrow cells can effectively reduce the tumor burden. The validity of this assumption has, in fact, already been shown for leukemic cells (15); similar therapeutic ratios are beginning to be applied to drug sensitivity determinations of solid tumors (11, 22).

The results of this preliminary study involving small numbers of patients indicate that the predictive accuracy of normalized in vitro comparison is at least as good as that of the conventional use of pharmacologically derived doses. Thus, in a sample of 21 patients, a concordance of 71% was obtained, which could be increased to 90% when account was taken of tumor bulk (Tables 3 to 5). Further, in the 6 patients suitable for testing at dose levels corresponding to conventional pharmacokinetically derived doses and to doses normalized to D50s for normal bone marrow, the respective concordance rates were 50 and 67% (Table 6). Although these patient numbers are too small to allow any definite conclusions, the results would encourage further study of normalized in vitro comparisons as an alternative predictive assay of tumor response.

In vitro efficacies of pharmacokinetically derived concentrations are unpredictable, and nonphysiological concentrations are of no clinical relevance. Therefore, the possibility of both false-positive predictions from testing at too high doses and false-negative predictions from testing at in vitro inactive doses can be circumvented by normalizing the drug activities.

It must be born in mind that attempts to correlate clinical response with in vitro sensitivity are affected by complicating factors, among which tumor mass and the interpretation of tumor response may be mentioned.

It has been suggested that tumor mass negatively influences the outcome of chemotherapy because of the development of resistant tumor cell lines (4). However, the negative influence of tumor mass (i.e., number of clonogenic tumor cells) also stems from the fact of proportionate killing, as indicated by log-linear dose-response curves (Chart 3). That is, the dose necessary to achieve a clinically complete response or cure increases with the number of clonogenic cells. Therefore, in addition to the sensitivities of the heterogeneous populations that comprise the clonogenic cells of the tumor, clinical response is limited by sheer cell number, whatever their sensitivities, and a confounding influence may be expected of it when using in vitro sensitivity as a predictive assay of tumor response, as is clear from Tables 3 to 5. There is extensive clinical evidence for the influence of tumor mass on tumor response, both in the area of chemotherapy (12) and of radiotherapy (2).

The interpretation of tumor response is complicated by the fact that it does not necessarily parallel intrinsic drug sensitivity. This is because "response" (volume change after treatment) depends not only on the number of cells killed, but also on tumor proliferation kinetics, tumor architecture, the mode of cell death, and the efficiency of clearance of dead cells from the tumor mass (16). Therefore, even if the confounding effect of tumor mass can be accounted for (as in Tables 3 to 5), attempts to correlate in vivo and in vitro "sensitivity" will inevitably be clouded by the uncertain connection between volume regression and effectiveness of treatment.

Bone marrow and tumor progenitor cells can be recovered in comparable in vitro culture conditions. The high and consistent plating efficiency of CM-CFU allows accurate normalization of in vitro activity of agents whose clinical dose-limiting factor is myelosuppression. Relative drug sensitivity determination of tumor versus bone marrow progenitors also has technical advantages. The mode of drug exposure becomes irrelevant in a relative test system, and continuous drug exposure, which requires the least amount of tumor tissue and is associated with the fewest technical errors, can be used throughout. Uniform handling of bone marrow and tumor progenitors make observations of single survival determinations obtained from small tumor samples more reliable. We were able to test an average of 4 drugs at 3 concentrations, and to evaluate sensitivity determinations from 80% of all tumor samples.

Dose-survival responses of bone marrow progenitors from different donors were reproducible over a cell kill range of 0 to 60% for all drugs tested, and did not change to a major degree after in vivo exposure to anticancer agents. Responses at higher dose ranges were more varied, and several factors can account for these observed variations. Coefficients of variations become larger as the number of surviving colonies decreases. Uptake and cytotoxic effects of anticancer agents are determined by cell
membrane properties and by cell cycle phase, and heterogeneity in cell cycle activity of individual samples can affect dose responses. Variability of response at higher exposure doses is even more pronounced in the case of tumors, because of clump contamination of the single-cell suspension, heterogeneity in cellular composition, and low plating efficiency (Table 1).

The in vitro effects on normal bone marrow progenitors of the 5 anticancer agents investigated were quite dissimilar, in spite of the structural relationship of 4 of them, and they did not correlate to any degree with their in vivo pharmacological parameters. Differences in tertiary structures and in net electrical charges result in variable degrees of drug binding to culture components and to plastic ware, thereby modifying the cellular drug exposure.

We conclude that normalization of in vitro drug activities by their effects on the dose-limiting normal host tissue allows comparison of the antitumor effects of newly developed agents to those of established clinical efficacy by means of a therapeutic ratio when tumor load is accounted for, and provides a basis to select rationally, among several drugs tested, the drug with the greatest likelihood of clinical activity.

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REFERENCES

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