A Quantitative Model for the Study of the Growth and Treatment of a Tumor and Its Metastases with Correlation between Proliferative State and Sensitivity to Cyclophosphamide

William D. DeWys

Department of Medicine, Division of Oncology, University of Rochester School of Medicine and Dentistry, Strong Memorial Hospital, and Rochester General Hospital, Rochester, New York 14642

SUMMARY

An experimental tumor model system has been designed to permit quantitative study of the growth of a solid tumor and its metastases and quantitative study of the response of this tumor and its metastases to chemotherapy. The Lewis lung carcinoma was transplanted i.m., and tumor growth was followed by serial diameter measurements; these data were then converted to tumor weight by means of a determined conversion equation. The total number of metastatic tumor cells in lung was determined by a quantitative transplant bioassay which involved comparison of tumor growth in bioassay recipients with tumor growth in a series of bioassay standards that had received graded doses of tumor cells under similar transplant conditions.

This model was used to study the influence of tumor growth rate and location on sensitivity to cyclophosphamide. The most rapidly growing tumor cells were most sensitive to cyclophosphamide, and the slowly growing tumor cells were least sensitive. A direct correlation was observed between tumor-doubling time and sensitivity to cyclophosphamide. When sensitivity was correlated with differences in tumor growth rate, there were only minor differences in sensitivity related to tumor location. Treatment of early tumors produced a dose-related increase in survival, but treatment of late tumors often shortened survival, especially at the highest doses tested. This evidence of increased toxicity for late treatment, together with the decreased tumor sensitivity with late treatment, may serve as a model for increased interest in chemotherapy of early cancer.

INTRODUCTION

In clinical studies of the chemotherapy of metastases developing after surgical removal of a primary tumor (adjuvant chemotherapy) there have been reported variable degrees of success. In some studies, postoperative chemotherapy has been of relatively little value (5, 19). In other studies, somewhat more encouraging results have been observed in selected subgroups (11, 13) or as a general trend (6, 18, 26). One important determinant of response is the cell type of the tumor being treated (14). Other as yet poorly understood factors may also influence the response of metastatic tumor cells to chemotherapy.

For an experimental study of those factors that may influence response of metastases to chemotherapy, an animal model system has been devised that uses a transplantable tumor in which lung metastases develop (23). This model system has been used for comparison of preoperative and postoperative adjuvant chemotherapy (22) and for a study of the effect of drug dose and treatment schedule on response of metastases to chemotherapy (20). Combinations of radiation therapy and chemotherapy have also been studied in this model system (21).

Several refinements in this model system have been developed for evaluation in greater detail of the relationships between the growth of a local tumor and its metastases, as well as the response of both a solid tumor and its metastases to chemotherapy. These refinements include alteration of the transplant technique for initiating the local tumor, development of a method of measuring quantitatively the growth of the local tumor on a weight basis, and development of methods of quantitating the growth of metastases. These methods were used for measurement of dose-response relationships for cyclophosphamide treatment of a solid tumor and its metastases. In a subsequent report, the interrelation between the growth rate of a solid tumor and the growth rate of its metastases (8) will be presented.

MATERIALS AND METHODS

Mice. Male C57BL/6 mice (Jackson Memorial Laboratories, Bar Harbor, Maine) were used at 8 to 12 weeks of age. They were housed 5 to 10/plastic cage and provided with Purina laboratory chow and tap water ad libitum.

Tumor. The Lewis lung carcinoma (29) is maintained in our laboratory by i.m. transplant every 2 weeks in C57BL/6 mice. For tumor transplantation, tumor tissue was removed...
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Chart 1. Relationship between tumor weight and the cube of tumor diameter based on measurement of 100 tumors of different sizes. Regression equation determined by computer analysis.

Table 1

Effect of normal tissue on transplantability of Lewis lung carcinoma

For measurement of the effect of normal tissue on bioassay sensitivity, bioassay standards were prepared by injecting tissue indicated into hind legs of mice, followed by injection of indicated number of tumor cells.

<table>
<thead>
<tr>
<th>No. of tumor cells implanted</th>
<th>Tissue transplanted prior to tumor transplant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
</tr>
<tr>
<td>$10^5$</td>
<td>5/5a</td>
</tr>
<tr>
<td>$10^4$</td>
<td>4/7</td>
</tr>
<tr>
<td>$10^3$</td>
<td>.0/8</td>
</tr>
<tr>
<td>$10^2$</td>
<td>.0/8</td>
</tr>
</tbody>
</table>

| $10^1$                      | .2/6 |       |       |        |

| $10^0$                      |       |       |       |        |

*a Number of “takes” over number of mice given injections. Data from 3 experiments are shown separated by commas, with individual experiment values aligned vertically.

Asectedly, cut into small pieces, pressed through a cytosieve (U.S. Standard Sieve Series, 100 meshes/inch, Dual Mfg. Co., Chicago, Ill.), and then suspended in Eagle's medium (minimum essential medium, Microbiological Associates, Inc., Bethesda, Md.). This suspension was then passed successively through wire cloths of 66- and 25-μm pore size (stainless steel wire cloth, 150 mesh/inch and 400 mesh/inch, Wire Cloth Enterprises, Inc., Pittsburgh, Pa.) to yield a suspension composed predominantly of single cells. Cells were counted with trypan blue exclusion and appropriate adjustments were made in the cell concentration. Experimental mice were given injections in the right hind leg muscles of $2 \times 10^5$ cells in 0.02 ml with a Hamilton microliter syringe and a 25-gauge needle.

Drug. Cyclophosphamide (NSC 26271), 2-bis(β-chlorethyl)amino-2H-1,3,2-oxazaphosphorinane, 2-oxide, received from Mead Johnson Co. (Evansville, Ind.) through the Cancer Chemotherapy National Service Center, was dissolved in sterile 0.9% NaCl solution immediately before use. The appropriate concentrations were injected i.p. in a volume of 0.01 ml/g body weight.

Tumor Measurement. For tumor growth data, tumor diameters were measured 3 times/week with calipers, and the mean of 2 measurements at right angles was recorded. For determination of the relationship between tumor diameter and tumor weight, tumor weight was derived as the difference between the weight of the excised tumor-bearing leg and the weight of the excised contralateral leg. Data comparing concomitant measurement of tumor diameters and tumor weight were subjected to regression analysis with an IBM 360 Computer.

Quantitative Tumor Cell Bioassay. Tissues from tumor-bearing mice were transplanted into the right hind leg of bioassay recipients with a tuberculin syringe and 19-gauge needle as follows. The tissue to be assayed was inserted into the syringe, followed by a small amount of Eagle's medium. The entire specimen was then forced through the needle into the leg muscle. Tumor growth in the bioassay recipients was measured 3 times/week with calipers. Tumor growth in bioassay recipients was compared with tumor growth in a series of bioassay standards. Bioassay standards for each experiment were prepared by first injecting 1 normal lung, 1 kidney, or 0.2 ml of liver tissue into the right hind leg and then injecting a known number of tumor cells into this transplanted tissue. This approach was used because technical difficulties prevented accurate mixing of tumor cells with normal tissue prior to injection.

Response to Drug Treatment. Response to cyclophosphamide for lung metastases and for the leg tumor while it was small was studied by quantitative transplant bioassay 24 hr after a single dose of cyclophosphamide. Response to cyclophosphamide for the tumor implant in the leg at 3 stages of growth was determined by following the

Chart 2. Effect of the presence of normal tissue on outgrowth of $10^5$ transplanted Lewis lung carcinoma cells. Control received tumor cells only while other groups received a transplant of designated tissue prior to transplant of tumor cells. Number of takes is shown as 1st set of data in Table 1.
outgrowth of the tumor after drug treatment. From this outgrowth curve, the number of tumor cells surviving treatment was estimated by a backward extrapolation to the time of treatment. Because tumor growth was not a simple exponential (8), an assumption has been made in this backward extrapolation. The assumption is that the cells surviving treatment recapitulate the growth rate of a similar number of cells in an untreated tumor. An early stage of the solid tumor was studied by both transplantation bioassay and the outgrowth method; and, by comparing these 2 studies, the reader may assess the validity of the assumption of backward extrapolation. The validity of backward extrapolation is also supported by work in other laboratories (24, 25, 31). During the latter stages of tumor growth in treated groups, the measured data did recapitulate the control tumor growth curve. For facilitation of comparison between various treatment groups, the appropriate control values were normalized, and the data were then presented in survival fraction form.

RESULTS

The correlation of mean tumor diameter and tumor weight for 100 tumors of various sizes is shown in Chart 1. The computed equation of the regression line was \( W = 0.82 \left( D^3 - 0.22 \right) \). The S.D. of the intercept was 0.0848, and the S.D. of the slope was 0.0266. The derived equation was used in subsequent experiments for conversion of tumor diameters to tumor weight.

Because previous investigators had shown that transplantation of normal tissue with tumor cells enhanced the growth of the tumor cells (27), the effect of concomitant transplantation of normal tissue with Lewis lung tumor cells was studied as a preliminary to transplantation bioassay. Normal tissue, specifically, 1 lung, 1 kidney, or 0.20 ml of liver, was transplanted into the right hind leg of groups of mice. Groups then received 10-fold dilutions of viable tumor cells into this transplanted tissue. The transplantability with different tissues is shown in Table 1, and the growth rate of a representative series of tumors is shown in Chart 2. There was a significant increase in the number of "takes" in groups that received a transplant of normal tissue prior to the transplant of tumor cells in comparison with groups receiving no tissue (Table 1). However, the type of normal tissue transplanted did not significantly affect the results (Table 1). The tumor growth pattern was not different for the several types of tissue transplanted (Chart 2). The presence of any of the tissues transplanted tended to accelerate the time of appearance of the tumor while the rate of growth was similar (Chart 2). Because these several tissues were similar in their effect, in subsequent experiments lung tissue was used in preparation of bioassay standards.

A representative set of bioassay standards is presented in Chart 3 showing tumor growth after graded doses of cells from \( 10^1 \) to \( 5 \times 10^6 \). These data were used for construction of a series of isodiametric lines, as shown in Chart 4. This form of the data could then be used to convert tumor size in experimental bioassay recipients to estimated number of viable tumor cells transplanted. This form also permits interpolation between the values of the log numbers of cells and, perhaps, extrapolation beyond the range of the bioassay standards.

After the above methodology was established, an experiment was designed for study of the sensitivity of the Lewis lung carcinoma to cyclophosphamide at different stages of tumor growth. In this experiment, the response of the leg tumor was measured by outgrowth of the tumor after
treatment, and the response of the lung metastases and of the leg tumor while it was small was measured by transplant bioassay 24 hr after a single dose of cyclophosphamide. A representative set of data illustrating tumor outgrowth and back extrapolation are presented in Chart 5.

The dose-response curves for the different treatment groups are presented in Chart 6. The dose-response curves for the Day 8 leg tumor obtained by the 2 methods show good agreement, thus supporting the validity of backward extrapolation. For the family of curves describing the response of the leg tumor to cyclophosphamide (Chart 6), there was a progressive decrease in antitumor effect with later treatment (Day 21) in comparison with earlier treatment (Day 15 or Day 8). Similarly, for the family of curves for treatment of lung metastases there was a decrease in sensitivity to cyclophosphamide with delay in treatment from Day 15 to Day 21.

Since a progressive change in growth rate as the tumor increased in size was observed for both the leg tumor and lung metastases (8), it seemed of interest to correlate this change in growth rate with the changes in drug sensitivity. A measure of the sensitivity to cyclophosphamide was obtained by reading from Chart 6 the dose of cyclophosphamide producing a surviving fraction of 0.10. The tumor growth rate at the time of each treatment was estimated as the slope of the tangent of the growth curves presented in the companion paper (8). The correlation of sensitivity with growth rate is presented in Chart 7. There was an apparent direct correlation between the dose of cyclophosphamide giving a 90% cell kill and tumor-doubling time (Chart 7).

The survival data for this experiment are presented in Table 10.

DISCUSSION

The model described herein has several refinements over that described previously for this same tumor (23). First, the pattern of tumor growth at the site of implantation, as well as the pattern of growth of metastases, can be determined.
Cyclophosphamide dose shown is that dose required to reduce tumor cell population by 90% according to the dose-response curves in Chart 6.

Several details of the present model merit comment. First, the concomitant transplantation of normal tissue or nonviable tumor tissue in the transplant bioassay may be expected to influence the outgrowth of tumor in the bioassay recipients (27). The bioassay standards were prepared with an amount of normal tissue equal to the amount of normal tissue transplanted with the experimental bioassay tumor, thus approximating closely the transplant conditions of the tumor cells being assayed. In assays of treated groups, the volume of nonviable tumor cells transplanted was considered to be small in comparison with the volume of normal tissue transplanted, and therefore the accelerating effect of this small amount of drug-killed tumor tissue may be expected to be minimal in comparison with the effects of the normal tissue. The mechanism of this acceleration of tumor growth by normal tissue is presently incompletely understood, but a nutritional medium may be provided to the tumor cells by the concomitantly transplanted normal tissue or nonviable tumor tissue (27).

A second aspect of this model is the range of sensitivity of transplant bioassay. The lower limit of the assay can be judged from Table 1, which shows that the majority of bioassay standards were positive with $10^3$ cells, slightly more than one-half were positive with $10^2$ cells, and a small number were positive with $10^1$ cells. The technical upper limit of assay standards is greater than $10^9$, so that the assay is sensitive over more than $5 \log_{10}$ of tumor cells. This is in contrast to previous studies in which bioassay was used as a qualitative assay only (15, 16, 23) or was sensitive over $3 \log_{10}$ tumor cells (12). The present method differs from that of Donelli et al. (12) in that it is not limited by the day of sacrifice of bioassay recipients, and therefore the range of sensitivity has been extended.

Bruce et al. (1) have suggested that the proliferative state of a cell population may be a determinant of sensitivity to cyclophosphamide. In their study, rapidly proliferating lymphoma cells were more sensitive to cyclophosphamide than were more slowly proliferating hematopoietic colony-forming cells. However, since their study used 2 different cell types, other factors could have influenced this observed difference in sensitivity. In a previous study from our laboratory (cell type (hematopoietic colony-forming cell) in different proliferative states was tested for sensitivity to cyclophosphamide (9). Rapidly growing hematopoietic colony-forming cells were most sensitive to cyclophosphamide, while slowly growing hematopoietic colony-forming cells were least sensitive. The present results are consistent with the conclusion that the proliferative state of a tumor cell population is a determinant of sensitivity to cyclophosphamide. The early treatment (Day 8 for solid tumor or Day 15 for lung metastases) was given while the tumor was in a state of rapid proliferation, and this treatment produced the most marked antitumor response (Chart 6). Later treatments were given to a tumor with a successively slower rate of proliferation, and these treatments required progressively larger doses of drug for a given degree of antitumor response (Charts 6 and 7).

The response of a tumor to chemotherapy must be considered in terms of tumor blood supply in addition to considering the role of the proliferative state of the tumor cells. In a histological study, Tannock and Steel (30) have reported a progressive reduction in the relative vascularity of a tumor as it increased in size. Similar findings were reported by Guillin (17) from tumor perfusion studies. From these studies, one concludes that a large tumor may receive proportionately less blood supply and therefore less drug on a
tumor weight basis, and this would lead to a requirement of a larger dose of drug for a given antitumor effect. Further study is needed to determine to what extent the changes in sensitivity of the leg tumor observed herein may be related to changes in relative blood supply, and to what extent these changes are related to changes in the proliferative state of the tumor population.

For the tumor cells in lung metastases, the change in growth rate is thought to be determined by systemic tumor-related factors and not by the local blood supply (8). Thus, the response of these lung metastases to cyclophosphamide may reflect the role of the proliferative state as a determinant of sensitivity to cyclophosphamide. The tumor cells in lung on Day 15 were in a rapid proliferative state and were more sensitive to cyclophosphamide, while the cells on Day 21 were in a less active proliferative state and were less sensitive to cyclophosphamide.

Possible clinical correlates of the present observations of a change in sensitivity to cyclophosphamide with changes in tumor size and growth rate have been reported for Burkitt’s tumor. Burkitt (2) reported total clinical regression in 6/6 patients with small Burkitt tumors, 10/27 patients with moderate-sized tumors, and 8/30 patients with large tumors. Similarly, Clift et al. (3) reported that Burkitt tumor response depended on the extent of the disease.

Previous workers have suggested that there may be a difference in sensitivity to cyclophosphamide related to the anatomical site of the tumor. Karrer et al. (23) suggested in a survival time study with the Lewis lung carcinoma that lung metastases may be more sensitive to cyclophosphamide than the same tumor implanted in the leg. In contrast, Conzelman and Springer (4) concluded from a study comparing survival time response of treated lung metastases with a tumor weight inhibition assay for a s.c. tumor SAH 1-1 that the s.c. tumor was more sensitive to cyclophosphamide than were lung metastases. The present results (Chart 7) suggest that lung metastases may be more sensitive than the leg tumor, but this difference is small compared with the difference in sensitivity related to differences in tumor doubling time. This apparently minor difference in sensitivity for different sites may be related to differences in tumor blood supply, as discussed above. The conflict between the conclusion of Karrer et al. and those of Conzelman and Springer may be due to differences in sensitivity related to tumor growth rate or to differences in the significance of the assay end point. Tumor weight inhibition does not always correlate well with survival time increase (10). The present results suggest that, when an appropriate assay is used and sensitivity is correlated with tumor growth rate, there is only a minor difference in sensitivity related to anatomic site. This generalization requires further testing and may not apply to certain privileged sites, such as across the blood-brain barrier (28).

Finally, the survival time response observed herein deserves comment. A dose of cyclophosphamide (240 mg/kg) which was nonlethal to tumor-free animals or to animals with early tumor killed the majority of animals bearing advanced tumors (Table 2). Since the majority of the deaths after treatment on Day 15 or Day 21 occurred within 6 days of treatment, the possibility of death from hematopoietic toxicity is suggested (9). Whether this reflects increased initial host toxicity or delay in the host recovery sequence is currently under study. It is possible that the systemic factors that may be contributing to slowing of tumor growth (8) may also slow the normal host recovery mechanisms. The findings of increased host toxicity for late treatment, together with the decreased antitumor effect for late treatment, may serve as a model, giving impetus to increased interest in vigorous chemotherapy of early cancer. The present results, together with the observations on changes in growth rate of metastases related to removal of the primary tumor (8), may be useful in planning future surgical adjuvant studies.

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