Changes in Growth Rate of an Experimental Solid Tumor Following Increasing Doses of Cyclophosphamide

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ABSTRACT

Well-defined times for minimum and maximum growth rates of rat hepatoma 3924A were found when cyclophosphamide doses were increased from 50 to 250 mg/kg. Minimum growth rates occurred 8 to 10 days after treatment and maximum growth rates occurred 13 to 16 days after treatment in 5 groups of 10 animals each that received 50, 100, 150, 200, and 250 mg of cyclophosphamide per kg. Tumor growth delay increased from 6 to 18 days when the dose was increased from 50 to 250 mg/kg. The accelerated growth rate of treated tumors on Day 14 after cyclophosphamide (150 mg/kg) was more than twice that of controls on the same day, 0.349 ± 0.030 (S.E.) (day^-1) and 0.156 ± 0.011 (day^-1), respectively. In addition, the accelerated growth rate of treated tumors on Day 14 was greater than that of controls at initiation of treatment, 0.286 ± 0.026 (day^-1) on Day 2. Increased DNA synthetic rate preceded increased tumor cell proliferation, which, in turn, increases tumor volume. Thus, biochemical studies demonstrating increased cellular proliferation confirm accelerated tumor growth following treatment.

INTRODUCTION

The change of tumor volume with time after treatment is one of the basic measurements for assessing therapeutic response. Analysis of tumor growth curves provides important theoretical and practical information for clinical and experimental oncology. We have developed a computer program to mathematically describe changes in tumor volume and growth rates following treatment (14). Tumor volumes are fitted with a polynomial by the method of least squares. Tumor growth rate at any time can be determined by taking the first derivative of the polynomial with respect to time.

Studies in patients with lung cancer have shown that an accelerated increase in tumor volume occurs 10 to 35 days after a reduction in tumor volume following single and fractionated radiotherapy (15). The time for minimum tumor volume was usually 5 to 7 days after radiation in patients with primary or metastatic disease. Studies on metastatic lung involvement from a variety of primary cancers in dogs showed a similar accelerating increase from 7 to 20 days in tumor volume after a reduction following radiation treatment (15). In dogs, the greatest volume reduction occurred 5 to 6 days after radiation. An acceleration of tumor volume changes was also demonstrated in mice with artificially induced metastases and following radiation of a primary rat rhabdomyosarcoma (3, 15).

Previous studies in this series have shown that the time for minimum tumor growth of 5 days in rat hepatoma 3924A was virtually unchanged when the 5-fluorouracil dose was increased from 50 to 250 mg/kg (5, 7). The time for maximum tumor growth of 12 days also was virtually unchanged. Following 1500 R of local tumor radiation to hepatoma 3924A, the time for minimum tumor growth rate was 8 days, and the time for maximum growth rate was 16 days (6).

For the current study, we have selected a chemotherapeutic agent from another major group of anticancer agents. Cyclophosphamide was selected from among the alkylating agents because of its broad spectrum of antitumor effectiveness and proven value in the clinical management of cancer patients (4).

MATERIALS AND METHODS

Solid Tumor Line. Repeated cell kinetic and growth studies over the past 10 years have shown hepatoma 3924A to be stable and reproducible. It is an undifferentiated tumor, and the parenchymal tumor cells are hypotetraploid. The kinetics of cell proliferation and tumor growth were as follows for tumors having volumes of approximately 200 cu mm (5): actual volume-doubling time, 96.3 hr; potential volume-doubling time, 42 hr; and cell cycle time, 27.4 hr. The different phases of the cycle were: T_G1, 14 hr; T_S, 9.3 hr; T_S, 3.7 hr; and T_U, 0.4 hr. The 1-hr thymidine-labeling index was 17.6, the growth fraction was 0.65, and the cell loss factor was 0.60. Tumors having volumes of approximately 3,000 and 13,000 cu mm yielded cell cycle times of 30.1 and 26.7 hr, respectively, with a somewhat longer G_1 and shorter S than for small tumors.3

Animals. Inbred ACI rats (Laboratory Supply Co., Indianapolis, Ind.), weighing usually 120 to 140 g, were used. Transplants of hepatoma 3924A (11) were performed by Dr. H. P. Morris, Howard University, Washington, D. C. The rats were caged individually in an air-conditioned room that was lighted from 8 a.m. to 8 p.m. and provided rat chow (Charles River Laboratories, Wilmington, Mass.) and water ad libitum. Ten rats each were used for treated and control groups.

Cyclophosphamide. Cyclophosphamide was supplied by Mead Johnson Research Center, Evansville, Ind. It was dissolved in 0.9% NaCl solution and given i.p. as a single injection.

Biochemical Studies. Groups of 3 rats were killed at various times between 1 hr and 21 days after cyclophosphamide (150 mg/kg) was given. One hr prior to killing, the rats were given i.p. injections of 50 μCi [methyl-^3H]thymidine (specific activity, 3 Ci/mmol). Tumors were chilled in cold 0.9% NaCl solution and weighed, and portions were taken for determination of.

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2 To whom requests for reprints should be addressed, at Box 392, University of Virginia Hospital, Charlottesville, Va. 22908.

3 C. J. Kovac and W. B. Looney, unpublished observations.

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DNA and \(^3\)H counts in the DNA fractions. RNA was eliminated from all samples by alkaline hydrolysis followed by washing with cold 10% trichloroacetic acid. DNA was extracted by heating at 90° for 20 min with 5% trichloroacetic acid and was measured by the method of Burton (2). Calf thymus DNA (Sigma Chemical Co., St. Louis, Mo.) was the standard. Radioactivity in the nucleic acid extracts was measured on a Beckman liquid scintillation spectrophotometer with external standardization.

**Tumor Volume Measurements and Tumor Growth Analyses.** The growth rate of the solid tumor was found through a combination of frequent tumor volume measurements and computer methods of curve fitting. Tumor volumes were determined from vernier caliper measurements of length, width, and height made daily for 1 to 2 weeks after treatment during the period of major changes in tumor growth rates. Tumors were then measured 3 times weekly until termination of the experiments. The tumor volume is well approximated by a hemiellipsoid, where volume = \(4\pi/3\cdot L/2\cdot W/2\cdot H/2\), which reduces to one-half the product of the length of its 3 axes, i.e., \(V = \frac{1}{2}LWH\). Experiments were initiated when tumor volumes reached approximately 200 to 300 cu mm. For example, tumor volumes in a typical group ranged from 188 to 287 cu mm, and no systematic relationship was found between these initial tumor volumes and other measured parameters.

A more detailed description of tumor response has been realized by introducing the concept of OTE\(^a\) for each of the 3 classes of tumor volume response: Class I, regression; Class II, pseudoregression; Class III, slowdown. OTE represents the magnitude of the tumor volume change immediately after treatment (13).

Tumor volumes are normalized to the volume on day of treatment and plotted on a semilogarithmic scale for each animal. The result is a series of graphs of

\[
\ln \left( \frac{V(t)}{V_0} \right)
\]

versus time where \(V(t)\) is the tumor volume at time \(t\) and \(V_0\) is the volume at time of treatment. These tumor volume growth curves are then fitted by the method of least squares with a polynomial of degree \(N\) whose functional form is

\[
\ln \left( \frac{V(t)}{V_0} \right) = a_0 + a_1t + a_2t^2 + \ldots + a_Nt^N
\]  

(A)

where \(N\) values of up to 6 are necessary for a proper fit. A "proper fit" is one whose \(\chi^2\) probability \([p(\chi^2)]\) falls between 5 and 95%, with the best case being a \(p(\chi^2)\) of around 50% (14). Polynomials are fit to the volume curves for each animal. Specific instantaneous tumor growth rate at any time can then be determined by taking the first derivative of the polynomial with respect to time. Equation A then becomes

\[
\frac{d}{dt} \ln \left( \frac{V(t)}{V_0} \right) = a_1 + 2a_2t + 3a_3t^2 + \ldots + Na_Nt^{N-1}
\]

In a plot of tumor growth rate over time done in this fashion, the ordinate is of the form

\[
\frac{1}{V(t)} \cdot \frac{dV(t)}{dt}
\]

Hence, the units of the ordinate are days\(^{-1}\), while the units of the abscissa are days. The value of the instantaneous growth rate on certain days is then found for each animal. The average value (±S.E.) of these measurements for the group is reported graphically (as in Charts 3 and 4). Alternatively, growth rates taken at the maximum of each individual tumor are averaged, and the data are reported in tabular form (as in Table 1).

**RESULTS**

Changes in mean tumor volumes for the 5 groups of animals given 50, 100, 150, 200, and 250 mg cyclophosphamide per kg are shown in Chart 1. Evaluation of response was made by "tumor growth delay" (the increased number of days for treated tumors to reach 1000 cu mm as compared to controls) and OTE, which quantitatively describes tumor volume changes following treatment (Table 1). The greatest change in both tumor growth delay and OTE occurred as the cyclophosphamide dose was increased from 50 to 150 mg/kg.

The mean tumor volume following 150 mg cyclophosphamide per kg was reduced below the mean volume of 228 ± 11

\[\text{DAYS AFTER INITIATION OF TREATMENT} \]

\[\text{Chart 1. Mean volume of hepatoma 3924A after treatment with 50 (O), 100 (T), 150 (O), 200 (A), and 250 (D) mg cyclophosphamide per kg. •, Controls. Points, mean for 10 animals, except when 250 mg/kg was given and only 6 rats survived; bars, S.E.}\]

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\[\text{which reduces to} \]

The abbreviation used is: OTE, overall treatment efficiency.
Solid Tumor Growth Rate Changes after Cyclophosphamide

The concentration of tumor DNA (mg of DNA per g of tumor) was reduced to approximately two-thirds of control values over the first 5 days after therapy (Chart 2A). DNA concentration remained depressed until Day 9 and then rapidly returned to control values by Day 11. Thus, the rapid increase in tumor DNA concentration after Day 9 paralleled the rapid increase in tumor volume between Days 9 and 12.

Changes in tumor growth rate following a single dose of cyclophosphamide (150 mg/kg) are shown in Chart 3. There was a marked depression in growth of treated tumors, which reached a nadir 5 days after treatment. This was followed by a marked increase in growth rate, which reached a maximum on Day 14. A second reduction in growth rate appeared to level off at 21 days after initiation of treatment. The gradual reduction in growth rate of control tumors is a reflection of the slowing of tumor growth with increasing tumor size. The experiment was terminated on Day 23; therefore, information is not available beyond this point. Tumor volume data on other experiments conducted for longer periods of time indicate that volume changes and, thus, growth rates for treated tumors eventually parallel those of controls.

Tumor DNA specific activity has been utilized in previous studies as an index of tumor cell proliferation following treatment. Tumor DNA specific activity was depressed 12 hr after cyclophosphamide (150 mg/kg) was administered (Chart 2B) and reached a nadir 3 to 4 days after treatment. Recovery began on Day 5. DNA specific activity reached a maximum on
Day 11, at which time it exceeded initial control values. The change in tumor DNA specific activity preceded the change in tumor growth rate. The nadir for negative tumor growth rate occurred on Day 5, 1 to 2 days after the nadir for tumor DNA specific activity. Maximum tumor DNA specific activity on Day 11 preceded the time for maximum tumor growth rate (Tmax) on Day 14.

The period during which the growth rates of treated tumors were above control levels (Chart 3) was examined in the following manner. (a) The average position and magnitude (at Tmax) of the growth rate peak resulting from a particular treatment was obtained. This was possible, since each animal in the treated group was fitted with its own individual growth rate curve, which has a particular value for the exact time of posttreatment growth rate maximum. (b) The duration of the period of accelerated growth rate was determined from the width of the growth rate curve obtained by averaging individual tumor growth rates for particular days. In order to characterize the width of the peak without ambiguity, we used the full width at half-maximum peak height.

For the 5 groups of animals used to determine the effects of increasing doses of cyclophosphamide on tumor growth rates (Chart 1), Tmax increased from 13 days following 50 mg/kg to 16 days following 250 mg/kg (Table 1). The time of minimum tumor volume (Tmax) was 8 to 10 days, except for 50 mg/kg which was 5 days. Although the growth rate peak following 50 mg/kg was not well defined and it was not possible to determine the full width at half-maximum peak height, the growth rate of this group exceeded control rates. Well-defined growth rate peaks were present following 100, 150, 200, and 250 mg/kg. Growth rate data are presented for 150 and 200 mg/kg in Charts 3 and 4. The full width at half-maximum peak height increased from 6.6 days after 100 mg/kg to 8.8 days after 250 mg/kg (Table 1).

The magnitude of the tumor growth rate at Tmax increased with increased doses of cyclophosphamide, from 0.289 ± 0.019 (day⁻¹) after 50 mg/kg to 0.383 ± 0.023 (day⁻¹) after 250 mg/kg. At the time of Tmax for the 250 mg/kg group, the growth rate was over 3 times control growth rate of 0.128 ± 0.014 (day⁻¹). During the period of maximum growth rate, the growth rate of treated tumors was significantly greater than that of time-matched controls and was similar to that of 0.298 ± 0.026 (day⁻¹) for controls at the beginning of the experiment.

**DISCUSSION**

Cyclophosphamide was more effective than 5-fluorouracil in controlling tumor growth in hepatoma 3924A. Our general experience in the AGI rat suggests that 150 mg/kg is approximately a 10% lethal dose for both cyclophosphamide and 5-fluorouracil. However, in this cyclophosphamide dose response, no animals died in the groups given 50, 100, 150, and 200 mg/kg, but 6 of 10 animals given 250 mg/kg died. Thus, tumor volume measurements in 4 of the 5 groups were unaffected by animal loss. The increased number of days for a treated group of tumors to reach a specific tumor volume as compared to the time for the control group to reach the same size has been defined as tumor growth delay (13). A 150 mg dose of cyclophosphamide per kg resulted in a 12.8 ± 0.49-day growth delay, and a 150 mg dose of 5-fluorouracil per kg resulted in a 7.8 ± 0.40-day growth delay.

We have introduced the term OTE to quantitatively describe the tumor volume changes following various forms of treatment. Both OTE and tumor growth delay were used in the analysis of results presented here. There was a 2-fold increase in both OTE and tumor growth delay when the cyclophosphamide dose was increased from 50 to 150 mg/kg. An incremental increase in OTE and tumor growth delay did not occur with increases in dosage to 200 and 250 mg/kg. Increasing the total chemotherapeutic dose beyond a certain level may provide little or no therapeutic gain in reduction of tumor volume.

A similar response was found in a rat mammary tumor, LMC. Incremental increases in tumor growth delay were greatest with smaller doses of cyclophosphamide (8). The curve was biphasic, with a smaller increase in the slope of the curve with doses of cyclophosphamide of 100 mg/kg or greater. This biphasic response was also shown by smaller incremental increases in surviving cells, with incremental increases in cyclophosphamide doses for the upper part of the tumor cell survival curve. This could be a result of such factors as increased toxicity of host, a fraction of the tumor cell population being more chemotherapeutically resistant than others, or that all "S" phase cells are killed at a certain dose level.

Parallel studies on cellular changes in hepatoma 3924A following cyclophosphamide and 5-fluorouracil have been carried out by Moore et al. (9). The cellular response of rat hepatoma 3924A to a single i.p. injection of 150 mg 5-fluorouracil per kg has been measured in respect to the spatial relationship of the cells to tumor microvasculature. In this tumor, the parenchyma is arranged in cords approximately 150 µm thick around the central capillaries. For untreated tumors, those cells at distances less than 80 µm from the capillary had a mean [³H]thymidine labeling index of 39% and a mitotic index of 2.1%, while for those cells more than 80 µm distant the values were 14 and 0.8%, respectively. Two days after 150 mg 5-fluorouracil per kg, mean cord thickness was reduced by 25% and did not recover to control levels until 11 days after treatment. This was also true for the mitotic index. Recovery of the labeling index was complete 2 days earlier. Although absolute values of parameters were different in the populations...
adjacent to and remote from the capillary, the time course of recovery was similar, with a "growth spurt" 7 to 9 days after treatment. The patterns of response to 5-fluorouracil and cyclophosphamide were different when measured in terms of cell density and cord radius. With 5-fluorouracil, the cords shrank promptly, but cell density was little altered, whereas with cyclophosphamide, the cords remained at control size for at least 9 days, but cell density declined sharply by 3 days (10). Recovery curves for the labeling index were different for the 2 drugs, but in each case values of the parameter labeling index and tumor DNA specific activity during recovery varied in a qualitatively similar manner.

The growth rate of treated tumors on Day 14 after 150 mg cyclophosphamide per kg was over twice that of controls on the same day, 0.349 ± 0.030 (day⁻¹) and 0.156 ± 0.011 (day⁻¹), respectively. The growth rate of controls decreases over time with increasing tumor size. Therefore, at the time of maximum growth rate of treated tumors, we are comparing growth rates of control and treated tumors of markedly different size. The fact remains, however, that there is a significant increase in the growth rate of treated tumors following treatment with cyclophosphamide. Furthermore, growth rates of some of the treated tumors on Day 14 are greater than those of control tumors at the beginning of treatment, 0.298 ± 0.026 (day⁻¹) on Day 2. Our finding of accelerated tumor growth differs from the general conclusion of Steel (12) that experimental tumors mostly have shown a smooth transition from regression to regrowth, without a steep rise in tumor volume after treatment. Possibly, the frequent volume measurements and criteria for proper fit in our experiments now allow the accelerated growth after treatment to be recognized.

If it is assumed that the magnitude of tumor growth rate can be related to the effectiveness of chemotherapy, it would be expected that proliferation-dependent agents such as 5-fluorouracil and cyclophosphamide would have their maximum effectiveness at the time of maximum tumor growth rate. Cyclophosphamide and 5-fluorouracil are Class III agents, defined by Bruce et al. (1) as agents which kill cells in all or most portions of the generation cycle and for which sensitivity of the cell population depends strongly on the fraction in the proliferative state. It remains to be determined if sequential administration of chemotherapeutic agents at Tmax would be more effective than if given at other times (such as Tmin). Increased DNA synthesis in the tumor after cyclophosphamide, as measured by [³H]thymidine incorporation, substantiates changes in growth rate taken from tumor volume measurements. Increased DNA-synthetic rate precedes increased tumor cell proliferation which, in turn, increases tumor volume. Thus, biochemical studies demonstrating increased cellular proliferation confirm the accelerated tumor growth following treatment.

The full width at half-maximum growth peak height of 7 to 9 days over the cyclophosphamide dose range of 100 to 250 mg/kg would provide flexibility for the sequential administration of chemotherapeutic agents once Tmax is established. Studies are in progress to determine the actual magnitude of therapeutic gain that could be realized by sequential administration of cyclophosphamide based on the magnitude and duration of Tmax induced by previous treatment.

Bone marrow has been shown to be the critical host organ with regard to sequential utilization of chemotherapeutic agents. The gastrointestinal tract recovers in approximately one-half the time of bone marrow. Studies with 5-fluorouracil have demonstrated that recovery of bone marrow from the effects of a large dose (150 mg/kg) in rats occurs 10 to 11 days after treatment and that maximal rate of tumor volume change occurs 12 days after treatment (5). The rate of proliferation (as demonstrated by tumor DNA specific activity) in the tumor is at a maximum 11 to 12 days after 5-fluorouracil. Parallel studies with cyclophosphamide that are comparable to the completed studies with 5-fluorouracil demonstrate that similar changes in tumor growth occur with a representative compound from another major class of cancer chemotherapeutic agents.

A therapeutic strategy for sequential therapy has been devised from results of these studies based on (a) recovery of host and critical organs of the host from the previous treatment series and (b) administration of a second and subsequent treatment series at times of maximum rate of tumor proliferation after the previous series (7).

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