Cell Kinetic-directed Sequential Chemotherapy with Cyclophosphamide and Adriamycin in T1699 Mammary Tumors

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ABSTRACT

The present studies were initiated to investigate the changes in \(^{3}H\)deoxythymidine labeling index, primer-dependent DNA polymerase labeling index, and S-G\(_2\) transition after treatment of T1699 transplantable mouse mammary tumors with Adriamycin (5 mg/kg) and cyclophosphamide (100 mg/kg). Treatment with these agents resulted in intervals of subnormal tumor cell proliferation as indicated by decreased \(^{3}H\)deoxythymidine labeling index, primer-dependent DNA polymerase labeling index, and S-G\(_2\) transition. Recovery, as indicated by increases in \(^{3}H\)deoxythymidine labeling index, primer-dependent DNA polymerase labeling index, and S-G\(_2\) transition, was observed three days after Adriamycin treatment and six to seven days after cyclophosphamide treatment. To evaluate the predictive nature of the kinetic changes for effective time sequencing, sequential combination chemotherapy protocols were designed and tested in T1699 tumor-bearing mice. The results from these studies showed that the most effective chemotherapy schedules were those in which the drugs were sequenced to coincide with the cell kinetic recovery from the single agents alone. These effective sequencing intervals were also found to be effective when used in multifraction sequential combination chemotherapy protocols. The results suggest that changes in cell kinetic parameters following drug perturbation can provide indications as to potentially efficacious as well as non efficacious sequencing intervals.

INTRODUCTION

CP\(^{3}\) and ADR are two of the most widely used and, in many cases, the most effective chemotherapy agents for a variety of human tumors (33). They have shown significant activity when used alone, but they are generally more effective when used together in combination (20, 23, 24). Previous studies with animal tumor models have shown that the effectiveness of sequential chemotherapy with these agents is dependent upon the sequencing intervals (9, 16, 28, 32). One way to optimize the sequential intervals is to utilize the changes in the cell kinetics after treatment to predict intervals during which the recovering tumor cell population would show increased sensitivity to a sequentially administered agent (6, 8, 9, 28).

The recovery kinetics following treatment with various cytotoxic drugs and radiation vary with the tumor system under study, the perturbing agent, and the dose of that agent (6, 9, 28). For example, in the 13762 rat mammary tumor system, recovery of the tumor cell proliferation after only 80 mg CP per kg was delayed for 7 to 10 days (9). In a less sensitive model, spontaneous mammary tumors in C3H/HeJ mice, recovery after much higher doses of CP was noted only 3 to 4 days after treatment (9, 11).

Utilizing these, as well as other drugs (6, 12) and radiation (6), we have shown that perturbations in the \(^{3}H\)dThd LI, the PDPI (in vitro growth fraction estimate), and the DLI (an estimate of S to G\(_2\) transition) can predict efficacious time sequencing intervals. Sequential therapy based on these kinetic changes can result in longer survival, better local tumor control, and tumor cure.

The present studies are an extension of previous observations (10) on the cell kinetic perturbations induced in T1699 mammary tumors by CP and ADR and, further, the application of this information to the design of sequential chemotherapy.

MATERIALS AND METHODS

Tumor and Animal Models. The T1699 transplantable mouse mammary tumor has been maintained in this laboratory for the past 6 years. This rapidly growing, immunogenic tumor model invariably "takes" when 5 \(\times\) 10\(^6\) cells are inoculated s.c. in syngeneic DBA/2J mice. Tumor-bearing mice were maintained on a 12-hr light-dark cycle, housed 12 to 15 mice/cage, and fed standard mouse chow (Ralston Purina, Evanston, Ill.) and water ad libitum. Tumor size measurements were made with calipers at frequent intervals throughout the study. Tumor-bearing mice were initiated into studies 14 days after tumor inoculation, when the calculated tumor mass was approximately 0.4 to 0.5 cu cm.

Cell Kinetic Measurements. The methods for in vitro single- and double-labeling of solid tumors have been previously described in detail (4). Briefly, tumor-bearing animals were killed by cervical dislocation. The tumors were resected and minced in McCoy's medium supplemented with fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.). The tumor cell suspensions were then either single-labeled with \(^{3}H\)dThd (15 to 17 Ci/mmol, 3 \(\mu\)Ci/ml; Amersham/Searle Corp., Arlington Heights, Ill.) for 1 hr or double-labeled, for the determination of T\(_s\), with \(^{3}H\)dThd as above and then for an additional 30 min with \(^{14}C\)dThd. All in vitro labeling was done at 37\(^\circ\) in a shaking water bath. Following the labeling procedure, viable cells were separated by Ficoll-Hypaque gradient centrifugation, and slide preparations were made for autoradiography as previously described (4, 5).

The primer-dependent DNA polymerase assay is an autoradiographic method to measure the simultaneous presence of nuclear DNA polymerase and nuclear DNA primer template activity in individual cell nuclei. This technique has also been...
previously described (5, 7, 25, 30). Briefly, imprints from a freshly cut tumor surface were made on microscope slides and air dried. The cytoplasm was stripped from the cells by dipping in a 0.125% agar solution at 40°C and then was air dried. This process serves to remove the cytoplasm, leaving only bare nuclei adherent to the slide. The slides were then fitted with a glass incubation chamber into which 0.5 ml of an incubation mixture containing adenosine, guanosine, and cytidine (0.2 mM); 5 mM MgCl2; Ficoll (Sigma Chemical Co., St. Louis, Mo.); and 5 μCi of [3H]dThd (specific activity, 54 Ci/mmol; New England Nuclear, Boston, Mass.) in 0.02 M Tris buffer (pH 7.4) were added. The preparations were incubated at 37°C for 45 min, at which time the reaction was stopped on ice. The incubation chambers were then removed, the slides were rinsed, and the cells were fixed in acidic formaldehyde and rinsed again prior to subsequent autoradiographic analysis.

**Autoradiography.** Kodak NTB-2 liquid photographic emulsion was used throughout. The emulsion was applied by dipping at 45°C, and the slides were exposed from 1 to 7 days in the dark at 4°C with Drierite. The exposed autoradiograms were developed by gold activation autoradiography, previously reported by us (4, 5), to reduce the autoradiographic exposure times required for adequate grain densities. Single-label [3H]dThd samples and primer-dependent DNA polymerase samples were exposed for 1 and 7 days, respectively, whereas for the double label samples, double emulsion autoradiography was used (4). The [3H]dThd LI's and PDPI's were determined by examining at least 300 labeled cells in double emulsion autoradiograms. Cells were considered labeled if they had 3 or more silver grains. Background was generally less than one grain per equivalent cell area, and mean grain counts were always in excess of 30 grains per labeled cell.

**Calculations.** The [3H]dThd LI and the PDPI are direct visual measurements of labeled fractions of a population. The T5 was calculated from:

\[ T_5 = \frac{T_a \times ^{14}C\text{-labeled cells}}{^{3}H\text{-only-labeled cells}} = \text{DLI} \]

where, \( T_a \) is the interval between administration of the labeled dThd’s (34).

In perturbed systems, the cell population may not be progressing asynchronously through the cell cycle. Thus, the above relationship may give artifactualy high or low \( T_s \) values depending on the flux of cells from S to G2 during labeling. In this study, the double label method was used as described; however, the ratio of \(^{14}C\)-labeled cells to \(^3H\)-only-labeled cells is referred to as the DLI and is a representation of the movement of cells from S to G2 at a point in time after treatment. Only in a steady-state condition will the DLI = \( T_s \) (12, 28).

In steady-state tumors, cell cycle parameters were calculated by Steel’s formulations (31), utilizing the PDPI as an estimate of the tumor growth fraction (30) as previously outlined (5). Student’s t test was used for statistical analysis of the data.

**Drug Treatments.** All drug treatments were initiated 14 days after tumor inoculation. In perturbed cell kinetic studies, tumor-bearing mice were weighed and administered ADR (5 mg/kg), CP (100 mg/kg), FUra (42 mg/kg), and MTX (6 mg/kg) were used. Drug dosages were selected on the basis of toxicity considerations. Treatment groups were comprised of 8 to 15 mice, and all agents were administered i.p. in sterile 0.9% NaCl solution. In the treatment studies, tumor shrinkage, complete remission (no palpable tumor), mean survival, and tumor cure (tumor-free for greater than 100 days) were used as end points.

**RESULTS**

Chart 1 shows the cell kinetic perturbations induced by ADR in T1699 mammary tumors. The [3H]dThd LI increased during the first 12 hr after treatment but subsequently decreased to less than 50% of control by about 60 hr. The DLI, a measure of the flux of cells into G2, increased significantly during the first 24 hr after treatment. This, together with the increased [3H]dThd LI seen during the first 12 hr, might suggest a temporary delay in S-phase transit to G2. Recovery of the [3H]dThd LI and the DLI was seen on Day 3. Partial recovery of the PDPI was noted at 60 hr; however, the PDPI remained subnormal throughout the study interval. Between Days 4 and 7, increases in DLI would imply a decrease in the rate of S to G2 transition and/or a decreased rate of DNA synthesis. These cell kinetic perturbations induced by 5 mg ADR per kg might suggest that subsequent therapy after ADR should be sequenced at a 3-day interval.

Cell kinetic data obtained from tumors 7 days after treatment with ADR, from age-matched untreated controls, and from 14-day pretreatment controls are shown in Table 1. The untreated
In untreated and ADR-treated Ti 699 mammary tumors, the rate of cell production is similar at both the 14- and 21-day study intervals. Inasmuch as the volumetric doubling time increased by more than a factor of 2, the cell loss rate must also have increased between 14 and 21 days. The major differences between tumors 4 days after ADR and their age-matched untreated controls was that after ADR the $T_s$ was prolonged and the fraction of actively proliferating cells (i.e., the PDPI) was decreased. Cell cycle times were similar in all groups. The cell production rate and cell loss rates, however, were considerably subnormal. Unlike age-matched control tumors, the volumetric doubling time ($T_o$) in ADR-treated tumors was not different than that for 14-day control tumors.

Chart 2 shows the results of studies to evaluate the cell kinetic perturbations induced in 14-day Ti 699 mammary tumors by 100 mg CP per kg. $[^3\text{H}]\text{dThd}$ LI's, although increased 6 hr after CP, decreased to approximately 4% by Day 4. Recovery of the $[^3\text{H}]\text{dThd}$ LI was observed between Days 5 and 7 after treatment. The PDPI decreased to less than 50% that of control by Day 6, with recovery noted between Days 7 and 10. The DLI was relatively stable during the study interval except for the large increase seen at 6 hr. Throughout the rest of the study interval, the DLI ratios were stable between 5 and 7. These data would suggest that recovery of tumor cell proliferation was initiated 4 to 5 days prior to the initiation of measurable tumor growth. The data would also suggest that, from a cell kinetic viewpoint, subsequent therapy should be sequenced at 6- to 7-day intervals.

Chart 3 shows the survival of Ti 699 tumor-bearing mice after treatment with various sequential schedules of ADR and CP. All treatments were started on Day 14 after inoculation. Simultaneous CP and ADR on Day 14 was no better than CP alone. Treatment with CP 24 hr after ADR resulted in considerable toxicity, and survival was no better than that for untreated tumors. The best survival was seen when CP was given.

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>$T_s$ (hr)</th>
<th>PDPI</th>
<th>$\lambda$</th>
<th>$T_C$ (hr)</th>
<th>Cell production rate/hr</th>
<th>Cell loss rate/hr</th>
<th>$T_o$ (hr)</th>
<th>Cell loss factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 14</td>
<td>23.8 ± 2.1</td>
<td>6.1 ± 0.3</td>
<td>63.2 ± 2.4</td>
<td>0.845</td>
<td>21.7</td>
<td>15.3</td>
<td>0.0319</td>
<td>0.0216</td>
</tr>
<tr>
<td>Day 21</td>
<td>22.5 ± 1.8</td>
<td>5.8 ± 0.2</td>
<td>64.0 ± 4.2</td>
<td>0.845</td>
<td>32.5</td>
<td>16.5</td>
<td>0.0318</td>
<td>0.0271</td>
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<tr>
<td>ADR (Day 21)$^b$</td>
<td>22.0 ± 2.1</td>
<td>9.1 ± 1.1</td>
<td>42.0 ± 2.8</td>
<td>0.786</td>
<td>21.8</td>
<td>16.5</td>
<td>0.0213</td>
<td>0.0117</td>
</tr>
</tbody>
</table>

$^a$ Mean ± S.E.

$^b$ Cell kinetics were measured on Day 21, 7 days after 5 mg ADR per kg.

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**Chart 2.** Effect of 100 mg CP per kg on the $[^3\text{H}]\text{dThd}$ LI, PDPI, DLI, and tumor volume, as a percentage of the pretreatment size ($V/V_0$), during the first 17 days after treatment. CP was given 14 days after tumor inoculation. Each point represents the mean for 4 to 5 tumors; bars, S.E.

**Chart 3.** Mean survival in days of tumor-bearing mice treated with 5 mg ADR per kg and then at various intervals thereafter with 100 mg CP per kg ($\Phi$). Survival of untreated controls ($\Box$) and controls treated with CP alone ($\Phi$, $\Delta$) are shown. Each point represents the mean for 8 to 10 mice; bars, S.E.
added to the protocol at Day 6 after CP. This was not unexpected, since the effective 3-day interval for ADR and the effective 6-day interval for CP were maintained.

Table 2 shows the results from studies to evaluate the dose-response relationship between tumor cure and the magnitude of a second CP treatment given 7 days after 100 mg CP per kg. These data show that tumor cure and remission frequency using a 7-day sequencing interval were dependent on the dose of the second CP treatment. CP (100 or 200 mg/kg) given acutely was ineffective. These results together with those presented in Chart 5 show that tumor cure with CP was not only sequence dependent but also dose dependent.

Chart 6 shows the results from studies in which MTX (6 mg/kg) and FUra (42 mg/kg) were given at various intervals after a single CP treatment. In these studies, FUra was given within 15 min after MTX. As in the previous studies, the best survival rates were seen when MTX and FUra were sequenced to take advantage of the recovery of cell proliferation after CP. In these studies, CP, MTX, and FUra all given together on Day 14 were no better than CP alone.

Chart 7 shows the results from sequential studies with ADR

### Table 2

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>CP dose (mg/kg)</th>
<th>Remissions b</th>
<th>Cures c</th>
<th>Cure (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days 14 + 21 a</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>200 + 0</td>
<td>1/5</td>
<td>0/5</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>100 + 100</td>
<td>12/12</td>
<td>12/12</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>100 + 75</td>
<td>6/8</td>
<td>6/8</td>
<td>75</td>
</tr>
<tr>
<td>4</td>
<td>100 + 50</td>
<td>4/8</td>
<td>3/8</td>
<td>38</td>
</tr>
<tr>
<td>5</td>
<td>100 + 25</td>
<td>2/15</td>
<td>1/15</td>
<td>6.6</td>
</tr>
<tr>
<td>6</td>
<td>100 + 0</td>
<td>0/8</td>
<td>0/8</td>
<td>0</td>
</tr>
</tbody>
</table>

a Treatment days post tumor inoculation.
b Number of animals with no palpable tumor/number of animals treated.
c Number of animals cured/number of animals treated.
and CP. In these studies, CP was given 3 days after ADR, and a second dose of CP was given at various intervals after the first. A single dose of CP 3 days after ADR resulted in a mean survival of approximately 46 days, whereas survival after a 200-mg/kg CP dose at 3 days after ADR resulted in decreased survival due to toxicity. The most efficacious treatment was the one in which the 2 CP doses were sequenced at a 6-day interval. In this group, the mean survival time was increased to about 76 days. Complete remissions were induced for all animals of this group, however, with only 40% of the animals in the group dying without tumor prior to the 100-day end point. The survival in all other groups was no better than after a single CP treatment 3 days after ADR.

Other more complicated sequential chemotherapy schedules could be designed for the T1699 mammary tumor utilizing the 3-day schedule interval for ADR and the 6-day schedule interval for CP. The results from one such study are shown in Table 3. In treatment Group 1, the 3-day treatment interval for ADR and the 6-day treatment interval for CP are maintained for 2 courses of therapy. The mean survival of nontreated mice was over 100 days, with a 100% remission rate and a 60% cure rate. In Group 2, the second ADR course was begun prematurely (i.e., only 4 days after CP). This schedule was not effective in producing either tumor cures or complete remissions, even though the second CP treatment was begun 3 days after the second ADR and the second CP was given 7 days after the first CP. The premature ADR treatment in some way modified the sequence that had previously been shown to be effective for the single agents alone.

**DISCUSSION**

The present studies were initiated to evaluate the effects of CP and ADR on the cell kinetics of T1699 mammary tumors and the prognostic value of these perturbations for the design of sequential chemotherapy. After treatment with 5 mg ADR per kg, recovery of the \[^3\text{H}\]dThd LI was noted by 72 hr. Partial recovery of the PDPI, however, preceeded LI recovery by approximately 12 hr. PDPI’s did not return to control levels and remained subnormal throughout the study interval. The significance of this observation is unclear, but it might suggest an impairment in the ability of noncycling cells to enter the division cycle.

The double label method has been used previously in perturbed systems to evaluate the flux of cells from the S phase into G2 (12, 26). In asynchronous populations, the DLI would be analogous to the transit time through DNA synthesis (7). Partial synchronization of the population would result in artificially short T5 values if the cohort is positioned at the S-G2 boundary and artificially long T5 values if the synchronized cohort is, for example, at the G2-S boundary. Thus, in a perturbed system, the flux from S to G2 at a point in time can be at least qualitatively assessed by this method.

The results after ADR treatment would suggest that, within the first 6 hr and lasting for at least another 42 hr, progression

### Table 3

<table>
<thead>
<tr>
<th>Treatment</th>
<th>(days posttransplantation)</th>
</tr>
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<tbody>
<tr>
<td>ADR (5 mg/kg/dose)</td>
<td>CP (100 mg/kg/dose)</td>
</tr>
<tr>
<td>Group 1#</td>
<td>14, 23</td>
</tr>
<tr>
<td>Group 2#</td>
<td>14, 21</td>
</tr>
</tbody>
</table>

# Ten mice/group.
of cells through S phase was inhibited. Although the DLI was within the normal range on Day 3, abnormally high values were noted between Days 4 and 7. Previous studies in the 13762 rat mammary tumor model indicated that recovery after 5 mg ADR per kg was initiated about Day 4 after treatment (28). In that system, there was little effect on the DLI throughout the study interval; however, entry into S phase was suggested to be subnormal shortly after ADR treatment. Previous studies have shown that cultured cells in the S phase of the cell cycle or at the G2-S transition may be most sensitive to ADR (3, 15, 17). Lethality, however, may result from an irreversible block in cell cycle progression in the G2 phase (1, 19, 22). Our results neither confirm nor deny a G2 progression delay in this tumor; however, the results are not inconsistent with that possibility.

The cell kinetic parameters for T1699 tumors 7 days after ADR suggest abnormally prolonged DNA synthesis times, subnormal cell production, and a subnormal fraction of proliferating tumor cells. Although we cannot rule out the possibility that the tumor cell population had not reestablished a steady state, recovery from the acute effects was indicated by Day 3. The 4-day interval between this recovery and the cell kinetic assays on Day 7 would have allowed 5 to 6 divisions for desynchronization.

Interestingly, the rate of cell loss was considerably reduced in ADR-treated tumors, even when compared to the 14-day value for the pretreatment group, a result perhaps of the immunosuppressive effects of ADR (14). It is tempting to speculate that the inhibition of CP-induced tumor shrinkage when CP and ADR were used in combination was related to the decreased cell loss. Similar but not nearly as profound effects on CP-induced tumor shrinkage were noted in 13762 rat mammary tumors treated with combinations of ADR and CP (28).

In treatment studies with sequential ADR and CP, the best survival results were seen when CP was sequenced 3 days after ADR. This correlates well with the timing of the recovery of cell proliferation after ADR. In previous studies with 13762 rat mammary tumors, sequential chemotherapy timed to coincide with the recovery phase after ADR also resulted in better responses than did treatments at kinetically unfavorable intervals (28).

The response of the T1699 mammary tumor to CP was somewhat different than the response after ADR. Recovery after CP was first evidenced by increases in the LI on Day 5 and recovery of the PDPI on or about Day 7. Unlike after ADR, the DLI after CP was relatively stable. Recovery of cell proliferation was noted at least 4 days prior to tumor regrowth. Therapy with CP was most effective when sequenced at a 6- to 7-day interval to coincide with the recovery of tumor cell proliferation. Delay of treatment until after tumor regrowth was evident proved to be too late for remission induction with a 2-dose schedule. Further, properly sequenced CP treatments were much more effective than the same total dose given acutely on Day 14. Cure frequency was found to be dependent on the magnitude of the second CP dose; however, even when the second CP dose was reduced 50%, the therapeutic result was substantially better than that with 200 mg/kg given acutely. Similar results with sequential CP treatments were observed with 13762 rat mammary tumors (9). In these studies, an effective sequencing interval for 80 mg CP per kg was found to be 9 days.

Sequential combination chemotherapy with CP, MTX, and FUra was also most effective when MTX and FUra were given on Day 5 or 6 after CP. Sequential combination chemotherapy with CP, ADR, FUra, and MTX in C3H/HeJ spontaneous mammary tumors was also most effective when MTX and FUra were sequenced to coincide with the proliferative recovery after CP and ADR (11). Previous reports have indicated that MTX and FUra can be synergistic when MTX is given before FUra (2, 13, 18). It is conceivable that the way in which MTX and FUra were administered in the present studies was suboptimal and that better responses might be possible with a more appropriate interval between MTX and FUra. Preliminary unpublished data, however, suggest no increased benefit of a 1- or 2-hr interval between the administration of MTX and FUra at the dose levels used in this tumor model.

Two CP treatments given 9 days apart were ineffective in producing remissions and cures; however, the 9-day CP interval could be made considerably more effective when ADR was administered on Day 6. The better response to this treatment may be accounted for by the fact that the 6-day and the 3-day intervals found to be effective for CP and ADR, respectively, were maintained. In C3H/HeJ mammary tumors, the cell kinetics after CP and ADR indicated recovery of cell proliferation between Days 3 and 4 after CP (9, 11). Thus, it would seem that the timing of proliferative recovery after drug perturbation may not only be host specific, as previously suggested (21), but also drug and tumor specific.

Kinetically defined effective sequencing intervals for CP and ADR were also effective when used in more complicated combination chemotherapy against T1699 tumors. The effectiveness was not compromised by the order of drug administration; however, a breach of the effective sequence for one agent could render the whole protocol ineffective even if the remaining treatments were given at the kinetically prescribed intervals. In T1699 tumors, the addition of ADR did little in the way of increasing the therapeutic effect of CP except in the instances where CP was administered at inappropriately long intervals. In this case, the judicious sequencing of ADR improved the therapeutic response when the kinetically defined sequencing intervals were maintained.

Studies in this and other tumor systems have shown that changes in cell kinetic parameters after drugs and radiation can indicate not only intervals during which the tumor cell populations are more sensitive to sequentially administered therapy but in addition unfavorable or less favorable sequence intervals (6—9, 11, 12, 21, 27—29).

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REFERENCES


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