Acute in Vivo Resistance in High-Dose Therapy

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ABSTRACT
In the design of sequential high-dose chemotherapy regimens, the selection of antitumor alkylating agents to be included in each intensification and the interval between the intensifications are critical to the design of the therapy. The tumor cell survival assay and tumor growth delay assay using the murine EMT-6 mammary carcinoma were used as a solid tumor model in which to address these issues. Tumor-bearing mice were treated with high-dose melphalan or cyclophosphamide followed 7 or 12 days later by melphalan, cyclophosphamide, thiopheta, or carboplatin. After treatment with melphalan both 7 and 12 days later, the tumor was resistant to each of the four drugs studied. After treatment with cyclophosphamide both 7 and 12 days later, the tumor was resistant to melphalan and thiopheta but was not resistant to cyclophosphamide or carboplatin. To extend the interval between high-dose treatments to 14 and 21 days, after the first intensification the tumor was transferred to second hosts that were either drug-treated or not drug treated. When high-dose melphalan-treated tumors were treated with a second high dose of melphalan, the tumors were very resistant with the 14-day interval and less resistant with the 21-day interval. This small effect was evident in the bone marrow colony-forming unit, granulocyte-macrophage (CFU-GM), except in the hosts pretreated with melphalan. When high-dose cyclophosphamide-treated tumors were treated with a second high dose of cyclophosphamide, drug resistance was observed both with the 14-day and 21-day interval if the host was non-pretreated or was pretreated with melphalan, but not if the host was pretreated with cyclophosphamide. The same was true in the bone marrow CFU-GM. Tumor growth delay studies supported these findings in that treatment with high-dose cyclophosphamide, melphalan, thiopheta, and carboplatin resulted in less than additive tumor growth delay, whereas treatment with high-dose cyclophosphamide prior to treatment with high-dose melphalan, cyclophosphamide, thiopheta, or carboplatin resulted in additivity to greater-than-additive tumor growth delay. High-dose combination regimens required dose reduction of the drugs, which resulted in decreased tumor growth delays.

INTRODUCTION
The clinical problem of drug resistance was recognized when in the 1940s Dr. Alfred Gilman's lymphoma patient failed to respond to a third course of nitrogen mustard after responding to the drug twice. Since then, elucidation of the mechanisms by which malignant cells develop a tolerance toward exposure to cytotoxic anticancer drugs has been a major area of investigation. Much of the research into drug resistance has been carried out in cell culture, most often using sublines cloned after repeated and/or chronic exposure of malignant cells to a specific agent. Although changes in cells developed in this manner were clear and often very well characterized, it has been more difficult to confirm that these changes correspond to the clinical problem.

One school of thought for overcoming drug resistance in the clinic is that drug resistance simply represents "undertreatment" of the disease (1). From this notion, treatment regimens consisting of high-dose combination chemotherapy with hematopoietic stem cell transplantation were developed and have been under clinical investigation for more than 10 years (2-11). Most recently, clinical protocols involving sequential high-dose regimens have been reported (12). The efficacy of this treatment approach, however, remains an open question. Preclinical in vivo modeling allows rigorous examination of many aspects of treatments, treatment combinations, sequences, and so on, that would be impossible to approach by clinical trial, because the number of variables involved would require a prohibitively large number of patients. The scientific study of cancer therapy relevant to the high-dose setting has required the development of preclinical models that go beyond the conventional dose end points of increase in life span and tumor regression/growth delay. High-dose therapy can be modeled using the tumor cell survival assay that allows tumor-bearing animals to be treated with "supralethal" doses of anticancer treatments with a quantitative measure of tumor cell killing (13, 14) and using a tumor growth delay assay with transplant of hematopoietic stem cells from syngeneic donors (15).

The current study was undertaken to examine the cyto- ductive potential and efficacy of sequential dose-intensive chemotherapy treatments in a murine model of breast cancer, EMT-6 mammary carcinoma.

MATERIALS AND METHODS
Drugs
Cyclophosphamide, melphalan, carboplatin, and paclitaxel were purchased as pure powders from Sigma Chemical Co. (St. Louis, MO). Thiopheta was purchased from the Dana-Farber Cancer Institute pharmacy. The drugs were administered by i.p. injection, except for paclitaxel, which was administered by i.v. injection. Melphalan was prepared as a stock solution in acidi-
fied ethanol (16). Paclitaxel was prepared as a stock solution in DMSO. For administration, all of the drugs were dissolved in 0.9% PBS, with concentrations of ethanol or DMSO limited to 1%.

Tumor

The EMT-6 murine mammary carcinoma was carried in female BALB/c mice (Taconic Farms, Germantown, NY). For experiments, $2 \times 10^6$ tumor cells prepared from a brei of several stock tumors were implanted s.c. into a hind leg of female BALB/c mice 8–10 weeks old (17).

Tumor Cell Survival

Experiment 1. EMT-6 tumor-bearing mice were treated on day 5 after tumor cell implantation with melphalan (30 mg/kg) or cyclophosphamide (400 mg/kg) or were untreated. These same animals were then treated on day 12 or 17 after tumor cell implantation with melphalan (20, 30, or 40 mg/kg), cyclophosphamide (100, 300, or 500 mg/kg), thiopeta (20, 30, or 40 mg/kg), or carboplatin (100, 250, or 400 mg/kg).

Experiment 2. EMT-6 tumor-bearing mice were treated on day 7 after tumor cell implantation with melphalan (30 mg/kg) or cyclophosphamide (400 mg/kg) and designated cohort I and tumors were transferred 24 h later. Non-tumor-bearing female BALB/c mice were untreated or were treated with melphalan (30 mg/kg), cyclophosphamide (400 mg/kg), paclitaxel (36 mg/kg, i.v.), or thiopeta (30 mg/kg) on day 0 and were designated cohort II. Cohort II day 7 and day 14 animals were implanted with tumor cells derived from tumors excised from the animals in cohort I. Tumors were allowed to grow in the cohort II animals. On day 7 after tumor implant, the cohort II animals were untreated or treated with melphalan (30 mg/kg) or cyclophosphamide (400 mg/kg).

A 24-h interval was incorporated before the mice were euthanized by cervical dislocation under anesthesia so as to allow for the full expression of drug cytotoxicity and repair of potentially lethal damage. Mice were immersed briefly in 95% ethanol, and the tumors were excised under sterile conditions in a laminar flow hood and minced to a fine brei with two scalps. Four tumors were pooled to make each treatment group. Approximately 300 mg of tumor brei were used to make each single-cell suspension. All reagents were sterilized with 0.22 μm Millipore filters and were added aseptically to the tumor cells.

Each sample was washed in 20 mL of Weymouth’s medium (151 Corp., Chicago, IL), after which the liquid was gently decanted and discarded. The samples were resuspended in 450 units of collagenase/ml (Sigma) and 0.1 mg DNase/ml (Sigma) and incubated for 10 min at 37°C in a shaking water bath. The samples were resuspended as described above and incubated for another 15 min at 37°C. Next, 1 mL of 1 mg/ml DNase was added, and incubation was continued for 5 min at 37°C. The samples were then filtered through two layers of sterile gauze. The samples were washed twice and then resuspended in Weymouth’s medium supplemented with 15% newborn calf serum. These single-cell suspensions were counted and plated at three different cell concentrations in duplicate for the colony forming assay. No significant difference was observed in the total cell yield from the pooled tumors in any treatment group. After 1 week, the plates were stained with crystal violet, and colonies of more than 50 cells were counted. The untreated tumor cell suspensions had a plating efficiency of 10–16%. The results were expressed as the surviving fraction (± SE) of cells from the treated groups as compared with untreated controls (16, 18).

Bone Marrow Toxicity

Bone marrow was taken from the same animals used for the tumor excision assay. A pool of marrow from the femurs of two animals was obtained by gently flushing the marrow through a 23-gauge needle, and the CFU-GM assay was carried out as described previously (16). Colonies of at least 50 cells were scored on an Accultle colony counter (Fisher Scientific, Springfield, NJ). The results of three experiments in which each group was measured at three cell concentrations in duplicate were averaged. The results were expressed as the surviving fraction of treated groups as compared with untreated controls.

Tumor Growth Delay Studies: Preparation and Harvesting of Donors for Collection of Peripheral Blood Stem Cells. Normal female BALB/c mice were treated with cyclophosphamide (200 mg/kg) by i.p. injection on day 0. On days 2–6, animals were treated with rhG-CSF (250 μg/kg) s.c. twice per day. On day 7, heparin (50 units) was injected i.v., and 20–30 min later, whole blood was collected by cardiac puncture and diluted into an equal volume of HBSS. By this method, one donor animal can provide $1 \times 10^7$ peripheral blood cells for six recipient animals. Peripheral blood cells were injected into the recipient animals i.v. (15).

High-Dose Treatment Recipient Animals

Experiment 1. Female BALB/c mice bearing EMT-6 mammary carcinomas were treated with a single dose of melphalan (30 mg/kg) or of cyclophosphamide (400 mg/kg) on day 5 after tumor cell implantation. Beginning on day 5 after the first high-dose drug administration and continuing through day 17, Lomotil (1.5 ml/100 ml) was added to the drinking water ad libitum. On day 6, 1 $\times 10^7$ peripheral blood cells collected from mobilized donors were injected i.v. into the tails of the treated animals. Also beginning on day 6 and continuing until day 21 posttreatment, the treated animals were injected i.p. twice per day with rhG-CSF (2 μg/kg). Animals were given Gatorade by gavage (0.2 ml) beginning on day 6, continuing as needed until the animals began drinking and eating. On day 12 after tumor cell implantation, the animals were either untreated or were treated with melphalan (30 mg/kg), cyclophosphamide (400 mg/kg), thiopeta (30 mg/kg), or carboplatin (250 mg/kg). On day 13, $1 \times 10^7$ peripheral blood cells collected from mobilized donors were injected i.v. into the tails of the treated animals.

Experiment 2. Female BALB/c mice bearing EMT-6 mammary carcinomas were treated with cyclophosphamide (400 mg/kg), thiopeta (30 mg/kg), or carboplatin (250 mg/kg) alone or in two drug combinations on day 7 after tumor cell...
implantation. Beginning on day 7 and continuing through day 12, Lomotil (1.5 ml/100 ml) was added to the drinking water ad libitum. On day 8, $1 \times 10^7$ peripheral blood cells collected from mobilized donors were injected i.v. into the tails of the treated animals. Also beginning on day 8 and continuing through day 12 posttreatment, the animals were injected i.p. twice per day with rhG-CSF (2 µg/kg). The treated animals were given Gatorade by gavage (0.2 ml) beginning on the first day posttreatment, continuing as needed until the animals began drinking and eating.

The progress of each tumor was measured three times weekly. Tumor growth delay was calculated as the days taken by each individual tumor to reach 500 mm$^3$ compared with untreated controls. Tumor volume was calculated as a hemispherical. Untreated EMT-6 tumors reached 500 mm$^3$ in 12.2 ± 0.4 days. Days of tumor regression and tumor growth delay were expressed as the means ± SE for each treatment group compared with controls (15).

**RESULTS**

The EMT-6 tumor is sensitive to the cytotoxic action of melphalan. Increasing doses of melphalan killed EMT-6 cells in tumors in a manner that increased logarithmically as determined by tumor cell survival assay (Fig. 1). Treatment of the tumor-bearing animals with melphalan (30 mg/kg) on day 5 after tumor cell implantation followed by various doses of melphalan (20, 30, or 40 mg/kg) on day 12 or on day 17 resulted in marked resistance of the tumor to the second melphalan treatment, so that the first exposure to melphalan (30 mg/kg) killed 2.5 logs of cells, but a second dose of the same drug given 7 days later killed <1 log of EMT-6 tumor cells and a dose given 12 days later killed about 1.5 logs of EMT-6 tumor cells. Administration of cyclophosphamide (400 mg/kg) kills about 2.5 logs of EMT-6 tumor cells. Treatment with a dosage range of melphalan after cyclophosphamide treatment also showed resistance to the melphalan therapy. With 7 days between administration of cyclophosphamide and melphalan (30 mg/kg), about 1.5 logs of EMT-6 tumor cells were killed by the melphalan, and with 12 days between administration of cyclophosphamide and melphalan (30 mg/kg), about 2 logs of EMT-6 cells were killed by the melphalan. When melphalan therapy was followed by a second administration of melphalan, the response of the bone marrow CFU-GM either was unchanged or showed a degree of resistance to the second dose of the drug. However, when melphalan administration was preceded by cyclophosphamide, there was an increase in the killing of the bone marrow CFU-GM compared with treatment with melphalan only.

Cyclophosphamide killed EMT-6 tumor cells in a logarithmic manner with increasing doses of the drug (Fig. 2). When administered to the animals, a dose of cyclophosphamide of 400 mg/kg killed about 2.5 logs of EMT-6 tumor cells. Treatment of the tumor-bearing animals with melphalan (30 mg/kg) either 7 or 12 days prior to treatment with cyclophosphamide resulted in <0.5 log and about 1 log of EMT-6 tumor cell killing by 400 mg/kg of cyclophosphamide. On the other hand, treatment of the tumor-bearing animals with cyclophosphamide (400 mg/kg) either 7 days or 12 days prior to treatment with a second dose of cyclophosphamide resulted in 3 logs and 2.5 logs of EMT-6 tumor cell killing by 400 mg/kg of cyclophosphamide. Preceding cyclophosphamide treatment with melphalan resulted in decreased killing of bone marrow CFU-GM by cyclophosphamide compared with cyclophosphamide alone, whereas preceding cyclophosphamide administration with a dose of cyclophosphamide did not alter the toxicity of the drug to the bone marrow CFU-GM at higher cyclophosphamide doses.

Administration of thiota to animals bearing the EMT-6 mammary carcinoma resulted in logarithmic killing of tumor cells with increasing dose of the drug (Fig. 3). Administration of melphalan (30 mg/kg) to the tumor-bearing animals either 7 or 12 days prior to treatment with thiota resulted in markedly decreased killing of EMT-6 tumor cells by thiota. When preceded by melphalan (30 mg/kg) on day 7 or day 12, a dose of 30 mg/kg of thiota produced about 0.5 log or about 1.5 logs of EMT-6 tumor cell killing, respectively, compared with 2.5 logs of EMT-6 tumor cell killing when thiota only was administered to the animals. Administration of cyclophosphamide (400 mg/kg) either 7 days or 12 days prior to thiota resulted in about 2 logs or 1.5 logs of EMT-6 tumor cell killing by thiota (30 mg/kg), respectively. Treatment with either melphalan (30 mg/kg) or cyclophosphamide (400 mg/kg) decreased the killing of bone marrow CFU-GM by a subsequent dose of thiota.

Carboplatin administered to EMT-6 tumor-bearing animals over a dosage range killed the tumor cells in a manner that increased logarithmically with increasing dose of the drug (Fig. 4). Preceding the administration of carboplatin with melphalan (30 mg/kg) by either 7 days or 14 days resulted in <1 log of EMT-6 tumor cell killing or about 1.5 logs of EMT-6 tumor cell killing by a dose of 250 mg/kg of carboplatin compared with 2.5 logs of EMT-6 tumor cell killing if only carboplatin was administered. Administration of cyclophosphamide (400 mg/kg) either 7 days or 12 days prior to carboplatin also decreased EMT-6 tumor cell killing by the drug to about 1.5 logs. Neither prior treatment with melphalan nor prior treatment with cyclophosphamide altered toxicity of carboplatin toward bone marrow CFU-GM.

To extend the interval between the intensification treatments, it was necessary to transfer treated tumors to second hosts. To determine whether prior chemotherapy would alter tumor growth in the second hosts, these animals either were untreated or were treated with melphalan, cyclophosphamide, paclitaxel, or thiota. Prior treatment with chemotherapy did not alter the growth of the tumors or tumor cell survival (plating efficacy) of the tumor cells from the melphalan-treated animals (Fig. 5). The survival of EMT-6 tumors from tumor-bearing animals treated with 30 mg/kg of melphalan was 0.0018 (Fig. 5, *NONE/NONE*). If the tumors from these animals are transferred to second hosts either untreated or treated 7 days earlier with chemotherapy, the administration of melphalan (30 mg/kg) 14 days after the prior exposure to melphalan resulted in about 1.5 logs less tumor cell killing than with the first administration of melphalan, except with thiota, in which there were 2 logs less EMT-6 cell killing upon the second administration of melphalan. When the interval between the first and second administration of melphalan was extended to 21 days, there was evidence of some reversion to melphalan sensitivity in the tumor with the least increase in tumor cell killing in the previously untreated.
Fig. 1 Survival of the EMT-6 tumor cells and bone marrow CFU-GM from the same animals with various doses of melphalan (PAM) on day (d) 7 (+); with melphalan (30 mg/kg) on day 5, followed by various doses of melphalan on day 12 (■) or day 17 (□); or with cyclophosphamide (CTX; 400 mg/kg) on day 5, followed by various doses of melphalan on day 12 (●) or day 17 (○) after tumor cell implantation. Points, means of three independent determinations; bars, SE.

Fig. 2 Survival of the EMT-6 tumor cells and bone marrow CFU-GM from the same animals with various doses of cyclophosphamide (CTX) on day (d) 7 (+); with melphalan (PAM: 30 mg/kg) on day 5, followed by various doses of cyclophosphamide on day 12 (■) or day 17 (□); or with cyclophosphamide (400 mg/kg) on day 5, followed by various doses of cyclophosphamide on day 12 (●) or day 17 (○) after tumor cell implantation. Points, means of three independent determinations; bars, SE.

Host and the greatest increase in the tumor cell killing in the host that had received cyclophosphamide 3 weeks earlier. A somewhat different pattern pertained in the bone marrow CFU-GM (Fig. 5). The bone marrow CFU-GM from hosts that had previously received melphalan was most sensitive to the second dose of melphalan, whereas the bone marrow CFU-GM from animals that were untreated prior to implantation of the melphalan-treated tumors were least sensitive to exposure to the drug.

For comparison, EMT-6 tumor-bearing animals were treated with cyclophosphamide (400 mg/kg), and those tumor cells were transferred to a second host that either was untreated or was treated with cyclophosphamide (400 mg/kg) or melphalan (30 mg/kg). The survival of EMT-6 tumor cells from the initial treatment with cyclophosphamide (400 mg/kg) was 0.005 (Fig. 6, NONE/NONE). Treatment of untreated hosts bearing the cyclophosphamide-treated tumor with a second dose of cyclophosphamide resulted in more than 0.5 log less tumor cell
Fig. 3 Survival of the EMT-6 tumor cells and bone marrow CFU-GM from the same animals with various doses of thiotepa (THIO) on day (d) 7 (+); with melphalan (PAM; 30 mg/kg) on day 5, followed by various doses of thiotepa on day 12 (■) or day 17 (□); or with cyclophosphamide (CTX; 400 mg/kg) on day 5, followed by various doses of thiotepa on day 12 (●) or day 17 (○) after tumor cell implantation. Points, means of three independent determinations; bars, SE.

Fig. 4 Survival of the EMT-6 tumor cells and bone marrow CFU-GM from the same animals with various doses of carboplatin (CARBO) on day (d) 7 (+); with melphalan (PAM; 30 mg/kg) on day 5, followed by various doses of carboplatin on day 12 (■) or day 17 (□); or with cyclophosphamide (CTX; 400 mg/kg) on day 5, followed by various doses of carboplatin on day 12 (●) or day 17 (○) after tumor cell implantation. Points, means of three independent determinations; bars, SE.

Killing than was obtained with the initial dose of the drug when the interval between the treatments was 14 days. However, treatment of cyclophosphamide-treated hosts bearing the cyclophosphamide-treated tumor with a second dose of cyclophosphamide 14 days later resulted in a 3-fold increase in EMT-6 tumor cell killing compared with the initial exposure to the drug. The greatest resistance to cyclophosphamide occurred when the host had been treated with melphalan prior to tumor implantation with the cyclophosphamide-treated tumor. When there was a 14-day interval between drug treatments, 1.5 logs less tumor cell killing was obtained with the second dose of cyclophosphamide than with the first exposure to the drug. Lengthening the interval between drug exposures to 21 days resulted in increased tumor cell killing in each of the three groups compared with that obtained with the 14-day interval. The tumor cell killing obtained with the second dose of cyclophosphamide in the host
that received no prior treatment and in the host that received prior treatment with melphalan remained less than that obtained with the first dose of cyclophosphamide. However, the tumor cell killing obtained with the second dose of cyclophosphamide in the host that received prior treatment with cyclophosphamide there was an 10-fold increase in the tumor cell killing obtained with the second drug exposure compared with the tumor cell killing obtained with the first cyclophosphamide treatment. There was less killing of bone marrow CFU-GM compared with the initial cyclophosphamide treatment with both the 14-day interval and the 21-day interval if the hosts bearing the cyclophosphamide-treated tumor were untreated or had received mel-
Fig. 6 Survival of EMT-6 tumor cells and bone marrow CFU-GM from the same animals after implantation of EMT-6 tumor cells from animals treated with cyclophosphamide (CTX; 400 mg/kg) into animals that were previously untreated or were treated with cyclophosphamide (400 mg/kg) or melphalan (PAM; 30 mg/kg) without further treatment or with a subsequent treatment with cyclophosphamide (400 mg/kg) either 14 or 21 days after the first cyclophosphamide exposure of the tumor. Columns, means of three determinations; bars, SE.

The bone marrow CFU-GM in the animals bearing the cyclophosphamide-treated tumors were as sensitive or more sensitive to the second dose of cyclophosphamide than to the original exposure to the drug in the first hosts.

A tumor growth delay assay was used to assess the response of the EMT-6 tumor remaining in situ to sequential high-dose drug treatments (Table 1). Mice bearing the EMT-6 tumor received high-dose melphalan (30 mg/kg) or high-dose cyclophosphamide (400 mg/kg) on day 5 and a second high-dose treatment on day 12. These animals received hematopoietic support consisting of peripheral blood cells from mobilized syngeneic donors on days 6 and 13 and rhG-CSF on days 6–22.
Table 1  Growth delay of the EMT-6 murine mammary carcinoma after two high-dose chemotherapy treatments with stem cell support

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Tumor growth delay (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melphalan (30 mg/kg), day 5</td>
<td>5.1 ± 0.4</td>
</tr>
<tr>
<td>Melphalan (30 mg/kg), day 5 → melphalan (30 mg/kg), day 12</td>
<td>7.2 ± 0.7</td>
</tr>
<tr>
<td>Melphalan (30 mg/kg), day 5 → cyclophosphamide (400 mg/kg), day 12</td>
<td>11.3 ± 1.4</td>
</tr>
<tr>
<td>Melphalan (30 mg/kg), day 5 → thiotepa (30 mg/kg), day 12</td>
<td>7.6 ± 0.5</td>
</tr>
<tr>
<td>Melphalan (30 mg/kg), day 5 → carboplatin (250 mg/kg), day 12</td>
<td>6.9 ± 0.8</td>
</tr>
<tr>
<td>Cyclophosphamide (400 mg/kg), day 5</td>
<td>19.6 ± 1.4</td>
</tr>
<tr>
<td>Cyclophosphamide (400 mg/kg), day 5 → melphalan (30 mg/kg), day 12</td>
<td>42.8 ± 2.3</td>
</tr>
<tr>
<td>Cyclophosphamide (400 mg/kg), day 5 → cyclophosphamide (400 mg/kg), day 12</td>
<td>46.4 ± 2.0</td>
</tr>
<tr>
<td>Cyclophosphamide (400 mg/kg), day 5 → thiotepa (30 mg/kg), day 12</td>
<td>29.8 ± 1.5</td>
</tr>
<tr>
<td>Cyclophosphamide (400 mg/kg), day 5 → carboplatin (250 mg/kg), day 12</td>
<td>30.8 ± 1.3</td>
</tr>
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* Tumor growth delay is the difference in days for treated versus control tumors to reach 500 mm³. Control tumors reach 500 mm³ in 12.2 ± 0.7 days after s.c. implantation.

* The tumor growth delays for the single drug treatments were: melphalan (30 mg/kg), 5.1 ± 0.4 days; cyclophosphamide (400 mg/kg), 19.6 ± 1.4 days; thiotepa (30 mg/kg), 4.2 ± 0.5 days; and carboplatin (250 mg/kg), 9.0 ± 1.1 days.

Tumor response to therapy was determined by tumor volume measurements. When animals bearing EMT-6 tumor were treated with melphalan (30 mg/kg) on day 5, a tumor growth delay of 5.1 days was produced. Following this dose of melphalan 7 days later with a second dose of melphalan or with high-dose cyclophosphamide, thiotepa, or carboplatin resulted in tumor growth delays between 6.9 and 11.3 days that were greater than for melphalan alone but less than expected for independent additive effects of the drugs. A single high-dose treatment with cyclophosphamide (400 mg/kg) on day 5 produced a tumor growth delay of 19.6 days. Treatment of these animals 7 days later with high-dose melphalan, cyclophosphamide, thiotepa, or carboplatin resulted in tumor growth delays of 42.8 days, 46.4 days, 29.8 days, and 30.8 days, respectively, which were additive to greater-than-additive for independent effects of the drugs.

Finally, the effect of two drug combinations in the high-dose setting was assessed by tumor growth delay (Table 2). The combination of high-dose cyclophosphamide and high-dose thiotepa was toxic, in that more than 50% of the animals died within 2 weeks after the treatment. The tumor growth delay for the remaining animals was 6.3 days, much less than expected for additivity of the drug treatments. Treatment with high-dose cyclophosphamide and high-dose carboplatin was toxic. When the doses of the drugs were reduced to allow survival of the treated animals, the resulting tumor growth delays were less than that obtained with a full dose of the most effective single agent in each combination regimen. The reason for this finding at least in part may be the severely depleted condition of these animals.

**DISCUSSION**

The high-dose setting is the most informative situation in which to examine the effect of drug sequence and drug combination, because it is in the high-dose setting where the greatest potential cell killing effects can be obtained. Several conclusions can be derived from the current study. The first is that administration of melphalan induced a metabolic condition in the host and in the tumor, which resulted in drug resistance that was slowly dissipating over a period of weeks. This finding is supported by a body of data from previous studies in cell culture (19–24) and in vivo (25, 26) that show that exposure to melphalan resulted in decreased sensitivity to subsequent drug treatment at least with antitumor alkylating agents. The sequential high-dose therapy tumor growth delay study clearly shows the diminished response of the EMT-6 tumor to treatment with high-dose melphalan, cyclophosphamide, thiotepa, or carboplatin 1 week after a prior dose of melphalan. The exception to this was the bone marrow CFU-GM of animals treated with melphalan, in which the sensitivity of this tissue to melphalan was increased when exposed to the drug again on day 21 but not on days 12, 14 or 17.

A second conclusion is that prior exposure to cyclophosphamide results in much less or no drug resistance to administration of a second drug. In fact, administration of a second dose of cyclophosphamide to animals that had already been treated with the drug can result in increased tumor cell killing compared...
with the initial treatment. This effect appears to be primarily an effect on the host, because when an untreated host or a host treated with melphalan bearing the cyclophosphamide tumor was exposed to cyclophosphamide, diminished sensitivity of the tumor to the drug was observed; only when the host was treated with cyclophosphamide was enhanced sensitivity to a second dose of cyclophosphamide observed. Although a much smaller effect, the melphalan-treated tumors in the hosts treated with cyclophosphamide were the most sensitive to the second dose of melphalan.

Exposure to cytotoxic therapy and especially to high-dose cytotoxic chemotherapy produces major metabolic responses in the host and in the tumor (17, 25, 26). Some of these responses are acute and short lived, but others may last weeks or longer. These metabolic changes, although not permanent genetic alterations, clearly affect the response of the host and the tumor to subsequent exposures to cytotoxic anticancer agents. In the design of sequential and combination high-dose treatment regimens, the response of the host and the tumor to prior therapy should be taken into account.

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