Rational Selection of Adjuvant Chemotherapy after Cytoreduction Surgery for Murine Neuroblastoma

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ABSTRACT

Improving the prognosis of advanced neuroblastoma remains an important yet unachieved goal of pediatric oncology, a fact which may be related to an insufficient analysis of the role played by cytoreductive surgery. Utilizing strain A mice bearing C-1300 syngeneic neuroblastoma, tumor biology and host immunocompetence were studied after cytoreduction surgery and adjuvant chemotherapy. Cell kinetic analysis in the residual tumor demonstrated an increase of the proliferative fraction 18 to 42 h after operation, but the same peak proliferation was delayed in bone marrow cells to 24 to 96 h. The potential for drug distribution to the tumor after cytoreduction surgery was assessed by injecting Na_2CrO_4 and measuring tumor uptake. There were two significant (P < 0.05) peaks of activity at 6 h and 3 days, suggesting local edema and neovascularity, respectively. Injection of both cell cycle specific and nonspecific adjuvant chemotherapeutic agents in a dosage of one-fourth of their 50% lethal dose at 24 or 72 h following surgical cytoreduction did not induce any antitumor activity at either injection time. However, when cyclophosphamide was given in this dose, the C-1300 tumor growth was impaired, an effect which was largely abrogated by first subjecting the tumor bearer to thymectomy and irradiation. The transfer of spleen cells from adjuvant cyclophosphamide-treated mice to tumor-inoculated normal mice significantly delayed tumor appearance when comparison was made with animals treated by operation alone, and such recipients also exhibited a more prolonged survival. These data suggest that the antitumor activity of cyclophosphamide following cytoreduction surgery of C-1300 neuroblastoma is mediated by both pharmacological and immunological mechanisms.

INTRODUCTION

Advanced neuroblastoma is one of the most lethal forms of cancer in children. Despite multimodality therapy including surgery, chemotherapy, immunotherapy, and/or irradiation therapy, the prognosis for patients with this disease remains poor, with a 30 to 35% overall survival rate. Neuroblastoma is known to be a tumor the cells of which are predominantly in the resting phase that might be resistant to chemotherapy (1). The cell kinetics of the tumor mass are variable and can potentially be manipulated to the advantage of the patient undergoing therapy (2). Additionally, the importance of host antitumor immunity is suggested by the fact that this tumor originates from the embryonal structure (3) and shows the highest incidence of spontaneous regression of any human cancer (4), and even partial resection often improves survival (5-7). Cytoreductive surgery may play a critical role in this sequence (8) and, therefore, it should be evaluated in relation to tumor cell kinetics, immunological manipulation, and adjuvant chemotherapeutic drug distribution to the remaining tumor mass. Furthermore, the suitable chemotherapeutic agent and its dosage schedule after surgical intervention could take advantage of the change in cell kinetics induced by surgery while minimizing postoperative risk and maximizing antitumor activity.

The present study was undertaken to analyze the synergistic antitumor activity of the combination of cytoreduction surgery and adjuvant chemotherapy for neuroblastoma using the C-1300 murine neuroblastoma model.

MATERIALS AND METHODS

Animals. Male strain A mice, 7 to 9 weeks old, were obtained from the Jackson Laboratory, Bar Harbor, ME.

Tumor. Uncloned C-1300 neuroblastoma was received from Dr. Harvey Schliesinger and maintained in our laboratory by serial subcutaneous inoculation into syngeneic strain A mice.

Chemotherapeutic Agents. CY (Mead Johnson & Co., Evansville, IN), ADR (Adria Laboratories, Inc., Columbus, OH), CDDP (Bristol Laboratories, Syracuse, NY), IdUrd (Calbiochemical, Los Angeles, CA), and VM-26 (Bristol Laboratories, Syracuse, NY) were reconstituted in sterile saline. VM-26 was reconstituted in 6-MO-2-thenylidene/3-D-glucopyranoside.

Flow Cytometric DNA Analysis. Tumor cell suspensions were obtained by mincing a solid tumor in phosphate-buffered saline. A suspen-

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Chart 1. Tumor cell (○) and bone marrow cell (○) kinetic change at sequential time intervals after cytoreduction surgery. Each point represents a mean value for 3 to 5 mice; bars, SE. Tumor cell kinetic changes between 12 and 24 or 36 h and between 36 and 72 h are significantly different (P < 0.01). Bone marrow cell kinetic changes between 12 and 48 h are significantly different (P < 0.001).

Chart 2. Concentration of 51Cr in the autochthonous residual tumor at varied intervals following cytoreduction surgery. Each point represents a mean value for 5 to 6 mice; bars, SE. The ratios at 6 h and 3 days are significantly different from the nonoperative control ratio (P < 0.05).

formula:

\[
\text{Residual tumor (S + G2M)} / \text{excised tumor (S + G2M)}
\]

Assessment of Tumor Vascularity. To assess the potential drug distribution to a smaller remaining tumor burden, Na\(^{35}\)CrO\(_4\), 0.1 μCi/0.5 ml, was injected through the mouse tail vein at various time intervals after operation by modifying Rogers' method (16). Fifteen min following these injections, the mice were killed with ether, and the incorporated \(^{35}\)Cr concentration in the residual tumor mass and the reference spleen organ were counted utilizing a γ-ray scintillation counter (Packard Instrument Co., Downers Grove, IL). The radioactivity of the tumor mass was quantified by the following ratio:

\[
\frac{\text{cpm/mg weight tumor}}{\text{cpm/mg weight spleen}}
\]

Immunodeficient Mice. Male 7- to 8-week-old mice were operatively thymectomized and, 7 days later, they received 450 rads of whole-body irradiation from a cesium source. All mice survived this pretreatment protocol.

Transferability of Immunological Activity. In order to assess the immunological activity of CY treated tumor-bearing mice, spleen cells from the 72-h adjuvant therapy group were harvested 3 days after CY injection. A suspension of spleen cells was subsequently administered intravenously in a dosage of \(4 \times 10^9\) cells/0.5 ml RPMI 1640 medium to recipient mice simultaneously receiving \(10^6\) C-1300 tumor cells in 0.1 ml into a subcutaneous site. The latency time of tumor appearance was then observed.

Statistical Analysis. The Student's t-test was used for statistical analysis of data, while assessment of mouse survival rate was analyzed by a log rank test (17).

RESULTS

Tumor Cell and Bone Marrow Cell Kinetic Change after Cytoreduction Surgery. Each mouse and its tumor had a different growth rate and a different DNA profile when compared with other tumor bearers. Therefore, to assess the influence on cell proliferation as measured by DNA production, a comparison was made of the ratios of the pre- and postoperative cell-cycle phase from the same animal's tumor mass and bone marrow harvested from a hind leg amputation. The DNA content of bone marrow cells before and after surgery was then calculated and compared in a similar manner. The preoperative DNA distribution of tumor cells was 41.4 ± 4.5% (SD) in G0 + G1 phase, 45.7 ± 4.9% in S phase, and 12.7 ± 2.4% in G2 + M phase; and the DNA distribution of bone marrow cells was 67.6 ± 4.6% in G0 + G1 phase, 24.6 ± 4.0% in S phase, and 7.7 ± 1.4% in G2 + M phase.

Chart 1 demonstrates that, from 18 to 42 h after operation, there is an increase in the proliferating population of tumor cells; however, 48 h after operation, this population decreases and,
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Charts C-1300 neuroblastoma growth after cytoreduction surgery on Day 0, either alone (x) or combined with early or late administered adjuvant chemotherapy: VM-26 at 24 h (Δ); VM-26 at 72 h (A); IdUrd at 24 h (O); or IdUrd at 72 h (●). Each point represents a mean value for 4 mice, and experiments were repeated. Bars, SE.

M CO OC SO-

100- •

50- •

12345 12345

DAYS AFTER OPERATION

Chart 4. C-1300 neuroblastoma growth after cytoreduction surgery on Day 0, either alone (x) or combined with early or late administered adjuvant chemotherapy: CY at 24 h (○); CY at 72 h (●); ADR at 24 h (A); ADR at 72 h (A); CDDP at 24 h (O); CDDP at 72 h (●). Each point represents a mean value for 4 mice; bars, SE.

by 96 h, it has returned to normal. At the same time, the bone marrow cells demonstrate a delayed increase of proliferative activity extending from 24 to 96 h after operation. These results suggest that, following cytoreductive surgery, an early administered chemotherapeutic agent might more selectively influence the tumor while sparing the bone marrow.

Isotopic $^{51}$Cr Distribution to the Residual Postoperative Tumor. When compared with intact tumors from nonoperated animals, the isotopic concentration in postoperative residual tumors was elevated at each postoperative analysis interval (Chart 2). At 6 h and 3 days, these increased concentrations of isotope were significantly higher than control values. These data may reflect early postoperative tissue edema followed by a relative hyperemia, perhaps due to tumor neovascularity. In any case, blood flow to the residual tumor was not impaired and, therefore, drug distribution to the residual autochthonous tumor should have been preserved following cytoreductive surgery.

Adjuvant Chemotherapy. On the basis of the above findings,
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Chart 5. C-1300 neuroblastoma growth after cytoreduction surgery alone on Day 0 (Δ); after CY alone on Day 0 (△); after cytoreduction surgery on Day 0, followed after 24 h by adjuvant CY (○); or after cytoreduction surgery on Day 0, followed after 72 h by adjuvant CY (•). Each point represents a mean value for 8 to 10 mice.

Chart 6. Percentage of mouse survival after treatment of C-1300 neuroblastoma with either cytoreduction surgery alone on Day 0 (Δ); CY alone on Day 0 (△); cytoreduction surgery on Day 0, followed after 24 h by adjuvant CY (○); or cytoreduction surgery on Day 0, followed after 72 h by adjuvant CY (•). Both early and late adjuvant therapy groups demonstrate a significantly better survival (P < 0.05 and P < 0.01, respectively) than seen with either cytoreduction surgery or CY alone.

both cell cycle specific (VM-26, IdUrd) and cell-cycle nonspecific (CY, CCDP, ADR) chemotherapeutic agents were administered either 24 or 72 h after operation to determine whether the antitumor effect of the drugs could be correlated with postoperative tumor and bone marrow cell cycle kinetic changes (Charts 3 and 4). Animals were assessed for serial tumor growth as well as ultimate survival. With the exception of CY, neither class of drugs, whether given early or late after operation, induced a significant tumor growth inhibition. However, CY did demonstrate tumor growth inhibition and better animal survival, but there was no difference in antitumor activity between early and late drug injection.

The tumor inhibition following CY administration (See Charts 5 and 6) suggests that the combination of cytoreductive surgery plus chemotherapy is clearly superior to either alone as measured by both a significant delay in tumor growth and by a prolonged mouse survival. Although the adjuvant effectiveness of CY is most pronounced in the presence of a smaller tumor burden, there was no advantage of either an early or a late postoperative administration schedule. The antitumor activity of CY in this model lacks a simple pharmacological explanation when one solely considers the short 6½-h half-life (18) of CY and the tumor
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15 DAYS AFTER OPERATION

Chart 7. C-1300 neuroblastoma growth in mice originally rendered immunodeficient by adult thymectomy and irradiation and treated by cytoreduction surgery alone on Day 0 (C); by CY alone on Day 0 (A); by cytoreduction surgery on Day 0, followed after 24 h by adjuvant CY (O); or by cytoreduction surgery on Day 0, followed after 72 h by adjuvant CY (•).

Table 1
Comparison of tumor growth between normal and immunodeficient hosts

Tumor sizes on Day 9 are listed. For details, refer to the legends of the Charts 6 and 7.

Tumor size (sq mm) on Day 9

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Normal (n=10)</th>
<th>Immunodeficient (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surgery alone</td>
<td>249.7 ± 90.8</td>
<td>239.7 ± 120.7</td>
</tr>
<tr>
<td>CY alone</td>
<td>238.7 ± 120.7</td>
<td>225.2 ± 31.7</td>
</tr>
<tr>
<td>Surgery + CY, 24 h</td>
<td>49.25 ± 25.2</td>
<td>108.7 ± 22.2</td>
</tr>
<tr>
<td>Surgery + CY, 72 h</td>
<td>56.4 ± 15.9</td>
<td>84.95 ± 42.16</td>
</tr>
</tbody>
</table>

Mean ± SD.

*0.01 < P < 0.05.

**P < 0.001.

Judg. 0.001 < P < 0.01.

and bone marrow cellular kinetics.

Cytoreductive Surgery and CY in Immunodeficient Mice. Since CY is known to influence the host immune response (7, 19, 20), a similar treatment protocol was repeated in adult mice rendered immunoincompetent by thymectomy and irradiation prior to tumor inoculation (Chart 7; Table 1). The subsequent analysis of tumor growth revealed that the antitumor effect of CY was largely abrogated in immunodeficient mice. These data suggest that an interaction occurred between the combination therapy and the tumor-bearing host’s immune response.

Adoptive Transfer of Antitumor Activity by the Passive Transfer of Spleen Cells from CY-treated Mice. After the transfer of spleen cells from tumor-bearing mice treated by cytoreductive surgery alone to mice simultaneously receiving C-1300 neuroblastoma, there was an acceleration of the subsequent tumor appearance in the recipient mice (16.7 day latency versus 19.8 days for controls) (Chart 8). However, when spleen cells were transferred from animals treated by the CY-plus-operation combination, tumor latency was not accelerated but was equal to that of the control RPMI 1640 medium inoculated mice (19.8 days for experiments versus 19.8 days for controls). This suggests that the tumor-bearing host has a transferable tumor growth-promoting cell population, possibly suppressor T-cells or suppressor macrophages and, while this population is not significantly influenced by surgery alone, it is very susceptible to the combination of cytoreduction surgery and CY therapy.

DISCUSSION

Previous reports have shown that cytoreductive surgery for advanced neoplasms increases the growth fraction of the residual tumor cell mass and shortens the survival of the tumor-bearing host (21–24). These reports described the growth pattern of distinct disease in these animal tumor models. Our study is the first to analyze the cell kinetics of residual tumor at the primary operative site. Such analysis is particularly important to neuroblastoma patients, most of whom present clinically with advanced local disease.

Our present study discloses the postoperative increase of the tumor cell growth fraction as being very transient, lasting from but 18 to 42 h after cytoreductive surgery. This is in accordance...
with Sordilla’s data on human neuroblastoma in nude mice (24), although the proliferative period is shorter than that described in other tumor models (21, 23). The differences may be explained by the fact that a remote tumor site with an intact vascular supply is not equivalent to a residual tumor mass with a disturbed blood supply such as was noted by the relative increased vascularity.

The increase in the proliferating population of bone marrow cells in contrast to the residual tumor cells was delayed and longer in duration, lasting from 24 to 96 h after operative intervention. This increase may be related to perioperative factors such as blood loss, anesthesia, hypoxia, and acidosis. This bone marrow cell kinetic change is critical, since the proliferating cell is more susceptible to chemotherapeutic agents and, thus, serious postoperative bone marrow suppression might be avoided by appropriate chemotherapeutic timing.

The chemotherapeutic drug concentrations in the residual tumor mass after cytoreductive surgery may be altered by low blood flow, tissue edema, arteriovenous fistulae, and tumor neovascularity. Although tissue concentrations of 51Cr in the remaining tumor mass may not be equivalent to drug concentrations, 51Cr distribution does reflect the perfusion of the residual tumor mass. Since tissue concentrations of isotope are higher after cytoreductive surgery, one may presume that drug concentrations would be parallel, a fact which is especially important for the treatment of a hypervascular tumor such as neuroblastoma.

Adjuvant chemotherapy with currently available agents has not yet been prescribed based on a knowledge of postoperative tumor cell-kinetic changes. In this study, the timing of drug injection based on tumor cell kinetics did not alter antitumor activity with either phase-specific or phase-nonspecific agents. Fisher (21) has reported that resection of a primary tumor did not influence the residual tumor sensitivity to postoperative CY, while at the same time assessing tumor kinetics and host antitumor activity, in order to achieve optimal antineoplastic therapeutic effects.

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REFERENCES

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