Potentiation of 1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea by Amphotericin B in Murine Ependymoblastoma

Guy Laurent, G. Atassi, and J. Hildebrand

INTRODUCTION

The use of nitrosoureas has greatly increased the number of investigations in the chemotherapy of both experimental and human brain tumors. Controlled studies have confirmed early results reporting the effectiveness of various nitrosoureas in treating malignant gliomas. However, despite wide agreement that nitrosoureas contribute to the treatment of malignant brain tumors, there is an urgent need for improvement of results achieved so far. The aim of this paper is to report a potentiation of CCNU effects by AMB in murine ependymoblastoma. This experimental model can possibly be applied to clinical trials.

MATERIALS AND METHODS

Experimental Ependymoblastoma. Male C57BL mice served as carriers of ependymoblastoma in all experiments except in studies of [3H]thymidine incorporation, in which male C57BL x DBA/2 F, mice were used. All animals were obtained from Charles River Breeding Laboratories, Wilmington, Mass. The ependymoblastoma, strain 01B11, was provided by Hazleton Laboratories, Vienna, Va. It was maintained by the s.c. transplantation every 14 days of approximately 3-cm fragments into the right flank of the animals. Fourteen-gauge spinal needles were used for this procedure. Chemotherapy was initiated 10 to 12 days after transplantation, when tumors became measurable. The volume of the tumor was assessed by the following formula: \[ V = \frac{4}{3} \pi A \cdot B^2 \] (16), in which A is the long axis and B the short one. The 2 dimensions were measured 2 to 3 times a week. Complete regression was defined as the disappearance of a tumor for at least 1 week. When complete regressions lasted for 2 months or more, they were considered as cures.

Drugs. Cold CCNU, supplied by the National Cancer Institute, Bethesda, Md., was dissolved in sesame oil and injected i.m. into the left thigh of the mice. 1-[2-Chloroethyl-(U-14C)]-3-cyclohexyl-1-nitrosourea (12 mCi/mmol) was a gift from Dr. R. R. Engle, Drug Research and Development Chemistry, National Cancer Institute. [3H]CCNU was dissolved in sesame oil and 10.4 \( \mu \)Ci were injected i.m. into each mouse. AMB was obtained as a mixture containing 0.8 mg of deoxycholate per mg of AMB from E. R. Squibb & Sons, Princeton, N. J. The drug was dissolved in 5% glucose just before the i.p. injection. Control animals received an equivalent amount of deoxycholate in 5% glucose.

Uptake of [14C]CCNU by the Tumors. Animals were killed by cervical dislocation at various intervals after injection of radioactive CCNU. Tumors were quickly removed and frozen. For determination of total radioactivity, part of the tissue containing the tumors was placed in Soluene (Packard Instrument Co., Downers Grove, Ill.) at 50°C until complete solubilization. The mixture was allowed to cool at room temperature, and 10 ml

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of toluene scintillation fluid were added for determination of radioactive activity.

Another fragment of the tumor was extracted with 20 ml of chloroform:methanol (2:1, v/v). The extract was filtered, dried in vacuum, redissolved in 10 ml of chloroform:methanol (2:1, v/v), and partitioned in two phases with 2 ml of 0.88% KCl according to the method of Folch et al. (7). Radioactivity of the upper and lower phases was measured in toluene scintillation fluid. Labeled CCNU, present only in the lower phase, was purified by thin-layer chromatography performed on silica gel plates with fluorescent indicator (Mackerey-Nagel, Düren, Germany). Plates were developed with chloroform:methanol:water (65:25:4, v/v/v). The spots corresponding to CCNU were localized under UV light. Silica gel was scraped off into scintillation vials, mixed with 3 ml of water and 10 ml of Aquasol (New England Nuclear, Boston, Mass.), and estimated for 

\[ ^{14}C \] radioactive activity. All measures of the \[^{14}C \] radioactivity were performed by liquid scintillation counting. Counting efficiency was measured by the internal standard method.

**Measure of \[^{3}H \] Thymidine Incorporation into Tumor DNA.** \[^{[methyl-3}H \] Thymidine \( (54 \text{ Ci/mmole}) \) was purchased from the Radiochemical Centre, Amersham, England. The animals received 2 \( \mu \)Ci of the isotope per g of body weight and were sacrificed 2 hr after injection. Tumors were quickly removed and homogenized in ice-cold 0.85% NaCl solution. DNA was extracted according to the Schmidt-Tannhauser-Schneider procedure and was determined by the method of Dische (18). Aliquots of DNA extract were mixed with Aquasol and \(^3H\) radioactivity was estimated by scintillation counting. Efficiency was measured by the external standard method.

**RESULTS**

**Antitumor Effects.** AMB given alone at the dosage of 25 mg/kg of body weight does not affect the rate of tumor growth (Chart 1). At this dosage, AMB does not produce a decrease in body weight or an increase in mortality. The antitumor effect of CCNU is related to the dosage of the nitrosourea (Table 1). With 2.5 mg of CCNU per kg of body weight, the rate of complete but temporary regressions is low and definite cures are rare. However, most of the tumors show a significant reduction in size, lasting from the beginning of the 2nd to the beginning of the 4th week following the administration of CCNU (Chart 2).

As shown in Table 2, the rate of complete regressions and cures is significantly increased when 25 mg of AMB per kg are given 10 hr prior to the administration of 2.5 or 10 mg of CCNU per kg. However, in tumors that underwent only partial regression, there is no difference between the group of animals treated with CCNU alone and those receiving the combination of AMB and CCNU (Chart 2).

**Effect of AMB on the Uptake of \[^{14}C \] CCNU by the Ependymoblastoma.** Chart 3 shows the radioactivity present in the total tumor tissue, chloroform:methanol (2:1, v/v) tumor extract, and the upper and lower phases obtained by partitioning the chloroform:methanol (2:1, v/v) extract according to the method of Folch et al. (7). Whereas the total radioactivity present in tumors increases for at least 8 hr after the administration of \[^{14}C \] CCNU, the lipophilic fraction, present in the lower phase after Folch's partition, decreases 1 hr after the injection of the nitrosourea. Radiochromatograms revealed the presence of CCNU only in this lower phase. However, the identification of the drug was achieved only by thin-layer chromatography and the radioactive peak could actually correspond to a mixture of CCNU and its hydroxylated derivatives. Thirty min after the injection of \[^{14}C \] CCNU, more than 40% of the radioactivity present in the lower phase was found to be CCNU, accounting for 20% of the radioactivity present in the total tissue. After 2 hr, CCNU represents less than 10% of the radioactivity present in the total tumor tissue. Therefore, the studies of the effect of AMB on CCNU penetration were performed during the 2 hr following the injection of the nitrosourea.

To test the sensitivity of these experiments, 5.2 or 15.6 \( \mu \)Ci of \[^{14}C \] CCNU per animal were given 1 hr before sacrifice. The radioactivity recovered in the total tumor tissue, chloroform:methanol (2:1) extract, and upper and lower phases was proportional to the amounts of the labeled drug injected, indicating that the test was sensitive for the amounts of labeled drug used in our experiments.

Chart 4 shows that the administration of 25 mg of AMB

**Table 1**

<table>
<thead>
<tr>
<th>CCNU (mg/kg body wt)</th>
<th>No. of animals</th>
<th>Complete regressions</th>
<th>Cures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>2.5</td>
<td>12</td>
<td>2</td>
<td>17</td>
</tr>
<tr>
<td>5</td>
<td>13</td>
<td>3</td>
<td>23</td>
</tr>
<tr>
<td>10</td>
<td>12</td>
<td>5</td>
<td>42</td>
</tr>
<tr>
<td>35</td>
<td>8</td>
<td>8</td>
<td>100</td>
</tr>
</tbody>
</table>

**Chart 1** Growth of a s.c. ependymoblastoma in animals that received a single dose of 25 mg of AMB per kg on Day 0 (9) and in controls (C). Tumor size is expressed as percentage of tumor volume on Day 0, on which volume is considered to be 100%. Each value is the mean of 9 determinations ± standard deviations.
Table 2

Effect of CCNU versus AMB + CCNU on the rate of complete regressions and cures of murine ependymoblastoma

The drugs were administered to mice 10 to 12 days after s.c. transplantation of ependymoblastoma. AMB was injected i.p. 10 hr prior to CCNU. Complete regression and cure are defined in Table 1.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg body wt)</th>
<th>No. of animals</th>
<th>Death by toxicity</th>
<th>Complete regressions</th>
<th>( \chi^2 ) test</th>
<th>Cures</th>
<th>( \chi^2 ) test</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCNU</td>
<td>2.5</td>
<td>73</td>
<td>1</td>
<td>5 (7)*</td>
<td>( p &lt; 0.01 )</td>
<td>4 (6)</td>
<td>( p &lt; 0.05 )</td>
</tr>
<tr>
<td>AMB + CCNU</td>
<td>25 + 2.5</td>
<td>71</td>
<td>3</td>
<td>16 (24)</td>
<td></td>
<td>12 (18)</td>
<td></td>
</tr>
<tr>
<td>CCNU</td>
<td>10</td>
<td>20</td>
<td>0</td>
<td>8 (40)</td>
<td>( p &lt; 0.05 )</td>
<td>3 (15)</td>
<td>( p &lt; 0.01 )</td>
</tr>
<tr>
<td>AMB + CCNU</td>
<td>25 + 10</td>
<td>20</td>
<td>1</td>
<td>14 (70)</td>
<td></td>
<td>11 (58)</td>
<td></td>
</tr>
</tbody>
</table>

* Numbers in parentheses, percentage.
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DISCUSSION

AMB is a polyene that forms complexes with cholesterol in mammalian plasma membranes and with ergosterol in fungal cell surfaces. These interactions result in an increased permeability of cells to various substances, such as hydrophilic nonelectrolytes or anions and cations (3), that may eventually lead to cell death (6). The ability of nontoxic doses of AMB to increase plasma membrane permeability has also been used to enhance the penetration into cells of various therapeutic agents. Indeed, Medoff et al. (15) demonstrated that AMB increases the incorporation of actinomycin D into HeLa cells. A similar mechanism could also be responsible for the additive effect of AMB and 5-fluorouracil in cryptococcal meningitis (9) and has been advocated for potentiation of other drugs by AMB (8, 10, 11, 13). More recently, Medoff et al. (14) showed a dramatic synergistic effect of AMB and BCNU against murine transplantable AKR leukemia.

This work extends this observation to solid tumors and another nitrosourea, CCNU. The rate of complete regressions and cures due to CCNU alone was significantly increased by AMB. However, in animals with partial regression of tumor volume, CCNU was no less effective than the combination of CCNU and AMB. These findings are consistent with data obtained by Medoff et al. on AKR leukemia. In that study, about 40% of the mice treated with AMB and BCNU were cured, whereas the survival time of the animals that received the combination of the 2 drugs but that died was not much longer than the survival time of the animals receiving BCNU alone. By increasing BCNU doses, Medoff et al. were able to prolong the survival time without producing any cure. This would indicate that the effect of AMB on membrane permeability does not account for their results. We came to the same conclusion by studying the penetration of labeled CCNU into s.c. ependymoblastoma. Under the conditions in which AMB stimulated antitumor activity of CCNU, it did not increase the amount of radioactivity found in the total tumor, chloroform:methanol extract, and the lower lipophilic phase of that extract, or under the form

**Table 3**  
[^4C]CCNU present in ependymoblastoma of mice treated with AMB and controls

[^4C]CCNU was injected i.m. to animals 12 or 14 days after transplantation of ependymoblastoma. Each animal received 10.4 μCi of[^4C]CCNU. AMB was injected i.p. 10 hr prior to[^4C]CCNU. The[^4C]-labeled drug was extracted from the tumor and purified by thin-layer chromatography as described in "Materials and Methods."

<table>
<thead>
<tr>
<th>Time interval</th>
<th>AMB (mg/kg body wt)</th>
<th>Radioactivity (dpm/100 mg tissue)</th>
<th>t test</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>0</td>
<td>1173 ± 472</td>
<td>NS*</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>815 ± 344</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>0</td>
<td>2084 ± 736</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>2716 ± 1208</td>
<td></td>
</tr>
</tbody>
</table>

* Mean ± S.D.

**Table 4**  
Incorporation of[^3H]thymidine into ependymoblastoma DNA in animals treated with AMB, CCNU; and the association of AMB and CCNU

The drugs were injected into mice 12 to 14 days after s.c. transplantation of ependymoblastoma. AMB was injected 10 hr prior to CCNU. Each animal received 2 μCi of[^3H]thymidine per g of body weight i.p. 24 hr after CCNU administration and was sacrificed 2 hr later.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg body wt)</th>
<th>No. of animals</th>
<th>Radioactivity (dpm/μg DNA)</th>
<th>t test</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>8</td>
<td>123 ± 25 (1) 77 ± 2 (2)</td>
<td>NS*</td>
</tr>
<tr>
<td>AMB</td>
<td>25</td>
<td>8</td>
<td>104 ± 40 (1)</td>
<td></td>
</tr>
<tr>
<td>CCNU</td>
<td>1</td>
<td>9</td>
<td>67 ± 17 (1)</td>
<td>NS</td>
</tr>
<tr>
<td>AMB + CCNU</td>
<td>25 + 1</td>
<td>9</td>
<td>66 ± 12 (1)</td>
<td></td>
</tr>
<tr>
<td>CCNU</td>
<td>10</td>
<td>3</td>
<td>29 ± 2 (2)</td>
<td>NS</td>
</tr>
<tr>
<td>AMB + CCNU</td>
<td>25 + 10</td>
<td>3</td>
<td>38 ± 7 (2)</td>
<td></td>
</tr>
</tbody>
</table>

* Mean ± S.D.

* Numbers in parentheses, separate experiments.

* NS: not significant.
of CCNU. Not only is the penetration of CCNU or its metabolites not affected by AMB, but the inhibition of DNA synthesis is unchanged by the dose of AMB used in this study. While the antitumor activity of CCNU on the ependymoblastoma is dose-related up to at least 40 mg/kg, an almost optimal inhibition of the DNA synthesis is already observed for 5 mg/kg. This suggests that other mechanisms perhaps related to the modification of proteins may be even more important than the inhibition by CCNU of DNA synthesis in murine ependymoblastoma. This hypothesis is further supported by Cheng et al. (2), who demonstrated that in leukemia L1210 cells metabolites of CCNU were bound more extensively to proteins than to nucleic acids.

The mechanism of potentiation of antitumor activity of CCNU by AMB in mouse ependymoblastoma needs further investigation. We were not able to demonstrate in preliminary studies that this potentiation was related, as in the case of AKR leukemia, to the time separating the administration of the 2 drugs, and we were unable to provide support for the hypothesis that the potentiation is immunological in nature as suggested by Medoff et al. (14) and Valeriote et al. (17). The therapeutic effect of the combination in which CCNU was given, even at 10 mg/kg, did not reach the curative rate obtained with 40 mg of CCNU per kg used alone. However, in humans the administration of CCNU is limited by the myelotoxicity of the drug. Thus the possibility of potentiation of relatively low doses of nitrosourea may be of great importance in clinical uses of those drugs. Although the doses of AMB used in this study may be too toxic to give to humans, our experimental model may still find a clinical application in the treatment of brain tumors. Indeed, AMB may be given intrathecally and may reach sufficiently high concentrations in the CSF (1). Since AMB does not leak readily from CSF, one may also expect to avoid an enhancement of systemic toxicity of nitrosoureas. If the potentiation observed in mice is not due to an enhancement of immunological reaction, this system may be particularly interesting in the treatment of meningeal carcinomatosis, where the problem of drug diffusion from CSF to tumor tissue is of limited importance. Such preliminary clinical assay is now in progress.

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REFERENCES

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