Hematological Toxicity of Repeated Injections of (Chloro-2-ethyl)-
ribofuranosyl-3-nitrosourea

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ABSTRACT

A previous study using a single injection of (chloro-2-ethyl)ribofuranosyl-3-nitrosourea has indicated the low acute hematotoxicity of this nitrosourea. However, because the hematotoxicity of nitrosourea is usually cumulative, we have studied the effect of injecting (chloro-2-ethyl)ribofuranosyl-3-nitrosourea (15 mg/kg) dissolved in 0.2 ml sterile oil C57BL x i.p. into DBA/2F, mice for 5 consecutive weeks. The dose per injection represents the minimal dose necessary to show the maximal therapeutic efficacy on L1210 leukemia.

Bone marrow cellularity and histology, spleen weight, bone marrow and spleen pluripotent stem cells, and colony-forming units committed to granulocyte-macrophage differentiation were measured 1, 2, 4, 7, and 14 days after the last injection in treated and control mice receiving oil only.

No morphological or histological changes were found in the spleen and bone marrow of treated mice. In both organs, the number of splenic pluripotent hematopoietic stem cells decreased by about 1 log 1 day after the last injection but rapidly returned to normal values on Days 4 to 14.

Granulocyte-macrophage-committed precursors were affected in both organs, at 20% of control values for bone marrow and 5% of control values for spleen on Day 1, with a transient and partial recovery on Day 4 followed by a second drop to 20 and 25% on Day 14.

This effect on granulocyte-macrophage precursors contrasts with the absence of significant effect when the same treatment is used on peripheral white blood cell counts.

Our results demonstrate that (chloro-2-ethyl)ribofuranosyl-3-nitrosourea belongs to the class of new nitrosoureas with low cumulative hematotoxicity.

INTRODUCTION

Chloroethylnitrosourea derivatives are oncostatic agents with a broad spectrum of activity (6, 14). Their high antitumor effect in some human cancers such as lymphomas and their capacity to cross the blood-brain barrier underline their clinical potential.

Most nitrosourea derivatives have shown a significant cumulative hematotoxicity in animals and in humans, which limits their use as single agents or in combination. The clinical use of BCNU and CCNU is usually limited by a major and long-lasting thrombocytopenia occurring most often after 5 to 6 administrations of the drug. This cumulative hematotoxicity may prevent further administration of cytotoxic agents for a long time.

Streptozotocin, a naturally occurring methylnitrosourea composed of 1-methylnitrosourea attached to a glucosamine carrier, is remarkable for its relative lack of myelosuppressive activity but has a narrow spectrum of antitumor activity (14, 16). Chlorozotocin is also attached to C-2 of a glucose carrier (1). Imbach and Montero (8) synthesized a series of derivatives in which the cyclohexyl group of CCNU was replaced by a sugar molecule. Two of these sugar derivatives, RFCNU and RPCNU, have demonstrated significant antitumor activity in our in vivo screening of transplanted tumors.

In a previous study (11), we compared the hematotoxicity induced in mice by a single injection of 3 nitrosourea, chlorozotocin, RFCNU, and RPCNU. The dose chosen for each compound corresponded to the minimal dose showing the maximum antitumor effect on L1210 leukemia. RPCNU showed significant hematotoxicity: a decrease both in WBC of peripheral blood and in the mean spleen weight; and a 2-log drop in the number of hematopoietic stem cells in the bone marrow and spleen of treated mice.

In contrast, chlorozotocin and RFCNU had no effect on peripheral blood and moderately decreased the number of CFUs and CFUc in the spleen (10% of control). The bone marrow cellularity and bone marrow CFUs were moderately decreased to, respectively, 60 and 25% of controls. All these changes were rapidly fully reversible.

In order to test the cumulative hematotoxicity of the 3 nitrosoureas, the compounds were injected weekly for 5 weeks, and the peripheral blood counts were measured during the 2 weeks following the last injection. This study (9) demonstrated the cumulative hematotoxicity of RPCNU, which induced a pronounced pancytopenia. Chlorozotocin showed a significant and preferential decrease in the number of platelets as in humans. (17, 18). With the RFCNU, no significant effect on peripheral blood count was observed at 15 mg/kg, and the number of platelets slightly decreased at Day 14 with a dose of 30 mg/kg.

It was therefore interesting to study, as we report here, the variations of CFUs and CFUc in the spleen and bone marrow of mice treated with 5 injections of RFCNU (15 mg/kg).

MATERIALS AND METHODS

Mice. Male specific-pathogen-free C57BL x DBA/2 F1 mice (8 weeks old and weighing 20 to 23 g), obtained from IFFA CREDO, Lyon, France, were used throughout the experiment.

Drug. RFCNU was dissolved in olive oil and administered i.p. at a weekly dose of 15 mg/kg in 0.2 ml for 5 weeks. Olive oil was injected i.p. into control mice following the same schedule. Five mice were used for each point.

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2 Sponsored by JSPS-INSERM-Cooperation Exchange Program.
3 To whom requests for reprints should be addressed.
4 The abbreviations used are: BCNU, 1,3-bis(2-chloroethyl)-3,1-nitrosourea; RFCNU, (chloro-2-ethyl)ribofuranosyl-3-nitrosourea; RPCNU, (chloro-2-ethyl)-ribopyranosyl-3-nitrosourea; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; CFUs, pluripotent hematopoietic stem cell; CFUc, granulocyte-macrophage-committed precursor.

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Table 1

<table>
<thead>
<tr>
<th>Time (days) after i.p. injection</th>
<th>Control</th>
<th>RFCNU</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of WBC/cu mm</td>
<td>% of lymphocytes</td>
</tr>
<tr>
<td>1</td>
<td>4410 ± 1235³</td>
<td>67</td>
</tr>
<tr>
<td>2</td>
<td>3388 ± 375</td>
<td>68</td>
</tr>
<tr>
<td>4</td>
<td>3600 ± 241</td>
<td>67</td>
</tr>
<tr>
<td>7</td>
<td>4225 ± 940</td>
<td>66</td>
</tr>
<tr>
<td>14</td>
<td>3970 ± 957</td>
<td>71</td>
</tr>
</tbody>
</table>

³ Mean ± S.D.

Table 2

<table>
<thead>
<tr>
<th>Time (days) after i.p. injection</th>
<th>Platelets (x 10³/cu mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>1</td>
<td>970 ± 139³</td>
</tr>
<tr>
<td>2</td>
<td>1288 ± 65</td>
</tr>
<tr>
<td>4</td>
<td>1344 ± 149</td>
</tr>
<tr>
<td>7</td>
<td>1382 ± 249</td>
</tr>
<tr>
<td>14</td>
<td>1050 ± 205</td>
</tr>
</tbody>
</table>

³ Mean ± S.D.

Blood. Blood samples were obtained by cardiac puncture on Days 1, 2, 4, 7, and 14 after the administration of the drug. Blood samples of control mice were obtained at the same time. RBC and WBC were determined with an automatic blood counter (Coulter Model S; Coulter Electronics). Platelet count and the percentage of granulocytes and lymphocytes were determined by conventional techniques.

Measure of Hematopoietic Precursors. Bone marrow cells were flushed with a syringe fitted with a 22-gauge needle into α-medium. After counting, the cells were diluted to appropriate concentrations and used for CFUs and CFUc assay. After the spleen was weighed, cells were obtained by gentle homogenization with a Potter-Elvehjem homogenizer in α-medium and were assayed for CFUs and CFUc.

The number of CFUs was determined according to the method of Till and McCulloch (19). Briefly, 2.5 × 10⁶ cells for spleen were injected i.v. into 10 lethally irradiated recipient mice, and the spleen colonies were counted 8 days later. Recipient mice for CFUs assay were irradiated shortly before the injection of the cell with 9.6 Gy using 137Cs at a dose of 8.5 Gy/min. The CFUc were assayed by the technique of Bradley and Metcalf (3). Two ml of 0.3% agar in α-medium supplemented with 20% horse serum (Biopro, Germany), containing 10⁶ cells, were plated in Petri dishes. Ten % lung-conditioned medium was used as a source of colony-stimulating activity. Four replicates were plated for each cell suspension. The plates were incubated at 37° in 5% CO₂ in humidified air, and the colonies were counted 7 days later.

Histology. The sternum was histologically analyzed. The hematoxylin-eosin and Gordon and Sweet techniques were used to specifically stain the reticulin network. Collagen fibers were stained by the picrofuscin technique of Van Gieson (20).

RESULTS

Effect on the Blood. As shown in Tables 1 and 2, RFCNU had no significant effect on WBC and platelet counts. We observed only a slight and reversible decrease of WBC on Days 2 and 4 after injection of the drug. The percentage of granulocytes and lymphocytes was never modified.

Effect on the Bone Marrow. As shown in Chart 1, the injections of oil alone or oil plus RFCNU induce a decrease in the cellularity of the femur, which is at a maximum on Day 7 and returns to normal on Day 14. There is no significant difference between treated and control mice; this variation is probably due to oil injections.

The bone marrow histological study did not show any significant effect of the drug on the cellularity or on the maturation of the erythrocytic, granulocytic, megakaryocytic, and lymphoid series.

Specific histological staining techniques did not demonstrate myelofibrosis in the treated animals.

Chart 2 shows that RFCNU induced a significant decrease in the CFUs on Day 1 after the last injection (30% of the control).
with a partial recovery on Day 4 and an overshoot on Day 14.

The effect of RFCNU on CFUs is shown in Chart 3. On Day 1, the CFUs of treated mice were 20% of those of controls. There was a transient recovery to 50% on Day 4, but the number of CFUs decreased again to 40 and 20% of that of the controls on Days 7 and 14, respectively, indicating an important disturbance in the CFU pool induced by the drug.

Effect on the Spleen. There was no effect on weight, as shown in Chart 4.

The drug induced a decrease in the CFUs with a rapid and complete recovery on Day 4, which persisted on Days 7 and 14 (Chart 2).

The effect of RFCNU on the spleen CFUs shown in Chart 5 is comparable to that obtained in bone marrow, with an initial decrease to 5% of control values on Day 1, partial recovery to 90% on Day 4, but a secondary decrease to 50 and 25% on Days 7 and 14, respectively.

DISCUSSION

RFCNU have been selected from among various sugar derivatives of nitrosourea because the compound exhibited the largest "plateau" of maximum antitumor effect expressed as the percentage of cure in a group of mice grafted i.p. with 10^6 L1210 leukemic cells and treated with a single i.p. dose of drug 1 day after the graft (7). This maximum antitumor effect without lethal toxicity was obtained with doses of RFCNU ranging from 15 to 30 mg/kg. Our previous study using a single injection confirmed the low and reversible toxicity of the minimal dose of the plateau.

We had previously shown the absence of a significant effect on peripheral blood cell count after 5 weekly injections of RFCNU (9). This experiment confirms the absence of significant toxicity on WBC, platelet counts, spleen weight, bone marrow, and spleen histology. The CFUs in both organs were not decreased further after 5 injections than they were after a single administration of the drug. The only evidence of cumulative toxicity was the rather long delay of full recovery of bone marrow CFUs. Splenic CFUs reached a normal value in 4 days. The significant and long-lasting decrease of bone marrow cellularity observed in this experiment is due to the repeated i.p. injections of oil which may induce a migration of bone marrow cells to other organs such as the spleen or peritoneum. The most striking effect of the 5 injections of RFCNU was observed in the compartment of bone marrow and splenic CFUs. Indeed, in these organs, RFCNU induced a significant depletion of CFUs which was not fully reversible on Day 14, although the WBC showed only a minor trend of leukopenia on Days 2 and 4. The difference in absolute numbers of granulocytes in control mice was not significant. It has been demonstrated (4) by serial transplantation of identical numbers of marrow cells carried out at a given interval which does not permit the repopulation of the bone marrow CFUs reserve that peripheral blood counts of mice recover after each successful transplant, even though there is a depletion of bone marrow CFUs. Morley and Blake (12) have described an apparent complete recovery of peripheral counts with mildly reduced marrow cellularity and markedly reduced CFUs in mice treated with 4 doses of busulfan. Therefore, the normal blood counts could indicate only that differentiation takes place at the expense of self-renewal when stem cell pools are chronically depleted (15). However, in the case of RFCNU, the good preservation of the CFUs compartment may also allow a good replenishment of CFUs after cessation of therapy. The latter hypothesis would fit the lack of effect on peripheral platelet counts, which are usually a good indicator of the cumulative hematotoxicity of nitrosourea.

RFCNU appeared less hematotoxic in humans than did RPCNU and chlorozotocin (13, 15, 17, 18). With RFCNU at doses ranging from 200 to 600 mg/sq m/month, a significant percentage of antitumor response was obtained in patients with gastrointestinal cancers (10). No severe hematological toxicity was observed among 32 previously untreated patients even at the highest doses of RFCNU and after repeated courses of treatment. In the whole series of patients, only 2% anemia and 4% severe neutropenia were observed. Thrombocytopenia below 100,000 platelets/cu mm occurred only in 18% of the patients, and the platelet count fell below 50,000/cu mm only in 6% of the patients. These patients had received prior chemotherapy. The dose per month of RFCNU used in this trial corresponds to the extrapolated dose used in our experiment in the mouse (5).

In the case of chlorozotocin, Phase I studies (17, 18) have shown that an i.v. bolus injection of 120 to 150 mg/sq m every 6 weeks produced minimal leukopenia with moderate levels of thrombocytopenia after 2 to 4 courses. At high doses, severe thrombocytopenia could be observed even in patients without prior treatment. Therefore, there was less hematotoxicity in humans with doses of RFCNU 4 to 5 times greater than chloro-
zotocin, while in the mouse we compared chlorozotocin (10 mg/kg) with RFCNU (15 mg/kg).

Obviously, the clinical data in humans are limited and do not originate from randomized trials, especially for the comparison of toxicity between the different drugs. Our study underlines the interest in hematologically detailed screening to compare the acute and chronic toxicity of antitumor agents in mice.

REFERENCES


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