In Vivo Study of Acute Hematotoxicity of Three Nitrosoureas, Chlorozotocin, (Chloro-2-ethyl)-ribofuranosyl-3-nitrosourea, and (Chloro-2-ethyl)-1-ribopyranosyl-3-nitrosourea

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ABSTRACT

The hematological effects of three sugar derivatives of nitrosoureas, chlorozotocin (2-(chloro-2-ethyl)-3-nitrosoureido)-D-glucopyranose), (chloro-2-ethyl)-1-(ribopyranosyl triacetate-2’,3’,4’)-3-nitrosourea, and (chloro-2-ethyl)-1-ribofuranosylisopropylidene-2’,3’-p-nitrobenzoate-5’)-3-nitrosourea, were studied in the bone marrow, spleen, and peripheral blood of mice. The three compounds were injected i.p., dissolved in sterile olive oil. A single dose, corresponding to the minimal dose showing the maximum therapeutic activity, was administered at Day 0; and erythrocytes, white blood cells, platelets, and hematocrit were counted at Days 1, 2, 3, 4, and 7. Spleen weight, bone marrow cellularity, and the hematopoietic precursors, peripheral hematopoietic stem cells (CFU-s) and splenic colony-forming unit committed to granulocyte-macrophage differentiation (CFU-c), were also measured in both organs.

Chlorozotocin and (chloro-2-ethyl)-1-ribofuranosylisopropylidene-2’,3’-p-nitrobenzoate-5’-3-nitrosourea had no effect on peripheral blood; the spleen weight was reduced at Day 4, and the number of hematopoietic stem cells (CFU-s) in the spleen was decreased by 2 logs with a complete recovery within 2 days, whereas the effect was significantly less on splenic granulocytic precursors (CFU-c). In the bone marrow, (chloro-2-ethyl)-1-(ribopyranosyl triacetate-2’,3’,4’)-3-nitrosourea also decreased the cellularity and the number of CFU-s but did not decrease the number of CFU-c.

Chlorozotocin and (chloro-2-ethyl)-1-ribofuranosylisopropylidene-2’,3’-p-nitrobenzoate-5’-3-nitrosourea had no effect on peripheral blood, decreased the number of CFU-s and CFU-c in the spleen to 10% in controls, and reduced bone marrow cellularity and the number of CFU-s to 60% of controls. Chlorozotocin affected mostly bone marrow CFU-c (25% of controls at Day 1) with a progressive return to normal level at Day 4. RFCNU has no toxicity on the bone marrow granulocyte precursors.

None of the three compounds produced thrombocytopenia, and all hematological effects were usually rapidly reversible.

INTRODUCTION

The chloroethyl nitrosoureas represent an important family of oncostatic agents. Many nitrosoureas derivatives (4) such as BCNU, CCNU, streptozotocin, chlorozotocin, RFCNU, and RFHNCU have demonstrated a significant antitumor activity (3, 9). BCNU and CCNU, the first nitrosourea derivatives subjected to a large Phase II clinical trial, exhibited a cumulative bone marrow toxicity in treated patients which necessitated discontinuation (2, 7), thus limiting the clinical use of these drugs. Streptozotocin, a naturally occurring methylnitrosourea which is a fermentation product of Streptomyces acromogenes, is composed of 1-methyl-1-nitrosourea attached to a glucoseamine carrier. Although this compound has a narrow spectrum of antitumor activity, it is remarkable for its relative lack of myelosuppressive activity (12). This important finding stimulated the search for new nitrosoureas with reduced hematotoxicity. Hansch et al. (6) predicted that nitrosoureas with greater solubility might possess greater antitumor activity and reduced toxicity. Johnston et al. (10) synthesized new water-soluble compounds with a glucose carrier of chloroethyl nitrosoureas.

Imbach and Montero (9) also 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 (Chart 1), have demonstrated a significant antitumor activity in our in vivo screening on transplanted tumors. Chlorozotocin, RFCNU, and RPCNU have also shown clinical activity in cancer patients (1).

The aim of the present study was to quantify the respective effects of these 3 derivatives on the 3 main hematopoietic compartments in mice, peripheral blood, spleen, and bone marrow.

Because we intended to compare the acute and chronic hematotoxicity of the 3 nitrosoureas simultaneously in 3 hematological compartments, we decided to select a single dose of each compound which would have a therapeutic significance. We studied the effect of the 3 drugs in DBA/2 × C57BL/10 F, mice grafted i.p. with 10⁵ L1210 leukemic cells and treated with a single i.p. dose of nitrosourea 1 day after the graft. The results of this study, represented in Chart 2, indicated that the 3 compounds showed a range of doses with a maximal antitumor effect expressed as the percentage of cured mice. The minimal dose showing this maximal antitumor effect was 10 mg/kg for chlorozotocin and RPCNU and 15 mg/kg for RFCNU. These results are in agreement with previ-
Acute Hematotoxicity of Nitrosoureas

Chart 1. Formulae of chlorozotocin, RFCNU, and RPCNU.

<table>
<thead>
<tr>
<th>RFCNU</th>
<th>RPCNU</th>
<th>CHLOROZOTOCIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>O_2_N-_C-_O-R</td>
<td>O-_AC</td>
<td>CH_2-OH</td>
</tr>
</tbody>
</table>

Chart 2. Groups of 10 DBA/2 × C57BL/10 F\_1 mice were inoculated i.p. with 10\^5 L1210 leukemic cells on Day 0. On Day 1, mice were treated with 5- to 50-mg/kg doses of RFCNU, RPCNU, or chlorozotocin, each mouse being weighed individually to determine dosage. Compounds were injected i.p. in olive oil suspension. Ten mice (control group) received i.p. the same volume of olive oil as placebo. On Days 5 and 9, drugs and placebo injections were repeated. The survival of mice was monitored daily, and dead animals were systematically autopsied to determine whether the cause of death was leukemia or toxicity. The oncoslastic effect of specified doses for each compound were expressed as T/C (oncoslastic index) = T/C × 100 (T representing the median survival of the treated mice and C the median survival of the control group), with value indicating that more than 50% of treated animals of the considered group were cured.

**RESULTS**

**Effect on Peripheral Blood.** As shown in Table 1, a single i.p. injection of 0.2 ml oil produced a significant variation of WBC in peripheral blood of control mice with a WBC peak at Day 4. Only RPCNU decreased the number of WBC at Day 4, which returned to normal at Day 7. RFCNU and chlorozotocin were not toxic at the dose used, and even the number of WBC was increased at Day 7 in animals treated with RFCNU.

None of the 3 drugs modified the number of platelets per cu mm (Table 2). No significant modification of hematocrit (results not shown) was detected in the control and treated groups.

**Effect on Spleen.** As shown in Table 3, the i.p. administration of oil resulted in an increase of the mean spleen weight at Days 4 and 7 (Table 3). RPCNU significantly decreased the mean spleen weight at Day 4, whereas the only effect of RFCNU and chlorozotocin consisted of the absence of increase of spleen weight at Day 7. Chart 3 shows that RPCNU induced a 2 log reduction of CFU-s at Day 1 with a complete recovery at Day 4, whereas RFCNU and chlorozotocin transiently decreased the number of CFU-s to 10% of control value at Day 1 with a

- **MATERIALS AND METHODS**

  Male specific-pathogen-free DBA/2 × C57BL/10 F\_1 mice obtained from IFFA CREDO, Lyon, France, 8 to 10 weeks old and weighing 20 to 23 g, were used throughout the experiment.

  Chlorozotocin, RPCNU, and RFCNU were dissolved in olive oil and administered i.p. as a single dose at a volume of 0.1 ml/10 g body weight. It has been shown previously that chlorozotocin in olive oil retained the same antitumor activity as that in water.

  Olive oil (0.2 ml) was injected i.p. into control mice. Five to 10 mice were used for each point.

  Blood samples were obtained by cardiac puncture at various days after the administration of the drug. Blood samples of normal control mice (without any treatment) were also obtained at Days 0 (before the drug injection), 1, 4, and 7, and the data were pooled together.

  RBC and WBC were determined with an automatic blood counter (Coulter S., Coultronics), and the number of platelets was determined by the conventional technique.

  Toxicity of the drug was assayed also on the pluripotent CFU-s and the monopotent CFU-c. Bone marrow cells were flushed out with a syringe fitted with a 22-gauge needle into Fischer's medium supplemented with penicillin (0.05 mg/ml), streptomycin (0.05 mg/ml), and neomycin (0.1 mg/ml). After the cell count, the cells were diluted to an appropriate concentration and used for CFU-s and CFU-c assay.

  Spleen cells were obtained by gently homogenizing the minced spleen pieces with a Potter homogenizer in Fischer's medium and were assayed for CFU-s and CFU-c.

  The number of CFU-s for femur and for spleen was determined according to the method of Till and McCulloch (13). Fifty to 500,000 femoral or spleen cells were injected i.v. into 10 lethally irradiated recipient mice, and the spleen colonies were counted 8 days later. Recipient mice for CFU-s assay were irradiated shortly before the injection of the cells with 960 rads using 137Cs (Gravatom Irradiator, England) at a dose rate of 85 rads/min.

  Ten % lung-conditioned medium was used as a source of colony-stimulating activity. Four replicate cultures were plated for each cell suspension. The plates were incubated at 37° in 5% CO\_2 in humidified air, and the colonies were counted 7 days later.
Table 1
Effect of chlorozotocin, RPCNU, and RFCNU on the number of WBC in peripheral blood

<table>
<thead>
<tr>
<th></th>
<th>No. of WBC/cu mm at indicated times after i.p. injection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 day</td>
</tr>
<tr>
<td>Control (normal)</td>
<td>6,850 ± 1,158</td>
</tr>
<tr>
<td>Control (0.2 ml oil)</td>
<td>6,225 ± 368</td>
</tr>
<tr>
<td>Chlorozotocin (10 mg/kg)</td>
<td>5,325 ± 2,066</td>
</tr>
<tr>
<td>RFCNU (15 mg/kg)</td>
<td>7,800 ± 2,271</td>
</tr>
</tbody>
</table>

Table 2
Effect of chlorozotocin, RPCNU, and RFCNU on the number of platelets in peripheral blood

<table>
<thead>
<tr>
<th></th>
<th>Platelets (x 10^7/cu mm) at following times after i.p. injection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 day</td>
</tr>
<tr>
<td>Control (normal)</td>
<td>1348 ± 132</td>
</tr>
<tr>
<td>Control (0.2 ml oil)</td>
<td>1118 ± 525</td>
</tr>
<tr>
<td>Chlorozotocin (10 mg/kg)</td>
<td>1323 ± 235</td>
</tr>
<tr>
<td>RPCNU (10 mg/kg)</td>
<td>1054 ± 85</td>
</tr>
<tr>
<td>RFCNU (15 mg/kg)</td>
<td>1288 ± 275</td>
</tr>
</tbody>
</table>

Table 3
Effect of chlorozotocin, RPCNU, and RFCNU on spleen weight

<table>
<thead>
<tr>
<th></th>
<th>Spleen wt (mg) at indicated times after i.p. injection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 day</td>
</tr>
<tr>
<td>Control (normal)</td>
<td>62.2 ± 5.6</td>
</tr>
<tr>
<td>Control (0.2 ml oil)</td>
<td>61.4 ± 5.9</td>
</tr>
<tr>
<td>Chlorozotocin (10 mg/kg)</td>
<td>63.6 ± 7.5</td>
</tr>
<tr>
<td>RPCNU (10 mg/kg)</td>
<td>55.0 ± 6.5</td>
</tr>
<tr>
<td>RFCNU (15 mg/kg)</td>
<td>62.8 ± 3.2</td>
</tr>
</tbody>
</table>

Chart 3. Effect of a single i.p. injection of 3 nitrosoureas on spleen CFU-s. Murine splenic colony formation of pluripotent hematopoietic precursors (CFU-s). From 50 to 500,000 spleen cells of control (with and without olive oil) and treated mice taken at various time intervals were injected i.v. into 10 lethally irradiated recipients per point. The spleen colonies were counted 8 days later. The results are expressed as percentage of control untreated mice. Chlorozo., chlorozotocin.

complete recovery at Day 2 (Table 3). Chart 4 shows the evolution of splenic CFU-c. Oil induced an increase of CFU-c from Day 2 to Day 7. RFCNU and chlorozotocin were the most toxic compounds at Day 1 with a rapid recovery at Day 2 for chlorozotocin and at Day 4 for RFCNU. RPCNU decreased the number of CFU-c less at Day 1; the overshoot of CFU-c recovery observed after the treatment with other drugs was not observed at Days 4 and 7.
Effect on Bone Marrow. The 3 nitrosourea derivatives decreased the bone marrow cellularity (down to 60% of normal controls) at Day 1 (Chart 5), with a complete recovery at Day 4 for RFCNU and chlorozotocin and at Day 7 for RPCNU. RPCNU was highly toxic for bone marrow CFU-s at Day 1 (Chart 6), whereas RFCNU and chlorozotocin decreased the number of CFU-s moderately. The maximum effect on bone marrow CFU-c (Chart 7) was obtained with chlorozotocin at Day 1, with a slow increase up to Day 7. RFCNU had no toxicity and a 2-fold increase of CFU-c was observed at Day 2 in mice treated with this compound. RPCNU had little effect on bone marrow CFU-c.

DISCUSSION

These experiments clearly demonstrate that RPCNU is more hematotoxic than are RFCNU and chlorozotocin and, therefore, that it is not sufficient to introduce sugar entities into the molecule of nitrosourea to suppress its hematotoxicity. On the other hand, it adds a new sugar derivative RFCNU to the list of the less hematotoxic nitrosoureas. It also shows that it is necessary, when oil is used as a carrier, to have control animals treated with oil. In our experiment, oil alone was capable of inducing significant hematopoietic modifications.

At doses used in this experiment, which are the minimal doses showing a maximum therapeutic activity, RPCNU affected CFU-s for 24 hr in bone marrow and for 48 hr in the spleen, whereas the effect on CFU-s of RFCNU and chlorozotocin was less marked and reversible within 24 hr. As expected, a single injection produced only a minor change in the peripheral blood. Among the 3 drugs, only RPCNU induced a significant drop in the number of WBC.

The hematocrit and the number of platelets were not modified. The major effect was found at the level of the CFU-s compartment in the spleen and bone marrow. RPCNU reduced the number of uncommitted stem cells in 2 logs whereas RFCNU and chlorozotocin reduce it by 1 log. As stated previously, the toxicity was rapidly reversible. The simultaneous decrease of the mean spleen weight and marrow cellularity also showed that RPCNU had a true cytotoxic effect on hematopoietic cells and did not merely modify their repartition in the organism. The effect was more profound in the spleen than in the bone marrow. The compounds also have differential effects on CFU-c. RPCNU mainly affected CFU-s and was less toxic for CFU-c. Chlorozotocin decreased splenic and femoral CFU-c significantly but was less toxic on CFU-s; RFCNU had also a minor toxicity on both CFU-s and CFU-c.

Our results with chlorozotocin are also in agreement with the results of Heal et al. (8) who showed that a single injection of chlorozotocin (15 mg/kg) had no lethal effect and did not modify the WBC in BALB/c × DBA/2 F2 mice, while keeping a good antitumoral effect on L1210. This would indicate that the use of oil instead of citrate buffer did not modify the biological effect of chlorozotocin, namely, its 50% lethal dose, hematological effects, and antitumoral effects.

We did not include in this study other nitrosoureas such as CCNU or BCNU. However, there are enough published studies comparing chlorozotocin with other more classical nitrosoureas, CCNU, BCNU, and trans-1-(2-chloroethyl)-3-(4-methylcyclohexy1)-1-nitrosourea, to compare with our results. Heal et al. (8) showed the lack of toxicity of chlorozotocin on the granulocytic cell lines compared to the known toxicity of CCNU (and BCNU). This reduced toxicity of chlorozotocin on human and murine hematopoietic precursor cells has been further documented by Schein et al. (11).

Our results are also in agreement with human studies (1, 7) which showed that the 3 studied nitrosoureas had an effect on
 peripheral blood only after repeated injections. Further experiments with repeated weekly injections of the same dose of compound have been undertaken in order to simulate the situation in humans where a cumulative hematotoxicity represents the major limiting factor of a long-term administration of nitrosoureas. Indeed, they show\(^5\) that 5 injections of RPCNU induce a long-lasting hematotoxicity with thrombopenia as in humans. The lack of cumulative toxicity of chlorozotocin on granulocytic precursors was also confirmed.

Further experiments on the effects of the 3 nitrosoureas on erythropoietic and megakaryocytic precursors are also in progress and will provide a complete overview of the comparative hematological effects in mice. It is hoped that this type of study may lead to a better understanding of the cumulative hematotoxicity of nitrosoureas and of their preferential thrombocytotoxicity, which constitutes the main limiting factor to their wider use in the treatment of human cancer.

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REFERENCES

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