Ovarian Reticular Cell Sarcoma of the Mouse (M5076) Made Resistant to Cyclophosphamide

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ABSTRACT

Resistance of mouse M5076 (M5) ovarian reticular cell sarcoma to cyclophosphamide (CTX) was obtained in vivo by repeated drug treatment followed by transplantation of the regrowing tumor. After 16 passages, we obtained an M5 subline resistant to CTX (M5-CTX-16R). Median survival times were approximately 29 and 39 days for M5 and M5-CTX-16R, respectively. Survival of M5-bearing mice given a single i.p. dose of 200 or 300 mg/kg was 160 and 168% of controls, whereas in M5-CTX-16R it was 103 and 123%, respectively. The resistance was not reversible after 14 additional passages with no further CTX treatment. M5 and M5-CTX-16R appear similar in histological features, pattern of metastasis formation, and DNA content, as assessed by flow cytometry (hypotetraploid). Metastases of M5-CTX-16R were also resistant to CTX. Flow cytometry studies 12 and 24 hr after CTX treatment revealed a block in S and G2-M phases in both tumors. After 48 hr and at subsequent times, no cytokinetic perturbation was evident in M5-CTX-16R, whereas in M5 marked accumulation of cells in G2-M was observed at 48, 72, 96, and 120 hr. Cross-resistance was found between CTX, L-phenylalanine mustard, chlorambucil, and hexamethylmelamine. M5-CTX-16R was sensitive, but less so than M5, to cis-platinum, 1,3-bis(2-chloroethyl)-1-nitrosourea, and imidazole-4-carboxamide, 5-(3,3-dimethyl-1-triazene). Adriamycin was equally active on M5 and M5-CTX-16R, while 4'-deme-

INTRODUCTION

CTX is an anticancer drug (4) clinically used as a single agent or in combination with other drugs for the therapy of different hematological and solid cancers. Unfortunately, as for other alkylating agents and for antitumoral drugs in general, even initial responsive tumors often become resistant after some courses of chemotherapy.

The development of resistance makes it impossible to complete the destruction of all neoplastic cells and therefore halts the patient’s cure. It is therefore important to clarify the mechanisms involved in such resistance by developing experimental animal tumors specifically resistant to single or to classes of anticancer agents.

We describe a murine reticular cell sarcoma of the ovary (M5076) made resistant to CTX. This appears to be a potentially useful model for investigating the nature of resistance to CTX and other bifunctional alkylating agents and for screening new drugs with different mechanisms of action.

MATERIALS AND METHODS

Animal. Female C57BL/6 mice [20 ± 2 (S.E.) g] obtained from Charles River, Calco, Italy, received an i.m. transplant of 5 × 10^6 viable cells of ovarian reticular cell sarcoma M5076 (M5) supplied by Mason Research Institute, DCT-Animal and Human Tumor Bank, Worchester, Mass. The tumor was maintained by i.m. passages in the same strain every 3 weeks. We obtained an M5 tumor line resistant to CTX (M5-CTX-16R) by repeated drug treatment followed by transplantation of the regrowing tumor for a total of 16 passages.

The mice were given i.p. doses of CTX, 20 mg/kg, daily for 10 consecutive days (20 to 29 days after tumor implantation) during the first 5 passages and then CTX, 200 mg/kg i.p., on Day 4 after tumor implantation for the subsequent 11 passages.

The development of resistance was followed by determining drug activity on the parental line and on the last line treated. We measured tumor sizes in treated and untreated mice with either M5 or M5-CTX-16R by recording maximum and minimum diameters with a vernier caliper at intervals of 2 to 5 days. We calculated tumor volume by the formula V = (LW^2)/2, where L is the average length in mm and W the width of the tumor; the volume is converted to weight in g assuming unit density. Data were then expressed as means ± S.D. of each group. The survival time was expressed as the median survival time of treated mice versus the median survival time of untreated mice x 100 (7).

In order to evaluate the effects of CTX on metastases of M5 and M5-CTX-16R, we performed autopsies 25 and 28 days, respectively, after transplant, and organs with metastases were inspected and weighed.

Drugs. L-PAM, CLB, HMM, BCNU, DTIC, and CDDP were kindly supplied by the National Cancer Institute, NIH, Bethesda, Md. CTX was supplied by Dr. Brock, Asta-Werke AG, Brackwede, West Germany; ADM was supplied by Dr. Arcamone, Farmitalia-Carlo Erba, Milan, Italy; VP16 was supplied by Dr. Lenaz, International Division, Bristol Meyers, New York, New York.

L-PAM, CLB, and HMM were suspended in hydroxypropyl cellulose; BCNU and VP16 were supplied in Tween 80.9% NaCl solution (1:10); DTIC was dissolved in 0.05 M citric acid; CTX, ADM, and CDDP were dissolved in 0.9% NaCl solution.

Flow Cytometry Analysis. The tumors were removed, washed in phosphate-buffered saline (KCl, 200 mg/liter; KH2PO4, 200 mg/liter; NaCl, 8000 mg/liter; NaH2PO4, 1150 mg/liter; Eurobio-Paris), and minced with scissors to remove the necrotic part from the vegetal part, which was used for DNA analysis. Cell suspensions for analysis by flow cytometry were obtained by forcing the tumor fragments through an 18.5-× 1.5-needle and resuspending them in Hanks’ solution (Flow Laboratories, U.K.) maintained at 4°C (19). The cells were stained with propidium iodide (Calbiochem-Behring, San Diego, Calif.) by adding 3 ml of propidium...
iodide solution (50 μg propidium iodide per ml in 0.1% sodium citrate plus 30 μl Nonidet P-40) to 100 to 200 μl of cell suspension and were stored at 4° for 30 min before DNA analysis.

Absence of aggregates and the suitability of the preparation were checked by fluorescence microscopy before the samples were run. Leukocytes from C57BL/6 mice were used as standard for DNA content ploidy. Normal cells in M5 or M5-CTX-16R tumors had the same DNA content in terms of fluorescence channel as did normal leukocytes. DNA analysis was performed using a 30L Cytofluorograph (Ortho Instruments, Westwood, Mass.). The fluorescence pulses were detected in a spectral range between 580 and 780 nm (to exclude the overlapping region of excitation and emission spectra or unbound propidium iodide) and then integrated. The coefficient of variation of standard leukocytes was between 1.5 and 2.5%, and the G1 peak of M5 and M5-CTX-16R tumors was between 4 and 6%. The results are the means of 4 animals/group.

RESULTS

CTX given in single doses of 200 or 300 mg/kg i.p. on Day 4 after tumor implantation slows the growth of the M5 tumor considerably (Chart 1, a and c) and increases the survival time of the M5-bearing mice (Table 1). When M5-bearing mice were treated with CTX according to the schedule described under “Materials and Methods,” the tumor gradually became resistant to the action of CTX. After 16 passages, M5 was completely resistant to the doses of CTX (200 and 300 mg/kg i.p.) which were effective in the sensitive tumor. Chart 1, b and d, summarizes this finding, showing the growth of the tumor; Table 1 indicates that the survival time of the animals bearing the resistant tumor (hereafter referred to as M5-CTX-16R) was not significantly affected by CTX treatment. Chart 2 gives more details of

<table>
<thead>
<tr>
<th>Agents</th>
<th>M5 T/Ca median × 100</th>
<th>M5-CTX-16R T/Ca median × 100</th>
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<tbody>
<tr>
<td>CTX</td>
<td>200 mg/kg i.p. (4)*</td>
<td>160</td>
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<td></td>
<td>300 mg/kg i.p. (4)</td>
<td>168</td>
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<tr>
<td>CLB</td>
<td>10 mg/kg i.p. (3, 7, 11)</td>
<td>139</td>
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<td>20 mg/kg i.p. (3, 7, 11)</td>
<td>153</td>
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<tr>
<td>L-PAM</td>
<td>6 mg/kg i.p. (3, 7, 11)</td>
<td>146</td>
</tr>
<tr>
<td></td>
<td>12 mg/kg i.p. (3, 7, 11)</td>
<td>71</td>
</tr>
<tr>
<td>HMM</td>
<td>40 mg/kg i.p. (9-17)</td>
<td>133</td>
</tr>
<tr>
<td>BCNU</td>
<td>30 mg/kg i.p. (4)</td>
<td>179</td>
</tr>
<tr>
<td>DTIC</td>
<td>36 mg/kg i.p. (7-15)</td>
<td>153</td>
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<tr>
<td>CDDP</td>
<td>3 mg/kg i.p. (12, 14, 16, 18)</td>
<td>175</td>
</tr>
<tr>
<td></td>
<td>4 mg/kg i.p. (12, 14, 16, 18)</td>
<td>75</td>
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<tr>
<td>ADM</td>
<td>12 mg/kg i.v. (5)</td>
<td>176</td>
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<tr>
<td>VP16</td>
<td>13 mg/kg i.p. (8, 11, 14)</td>
<td>120</td>
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* T/C, median survival time of treated mice versus median survival time of untreated controls.

Numbers in parentheses, day(s) of treatment after tumor implant.

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<tr>
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<th>% Survivors</th>
<th>% Survivors</th>
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<td>Days after tumor implant</td>
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<tr>
<td>M5</td>
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<td>CTX</td>
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<tr>
<td>M5-CTX-16R</td>
<td>e</td>
<td>f</td>
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<tr>
<td>CTX</td>
<td>g</td>
<td>h</td>
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Chart 1. Effect of CTX on the growth of M5 (a and c) and M5-CTX-16R (b and d) tumors in mice. a and b: , controls (0.9% NaCl solution); , CTX, 200 mg/kg i.p. (Day 4). c and d: , controls (0.9% NaCl solution); , , CTX, 300 mg/kg i.p. (Day 4). Experiments in b and d were made after 14 and 7 passages, respectively, of M5-CTX-16R during which no CTX treatment was given. Both tumors were implanted, i.m. with an inoculum of 5 × 10⁶ cells. Numbers in parentheses, surviving animals at that time.

Chart 2. Effect of CTX on survival of M5 (a and b)- and M5-CTX-16R (c and d)-bearing mice. a and c: ; , controls (0.9% NaCl solution); , , CTX, 200 mg/kg i.p. (Day 4). b and d: , , controls (0.9% NaCl solution); , , CTX, 300 mg/kg i.p. (Day 4). Experiments in c and d were made after 14 and 7 passages, respectively, of M5-CTX-16R during which no CTX treatment was given.

Table 2 shows the results of autopsies 25 and 28 days, respectively, after transplantation of M5 and M5-CTX-16R in animals treated or untreated with CTX. The pattern of metastasis growth was similar; however, unlike the metastases in the pri-
Table 2
Antitumor and antimetastatic activity of CTX on M5 and M5-CTX-16R
Autopsy was carried out 25 days after tumor implant for M5 and 28 days for M5-CTX-16R.

| Tumor        | Body wt (g) | Tumor wt (g) | Liver wt (g) | Spleen wt (g) | Metastases (%)
|--------------|-------------|--------------|--------------|---------------|----------------
| M5 Controls  | 20.3 ± 0.99 | 4.28 ± 0.71  | 1.94 ± 0.27  | 0.18 ± 0.02   | 80             
| CTX, 200 mg/kg i.p. | 18.6 ± 0.34  | 1.24 ± 0.10^a | 1.05 ± 0.05  | 0.10 ± 0.006   | 0              
| M5-CTX-16R Controls | 21.0 ± 2.80  | 5.06 ± 0.42  | 2.57 ± 0.31  | 0.22 ± 0.02   | 88             
| CTX, 200 mg/kg i.p. | 22.67 ± 0.87 | 4.36 ± 0.31  | 2.34 ± 0.28  | 0.21 ± 0.01   | 89             

* Mostly in liver, ovary, and spleen.
 Numbers in parentheses, day of treatment after tumor implant.
 ^p < 0.01 by Student’s t test.
 ^p < 0.025 by Student’s t test.

Chart 3. Flow cytometry DNA histograms of M5 and M5-CTX-16R without CTX treatment (control) and at intervals (12, 24, 48, 72, 96, and 120 hr) after CTX treatment (200 mg/kg i.p. 14 days after tumor transplantation). The percentages of G1-S and G2-M are the means of 4 samples.

mary tumor, those in M5-CTX-16R were not responsive to treatment with CTX. Macroscopic inspection showed that M5-CTX-16R was less necrotic than M5. Histological examination of M5 and M5-CTX-16R did not reveal any significant difference between the 2 tumors which proved to be reticular cell sarcomas. That M5-CTX-16R was irreversibly resistant to CTX was shown by the fact that CTX was ineffective even when tested in mice bearing M5-CTX-16R not treated with CTX during the 14
passages after the onset of resistance (Chart 1b). M5-CTX-16R shows the peculiar characteristic that it grows more than its parent tumor M5 before it kills its host. In fact, in a series of tests, it was found that the average survival time of animals with M5 was 28.9 ± 0.9 (S.E.) days, while for M5-CTX-16R it was 39.4 ± 1.1 days (p < 0.001 according to Student's t test). Animals bearing M5 died with a smaller tumor than those bearing M5-CTX-16R (3.76 ± 0.14 versus 5.61 ± 0.29 g) (p < 0.001). Therefore, the onset of resistance to CTX appears linked to a selection of M5 cells which are less toxic for the host. Chart 3 shows the DNA patterns (flow cytometry) of M5 and M5-CTX-16R before and 12, 24, 48, 72, 96, and 120 hr after CTX treatment. The DNA content of cells from M5 and M5-CTX-16R was virtually identical, the peak of fluorescence of neoplastic cells in G1 corresponding to Channel 75 (hypotetraploid). Twelve hr after CTX treatment, an accumulation of cells in S phase was observed in M5 and to a slightly lesser extent in M5-CTX-16R. At 24 hr, there was a slightly higher percentage of cells in G2-M in M5-CTX-16R, whereas in M5 a striking block in G2-M was still evident even 72 and 96 hr after CTX treatment. By 120 hr, neoplastic cells were so few as to make the analysis of distribution unreliable under our conditions. It looks, therefore, as though repair of the resistant cells is more effective than that of the sensitive M5.

We also investigated whether there was cross-resistance between CTX and other anticancer agents selected on the basis of their supposed mechanisms of action. As can be seen in Chart 4, CLB, 10 mg/kg, did not apparently affect the growth of M5-CTX-16R but it markedly reduced the growth of M5. A dose of 20 mg/kg had a marginal effect on M5-CTX-16R but a striking effect on M5.

L-PAM gave measurable inhibition of tumor growth of M5-CTX-16R at 12 mg/kg, a dose that was not tolerated in M5-bearing mice (all animals died before the tumor was measurable); a dose of 6 mg/kg had only marginal activity in M5-CTX-16R but very marked reduction of M5 tumoral masses was observed.
M. D'Incalci et al.

Chart 6. Effect of CDDP on M5 (a and c) and M5-CTX-16R (b and d) tumors in mice. a and b: controls (0.9% NaCl solution); ---, CDDP, 3 mg/kg i.p. (Days 12, 14, 16, and 18). c and d: controls (0.9% NaCl solution); ---, CDDP, 4 mg/kg i.p. (Days 12, 14, 16, and 18). Experiments in b and d were made after 7 and 2 passages, respectively, of M5-CTX-16R during which no CTX treatment was given.

Chart 7. Effect of ADM and VP16 on M5 (a and c) and M5-CTX-16R (b and d) tumors in mice. a and b: controls (0.9% NaCl solution); ---, ADM, 12 mg/kg i.v. (Day 5). c and d: controls (Tween 80, 10% in 0.9% NaCl solution); ---, VP16, 13 mg/kg i.p. (Days 8, 11, and 14). Experiments in b and d were made after 2 and 5 passages, respectively, of M5-CTX-16R during which no CTX treatment was given.

The effect of HMM on growth of M5-CTX-16R was negligible compared to that on M5. The survival times of M5-bearing mice were significantly prolonged by CLB, L-PAM (only at the dose of 6 mg/kg), and HMM, but these treatments were ineffective on M5-CTX-16R (Table 1). Chart 5 shows the activity of BCNU on growth of M5 and M5-CTX-16R. The 2 tumors appeared equally sensitive to this treatment (30 mg/kg i.p. on Day 4 after transplantation), as shown by the fact that survival time was increased in animals bearing the sensitive and the resistant M5 tumor (Table 1). Likewise, DTIC retained almost normal effectiveness in inhibiting the growth of M5 and M5-CTX-16R tumors and prolonging survival (Table 1).

Chart 6 depicts the effect of CDDP at 2 doses, 3 or 4 mg/kg...
i.p. 12, 14, 16, and 18 days after tumor transplantation. The results are not clear-cut because, at the 3-mg/kg dose, CDDP delayed tumor growth in both sensitive and CTX-resistant M5 lines; however, survival time was prolonged only with the sensitive line (Table 1). At the 4-mg/kg dose, CDDP was very toxic for the sensitive line but not for the CTX-resistant line, and accordingly survival time was prolonged only in animals bearing the CTX-resistant but not the CTX-sensitive M5 tumor.

Finally, Chart 7 indicates that ADM was equally sensitive in inhibiting the growth of both M5 tumor lines as well as in prolonging survival time (Table 1). VP16 was inactive on both tumor lines as indicated by tumoral growth (Chart 7) and survival time (Table 1).

**DISCUSSION**

The line (M5-CTX-16R) of a murine ovarian reticular cell carcinoma (M5) described here shows complete resistance to CTX, which is apparently not reversible when CTX was discontinued for a period of 14 serial transplantations in syngeneic mice. The development of resistance was assessed from the growth of the tumor and the average survival time of the tumor-bearing animals after a single large dose of CTX (200 or 300 mg/kg i.p.), which was effective on the sensitive tumor. The mechanism at the basis of this resistance has still to be elucidated. The data obtained thus far suggest that the mechanism of resistance does not involve a lack of uptake of active metabolites of CTX. In fact, after 12 or 24 hr, there is a cytokinetic perturbation (accumulation of cells in S and G2 phase) both in M5 and in M5-CTX-16R, indicating that the active chemical species had entered the cells and had interacted with their target macromolecules. This cytokinetic perturbation, however, was rapidly reversed in M5-CTX-16R but was more marked and longer-lasting in M5. This suggests the hypothesis that M5-CTX-16R may have developed a more efficient repair mechanism for damage caused by CTX.

The resistant tumor M5-CTX-16R has the same histological features as did the M5 parent line but definitely less necrotic material. Other biological features characterize the M5-CTX-16R tumor when transplanted i.m.; its growth appeared somewhat slower than that of the sensitive parent line M5 as judged by the time necessary to obtain palpable tumors. In addition, the survival time of M5-CTX-16R-bearing mice was longer (38.4 versus 28.9 days), and the resistant tumor was somewhat less toxic for the host judging from the fact that M5-CTX-16R-bearing mice died with larger tumors than did those with the M5 parent line (5.6 versus 3.8 g). The apparently lower virulence of the M5-CTX-16R tumor is an interesting finding which may have important implications.

The fact that the M5-CTX-16R tumor has developed new biological characteristics made study of its sensitivity to a number of anticancer drugs particularly interesting. We selected alkylating agents with the same alkylating group, L-PAM and CLB; other different alkylators, such as BCNU, DTIC, CDDP, and HMM; ADM, believed to act primarily as an intercalating agent; and VP16, the mechanism of action of which is still not well defined. While all the other compounds are active on M5, VP16 shows only borderline activity.

Cross-resistance could be expected between CTX, L-PAM, and CLB, inasmuch as they all have the same bifunctional alkylating group. Although there are some selected experimental models in which this cross-resistance is not observed (13, 15, 22), there is ample experimental and clinical evidence that in most cases these drugs are cross-resistant (5, 10, 16, 18).

The finding that HMM was cross-resistant with CTX appears interesting as its real mechanism of action is still a matter of debate. The hypothesis that it acts as an alkylating agent through the formation of N-methylols (8) known to covalently bind DNA and proteins (1) may be supported by the fact that HMM is inactive in M5-CTX-16R. This is also in line with recent clinical observations that ovarian cancer patients refractory to alkylating agents (3, 9) did not respond to HMM.

As reported previously on different CTX-resistant murine tumors, BCNU is not cross-resistant (12), thus confirming that this alkylating agent probably acts by a different mechanism. It should be noted that BCNU, in addition to alkylating activity induces carbamylation of proteins (12).

Lack of cross-resistance between CTX and CDDP has been reported previously in other murine tumors (14). The fact that CDDP is active on M5-CTX-16R but not as active as it is on the M5 is in line with clinical data indicating that CDDP is still active, but much less so, in patients who have become resistant to classical alkylating agents (11, 23).

DTIC also appears to have no cross-resistance to CTX, and this confirms previous findings in mouse leukemias (21).

That ADM was not cross-resistant to CTX could be expected on the basis of available knowledge on its mode of action. Although it has been shown that ADM, by metabolic reduction, forms free radicals that covalently bind DNA (17) and that it kills cancer cells even though it does not cross the cell membrane (20), the main mechanism of action for its antitumor activity is believed to be related to intercalation in DNA (6).

VP16 was tested to see whether overgrowing cells, mutant for the resistance to CTX, could become sensitive to a drug that was previously inactive, but this was not the case and VP16 was equally ineffective on M5 and M5-CTX-16R.

A point that is not clear is why L-PAM, CLB, and CDDP at high doses were toxic in M5- but not in M5-CTX-16R-bearing mice. A tentative explanation is that the drug toxicity is added to the toxicity due to the tumor which, as discussed above, seems greater for M5 than for M5-CTX-16R.

In conclusion, M5-CTX-16R appears to be a model suitable for studies aimed at elucidating the mechanism of resistance to CTX and similar alkylating agents and at screening for non-cross-resistant drugs.

**REFERENCES**

M. D’Incaici et al.


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