Curative Effect of DL-2-Difluoromethylornithine on Mice Bearing Mutant L1210 Leukemia Cells Deficient in Polyamine Uptake

Lo Persson, Ingvar Holm, Anders Ask, and Olle Heby

Departments of Physiology [L. P.], Zoophysiology [I. H., O. H.], and Oncology [A. A.], University of Lund, S-223 62 Lund, Sweden

ABSTRACT

The objective of the present investigation was to determine to what extent polyamine uptake from the host contributes to the ability of tumor cells in overcoming the antiproliferative effect of a polyamine synthesis inhibitor. A mutant L1210 leukemia cell line deficient in polyamine transport was isolated by selection for resistance to methylglyoxal bis(guanylhydrazone), an extremely cytotoxic agent which is taken up by the same transport system as the polyamines. C57BL/6 × DBA/2 F1 mice inoculated with mutant L1210 cells survived on the average 60 to 70% longer than mice inoculated with the parental cells. The therapeutic effect of a polyamine synthesis inhibitor, DL-2-difluoromethylornithine (3% in the drinking water), was much greater on mice bearing mutant L1210 cells (87% increase in median survival time; 13 of 40 mice cured) than on mice inoculated with parental cells (22% increase in median survival time). Similar results, although not as striking, were obtained using athymic nude mice, indicating that the therapeutic difference is not merely due to increased immunogenicity of the mutant cells.

MATERIALS AND METHODS

Experimental Animals. Male C57BL/6 × DBA/2 F1 (hereafter called B6D2F1) mice, weighing 18 to 20 g, were purchased from Gl. Bomhol, Ry, Denmark. The mice were given standard laboratory feed and tap water ad libitum.

Nude BALB/c (nu/nu) mice were bred by mating heterozygous (nu/+) females with homozygous (nu/nu) males. The mouse colony was kept under sterile but not specific-pathogen-free conditions. The mice were held in sterile cages under filter tops and with coarse sawdust bedding. They obtained sterilized standard laboratory feed and acidified (pH 2.5 to 2.7) water ad libitum. The nude mice used in the experiments were males weighing 18 to 20 g.

Cell Culture. L1210 cells were grown in RPMI Medium 1640 supplemented with 10% fetal calf serum, 50 units/ml of penicillin, 50 µg/ml of streptomycin, and 50 µM 2-mercaptoethanol. The cells were routinely subcultured (1.0 × 10⁵ cells/ml) every 3 or 4 days.

Mutagenesis. L1210 cells resistant to MGBG were isolated essentially as described by Mandel and Flintoff (25). Thus, logarithmically growing cells were treated with ethyl methanesulfonate for 18 h at 37°C, centrifuged, and resuspended in fresh medium. The mutagenized cells were grown in the absence of drug for 4 days and were then reseeded in medium supplemented with various concentrations of MGBG (1 to 50 µM). In the medium containing 1 µM MGBG, a resistant cell population emerged. These cells were then subjected to stepwise increases in the concentration of MGBG (5, 10, 50, 200 µM) over a period of 2 mo. Then the mutant cells grew almost as well in 200 µM MGBG as did the parental cells in drug-free medium. The mutant cell line was designated L1210-MGBG' and was maintained under the same conditions as the parental cells with the exception that the medium was supplemented with 200 µM MGBG. [3H]Spermidine Uptake. For time-dependent uptake, L1210 and L1210-MGBG' cells were seeded at a density of 2.0 × 10⁵ cells/ml in drug-free medium. [3H]Spermidine (1 Ci/mmol) was added 24 h after seeding to a final concentration of 2 µM and the cells were then incubated at 37°C for various time periods. Incubation was terminated by rapidly chilling the cells on ice followed by centrifugation at 3000 × g for 5 min at 4°C. The cells were washed in ice-cold Ca²⁺- and Mg²⁺-free Dulbecco's phosphate-buffered saline containing 10 mM unlabeled spermidine. Parallel cultures were incubated at 0°C to determine nonspecific binding of [3H]spermidine. The cells were then dissolved in 300 µl of Soluene 350 (Packard Instruments), and the amount of radioactivity was determined in a liquid scintillation spectrometer.

For Lineweaver-Burke analysis, L1210 and L1210-MGBG' cells that had been grown in drug-free medium were centrifuged at 500 × g, 5 min, and resuspended in prewarmed phosphate-buffered saline to a density of 5.0 × 10⁵ cells/ml. Various concentrations of [3H]spermidine were added, and uptake was determined as described above. Results were plotted according to the method of Lineweaver and Bur-ke (26). Uptake was determined in untreated cultures as well as in cultures treated with the inhibitor. The Lineweaver-Burke plots were used to estimate the dissociation constant Kᵣ and the maximal velocity Vₐ₅₀ of the transporter.
L1210-MGBG': A POLYAMINE TRANSPORT MUTANT

(1.0 Ci/mmol) were added, and the cells were then incubated at 37°C for 10 min. Uptake and nonspecific binding of [3H]spermidine were assayed as described above.

Tumor Models and Drug Regimen. B6D2F1 and athymic nude mice were inoculated i.p. with 1.0 x 10^6 mutant or parental L1210 cells (in 0.2 ml of 0.9% NaCl solution). DFMO was included in the drinking water (3%), beginning 1 day after tumor inoculation. The average DFMO intake corresponded to a dose of 4.2 to 4.4 g/kg each day.

Statistical Analysis. Survival data were evaluated according to Mantel (28).

RESULTS

By exposing mutagenized L1210 cells to MGBG at stepwise increasing concentrations, an MGBG-resistant cell line was isolated. The mutant cells were able to grow in the presence of 200 μM MGBG, whereas the parental cells grew poorly with significant cell death at an MGBG concentration of 1 μM. The resistance to MGBG was due to a marked deficiency in the transport system as evaluated by measuring the uptake of radioactive spermidine (Fig. 1). A kinetic analysis of the spermidine uptake revealed a decrease in Vmax and an increase in Km (Fig. 2). As to the cellular polyamine content, there was no major difference between the L1210-MGBG' and the parental cells, with the exception of a small decrease in spermidine content in the mutant cells (Table 1).

As shown in Fig. 3, the growth curve for the MGBG-resistant cells was similar to that of the parental cells. Notably, the change in polyamine uptake did not affect the sensitivity of the cells to DFMO treatment in culture (Fig. 3). A similar decrease in the cellular polyamine content was observed in the mutant and parental cells as a result of DFMO treatment (Table 1). This treatment stimulated spermidine uptake into MGBG-resistant cells and parental cells by 2.6- and 4.6-fold, respectively. Despite this increase the rate of spermidine uptake was extremely low in the mutant cells.

To investigate the physiological importance of the cellular polyamine uptake system, the survival of B6D2F1 mice inoculated with either parental or mutant L1210 cells was studied (Fig. 4). The median survival time of mice bearing the parental L1210 cells was 9 days. Mice inoculated with L1210-MGBG' cells exhibited a median survival time that was 6 days longer (P < 0.001). The therapeutic effect of DFMO was dramatically greater in mice inoculated with the mutant L1210 cells than in mice bearing the parental cells (Fig. 4). Mice inoculated with parental L1210 cells survived on the average 2 days longer when treated with DFMO (P < 0.01). DFMO treatment of mice inoculated with the mutant cell line gave rise to an 87% increase in median survival time (P < 0.001) with a 33% cure; i.e., 13 of 40 mice survived for more than 100 days (Fig. 4) and showed no sign of tumor presence.

Table 1 Polyamine content of MGBG-resistant (L1210-MGBG') and parental LI210 cells in the absence or presence of DFMO

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Putrescine (nmol/10⁶ cells)</th>
<th>Spermidine (nmol/10⁶ cells)</th>
<th>Spermine (nmol/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1210</td>
<td>0.32 ± 0.08</td>
<td>2.33 ± 0.21</td>
<td>0.78 ± 0.12</td>
</tr>
<tr>
<td>L1210 + DFMO</td>
<td>&lt;0.005</td>
<td>0.33 ± 0.10</td>
<td>1.30 ± 0.11</td>
</tr>
<tr>
<td>L1210-MGBG'</td>
<td>0.23 ± 0.07</td>
<td>1.54 ± 0.33</td>
<td>0.83 ± 0.14</td>
</tr>
<tr>
<td>L1210-MGBG' + DFMO</td>
<td>&lt;0.005</td>
<td>0.15 ± 0.03</td>
<td>1.65 ± 0.32</td>
</tr>
</tbody>
</table>

* Mean ± SD (n = 3).
L1210-MGBG\textsuperscript{r}: A POLYAMINE TRANSPORT MUTANT

Fig. 4. Effects of DFMO on the survival of B6D2F\textsubscript{1} mice inoculated with 1.0 \times 10\textsuperscript{6} MGBG-resistant (L1210-MGBG\textsuperscript{r}) or parental L1210 cells. One hundred % corresponds to 20 (L1210), 31 (L1210 + DFMO), 40 (L1210-MGBG\textsuperscript{r}), and 40 (L1210-MGBG\textsuperscript{r} + DFMO) mice. DFMO was given in the drinking water (3\%) from Day 1 on. The treatment was discontinued after 50 to 60 days.

Fig. 5. Effects of DFMO on the survival of athymic nude mice inoculated with 1.0 \times 10\textsuperscript{6} MGBG-resistant (L1210-MGBG\textsuperscript{r}) or parental L1210 cells. One hundred % corresponds to 10 mice. DFMO was included in the drinking water (3\%) from Day 1 on.

Results from experiments made by Mihich and coworkers (29, 30) indicate that L1210 leukemia sublines may exhibit differences in immunogenicity which affect the survival of their host. Thus, the difference in therapeutic effect of DFMO against the parental and the mutant L1210 cell lines might be due to altered immunogenicity. This possibility was investigated using athymic nude mice. Parental as well as mutant L1210 cells grew well in nude mice, resulting in host survival times corresponding to those observed when using B6D2F\textsubscript{1} mice (Fig. 5). As observed for the B6D2F\textsubscript{1} mice, nude mice survived a few days longer ($P < 0.01$) when inoculated with mutant cells than with parental L1210 cells (Fig. 5). Also in the nude mice DFMO did have a greater effect on the mutant cells than on the parental L1210 cells. However, the response to DFMO was remarkably less pronounced. In nude mice bearing parental L1210 cells, DFMO treatment caused no significant prolongation of survival, whereas mice bearing mutant cells survived on the average 42\% longer ($P < 0.001$) when treated with DFMO (Fig. 5). No cure of leukemia in nude mice was obtained by DFMO treatment.

DISCUSSION

All mammalian cells appear to be able to take up polyamines from their environment. The knowledge of this transport system is meager. It is an energy-dependent process capable of accumulating intracellular polyamines in the millimolar range from micromolar concentrations extracellularly (31–33). Whether the polyamine uptake system is of any major physiological significance is not known. Normally, cells are capable of producing the amount of polyamines they need. However, in cases when the biosynthesis of polyamines is impaired, due to lack of substrates or due to inhibition of biosynthetic enzymes, cells might be more dependent on extracellular polyamines. In fact, the uptake of extracellular polyamines increases significantly in DFMO-treated cells (19, 20). The supply of polyamines in the blood is relatively large (22–24). It is conceivable that these polyamines can be used whenever needed and thus reduce the antiproliferative effect of polyamine synthesis inhibitors on tumors in vivo.

The importance of the cellular polyamine transport system was investigated in this paper using a mutant leukemic cell line deficient in polyamine uptake. The fact that these cells were isolated by selection for resistance to MGBG confirms the presence of a mutual transport system for MGBG and polyamines (19, 20, 25, 26). It should be noted that not all MGBG-
resistant cell lines are deficient in polyamine transport. Thus, resistance of some human cell lines to MGBG has been explained by reduced accumulation of the drug due to changes in its intracellular binding sites (34). As to the L1210-MGBG' cells, however, the major cause of resistance is an uptake deficiency. Thus, a major decrease in spermidine uptake (as measured in short-term experiments) was observed for these cells (Fig. 1), whereas the uptake rates of the MGBG-resistant human cell lines did not differ from that of the parental cells (34). In addition, a much greater concentration of putrescine (about 10-fold) was required to reverse the antiproliferative effect of DFMO on the L1210-MGBG' cells than on the parental cells (results not shown).

MGBG has many effects on polyamine metabolism potentially related to its cytotoxic mechanism of action, such as induction of spermidine/spermine N'-acetyltransferase (35) and S-adenosylmethionine decarboxylase (36). However, no major differences in the activities of these enzymes (results not shown) or in the concentrations of the polyamines were found when comparing parental and mutant L1210 cells.

The tumorigenicity of the mutant cells in B6D2F1 mice was reduced as compared to that of the parental cells. However, tumorigenicity was not completely lost as described for some adenovirus-transformed cell lines selected for resistance to MGBG (37, 38). Interestingly, the therapeutic effect of DFMO was much greater against leukemia caused by the mutant cells than that caused by the parental L1210 cells. Thus DFMO treatment cured 33% of the B6D2F1 mice that had been inoculated with mutant L1210 cells. This is the strongest therapeutic effect ever observed for DFMO in an in vivo tumor system. Whether the effect is entirely a function of decreased polyamine uptake remains to be established.

Mihich and Kitano (29) demonstrated that mice inoculated with another MGBG-resistant subline of L1210 leukemic cells survived longer than mice that had received cells from the parental line. Furthermore, the therapeutic effect of various anticancer drugs was greater in mice bearing the resistant cells than in mice inoculated with the parental L1210 cells. The fact that these differences in survival were reduced or abolished if the mice were given total X-radiation indicates a difference in immunogenicity of ornithine decarboxylase. Biochem. Biophys. Res. Commun., 81: 58–66, 1978.


Curative Effect of dl-2-Difluoromethylornithine on Mice Bearing Mutant L1210 Leukemia Cells Deficient in Polyamine Uptake

Lo Persson, Ingvar Holm, Anders Ask, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/48/17/4807