Reversal of Methotrexate Inhibition of Colony Growth of L1210 Leukemia Cells in Semisolid Medium

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ABSTRACT

L1210 mouse leukemia cells were grown in a methylcellulose-based medium, and the inhibitory effect of methotrexate (MTX) on colony formation and its reversal were examined. The effect on colony formation was studied in order to compare the results with those obtained with normal mouse bone marrow cells grown in a similar manner in previous studies and in additional experiments presented. Light microscopy could not be used for colony counting of L1210 cells because MTX did not inhibit colony formation and only affected further colony growth. Therefore, it was necessary to evaluate the toxic effect of MTX by analysis of colony size distribution using an electronic image analyzer. Results show that the reversal of MTX toxicity to L1210 cells with leucovorin is competitive and is similar to that with normal mouse bone marrow cells. Thymidine in combination with a purine prevents MTX toxicity as well. The optimal concentration of thymidine is $10^{-7}$ M, whereas at least $10^{-6}$ M purine is required. Reversal of MTX toxicity by thymidine and purines is independent of the MTX concentration and is possible at drug concentrations as high as $10^{-4}$ M. Compared to mouse bone marrow cells, L1210 cells appear to require more purines to prevent the toxic effects of MTX. MTX toxicity towards bone marrow myeloid precursor cells can be reversed by $10^{-4}$ M inosine alone. These cells are better protected against MTX toxicity when $10^{-6}$ to $10^{-5}$ M thymidine is added. The results suggest that the use of a high-purine-low-thymidine combination has advantages over the use of leucovorin in controlling toxicity over a wide range of MTX concentrations and in providing some degree of selective protection to normal proliferating cells.

INTRODUCTION

The administration of 'rescue' agents to patients treated with MTX\(^4\) is widely practiced in cancer chemotherapy (2). The aim of these rescue techniques is to increase the dose of MTX without increasing toxicity to normal tissues in an attempt to improve the therapeutic effectiveness of MTX. The conventional rescue agent has been leucovorin (N\(^5\)-formyltetrahydrofolate) which replenishes pools of reduced folates depleted by the MTX-mediated inhibition of dihydrofolate reductase. Although no clear evidence can be given that normal tissues are preferentially protected from MTX toxicity by leucovorin (12), it has been shown that delaying the leucovorin administration after treatment with MTX enhances the antitumor effect of MTX in vivo (6, 18), possibly by allowing exposure of tumor cells to MTX at higher concentrations and for longer periods of time.

The inhibition of the de novo synthesis of thymine and purine nucleotides by MTX-mediated depletion of reduced folates can be circumvented by the reutilization of nucleobases and nucleosides via the salvage pathways. Reversal of MTX cytotoxicity to normal mouse tissues by bolus injections of dThd or dThd plus a purine with (17) and without (9, 19) loss of antitumor activity has been reported. Although indications are available that the administration of these nucleotide precursors might result in selective rescue from MTX toxicity, the factors responsible for this differential rescue are not yet established. Recently, these studies have been extended to patients and have demonstrated the effectiveness of dThd administration alone in preventing MTX toxicity, although no definite evidence of enhanced antitumor activity could be shown (5, 10, 16).

In an earlier study, leucovorin and equimolar dThd-purine combinations were examined for their ability to reverse the inhibitory effect of MTX on colony formation of normal mouse bone marrow cells grown in semisolid medium (15). Studies by others of various cells in culture, predominantly of tumor origin, have shown a wide variation in the reversibility of MTX toxicity by pyrimidines and purines (3, 7, 8). The present investigation concerns MTX reversal in mouse L1210 leukemia cells and was carried out using cell culture methods similar to those used for the study with mouse bone marrow cells. Initiation of colony formation of L1210 cells in semisolid medium was found to be unaffected by MTX, and only further growth of the colonies was inhibited. Therefore, it was necessary to use electronic counting of colonies according to size in order to assess drug toxicity. The results suggest that, under the conditions used, a dThd-purine combination may be more effective than leucovorin in maintaining selective MTX cytotoxicity to L1210 cells.

MATERIALS AND METHODS

Materials. Powdered Roswell Park Memorial Institute Medium 1640, McCoy's powdered medium, and dialyzed FCS were obtained from Grand Island Biological Co. (Grand Island, N. Y.). Methylcellulose was purchased from Dow Chemicals USA (Midland, Mich.), and bovine serum albumin (Fraction V) was from Armour Pharmaceutical Co. (Eastbourne, England). dThd, inosine, hypoxanthine, and adenosine were acquired from Sigma Chemical Co. (St. Louis, Mo.), and leucovorin (dL-folic acid, calcium salt) and MTX were from Lederle Laboratories (Pearl River, N. Y.). N-2-Hydroxymethylpiperazine-N-2-ethanesulfonic acid was purchased from Serva Feinbiochemica (Heidelberg, W. Germany). All other chemicals used were of high reagent grade quality and were obtained commercially.

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4 The abbreviations used are: MTX, methotrexate; dThd, thymidine; FCS, fetal calf serum; CFU-C, colony-forming units-culture.

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Dialyzed FCS was heat inactivated at 56° for 30 min before use. The concentration of leucovorin used was based on the concentration of the active L isomer.

Cell Culture. L1210 cells were maintained in liquid suspension culture in Roswell Park Memorial Institute Medium 1640 containing 10% dialyzed FCS, 60 μM 2-mercaptoethanol, 25 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid, penicillin (100 units/ml), and streptomycin (100 μg/ml), with the pH adjusted to 7.4 with NaOH. Cells were grown in 75-cm² plastic disposable culture flasks (Nunc AS, Roskilde, Denmark), loosely capped, in a 37° incubator with a 7.5% CO₂ humidified atmosphere. Every 2 or 3 days, cultures were diluted with fresh medium to a density of 5 x 10⁵ cells/ml. Under these conditions, cell generation time was 12 hr. Cell counting was performed with a hemacytometer. During logarithmic growth, L1210 cells were used for plating in semisolid medium to study the effect of MTX on colony formation. The plating technique was similar to that described earlier for mouse bone marrow cells (4, 15). The plating medium was prepared by mixing 1.6 ml McCoy's + medium [the composition of McCoy's + medium has been described in detail elsewhere (15)]. 2.5 ml 1.8% methylcellulose, 0.5 ml dialyzed FCS, 0.3 ml 20% (w/v) bovine serum albumin, 0.35 ml 1 mM 2-mercaptoethanol, and 0.25 ml McCoy's + medium containing 10⁴ cells/ml. MTX, leucovorin, dThd, or purines, when used, were included in the 1.6-ml portion of McCoy's medium. No colony-stimulating factor was used. The cell suspension in the methylcellulose-based medium was divided into four 35-mm-diameter plastic Petri dishes, each receiving a volume of 1.1 ml. The plated cells were incubated as described above for 10 to 12 days. The CFU-C assay for the bone marrow cells was performed essentially as described before (15) using both equimolar and nonequimolar concentrations of dThd and inosine. Bone marrow cells were obtained from female DBA/2 mice (IFFA/CREDO, Saint-Germain-sur-l Arbre, France) femurs.

Analysis of Colony Formation. Colonies were counted, and their size distribution was determined using a Quantimet 720 computerized image analyzer (Cambridge Instrument, England). Light from an incandescent source was transmitted through the culture dish, and the image of the dish was projected onto a monitor screen which facilitated control of counting. The image of the dish and colonies was digitalized by a computer on the basis of discrimination between areas of different light intensity. Detection of colonies was dependent on the density of cells within the colonies; detection sensitivity was adjusted to give maximal colony counting with minimal background from light scattering near the edge of the culture dish. Colonies were counted at different size values ranging from 1 to 15 relative size units. In our experiments, 1 size unit corresponded to 0.05 mm. The number of colonies at each size level was recorded and printed by a Hewlett-Packard 9866 A calculator. The data gave an indication of the size distribution of colonies ≥0.05 mm in diameter.

CFU-C colonies were counted under a light microscope. Since our control colonies were much larger (300 to 5000 cells) than in experiments reported previously, our cutoff point for a colony in the present experiments was taken at 120 cells.

RESULTS

Effect of MTX on L1210 Formation. L1210 cells when plated in semisolid medium in the manner previously used for mouse bone marrow cells were found to be sensitive to the presence of MTX; however, the effect of MTX could not be easily studied with light microscopy. The initiation of colony formation was only slightly affected by MTX even at concentrations as high as 10⁻⁴ M. Conversely, doubling of L1210 cells in liquid suspension culture was inhibited completely by 10⁻⁷ M MTX. A more apparent effect of MTX in semisolid culture was a marked decrease in the size and density of colonies formed. Using the electronic analysis of colony formation described in “Materials and Methods,” it was possible to quantitate changes in the diameter size of colonies or changes in colony growth. As an example, Chart 1 shows the effect of MTX on colony diameter. The results are plotted as the number of colonies with diameters greater than or equal to those indicated on the abscissa; i.e., the actual diameter size distribution curves are the first derivatives of the curves shown. The number of colonies is represented as the percentage of the total number of control colonies with diameters greater than 0.05 mm. The data show that, in the presence of MTX, colonies formed were decreased in number and size. By light microscopy, 100 to 200 colonies could be counted (20 to 40% plating efficiency) in control dishes. The number of colonies in control cultures counted with the image analyzer differed less than 10% with values obtained by microscopic counting. When cells were plated in the presence of 10⁻⁸ or 10⁻⁸ M MTX, the colony count using light microscopy decreased by 11 and 19%, respectively, compared to controls. When counting was performed with the image analyzer, thereby limiting the examination of the effect of MTX colony formation to colonies ≥0.05 mm, 70 and 90% inhibition was observed. When counting was limited to colonies with larger diameters, greater inhibition by MTX of colony formation could be noted. This inhibitory effect may also be viewed in Chart 1 as a shift to the left of the colony diameter size distribution curve. These findings demonstrated that the study of MTX effects on L1210 colony formation in semisolid medium could be better conducted by counting colonies with a diameter of at least 0.05 mm, a cutoff diameter of 0.1 mm giving a considerable number of colonies in control plates and a marked reduction of colony number in MTX-treated plates.

Reversal of MTX Cytotoxicity by Leucovorin. The addition of leucovorin to the plating medium reduced the adverse effect of MTX on L1210 colony formation. Chart 1 illustrates that, in the presence of both 10⁻⁸ M MTX and 10⁻⁷ to 10⁻⁴ M leucovorin, the shift in the colony diameter size distribution curve to

![Chart 1. Decrease in L1210 colony size by MTX and its reversal by leucovorin. Cells were cultured in the absence (-----) and presence (----) of 10⁻⁷ M MTX, and leucovorin was added at the following final concentrations: 10⁻⁴ M (○), 10⁻⁵ M (●), 10⁻⁶ M (▲), 10⁻⁷ M (△), 0 (■).](image-url)
the left is less than for those cultures with only MTX added. These data also show a concentration-dependent reversal by leucovorin of the MTX-mediated reduction in colony growth. This observation could also be made examining those colony counts obtained at 0.05-, 0.10-, or 0.15-mm-diameter sensitivities. Colony counts approximately 75% of control at 0.05- and 0.10-mm sensitivities indicate that with $10^{-4}$ M leucovorin and $10^{-5}$ M MTX there is near-complete prevention of MTX toxicity to L1210 cells. Evaluation of colony formation in this manner offered a convenient method of comparing the effects of various agents at different concentrations on colony formation. Subsequent data presented here are based on colony counts made at instrument settings for the detection of colonies with minimal diameter sizes of 0.10 mm.

The effectiveness of leucovorin in preventing the MTX inhibitory effect on L1210 colony formation is further illustrated in Chart 2. At $10^{-6}$ M MTX (which is 50% inhibitory), the presence of equimolar leucovorin resulted in complete prevention of MTX toxicity; at $10^{-7}$ M MTX, equimolar leucovorin resulted in a colony count of 65%, whereas with a 10-fold higher leucovorin: MTX ratio prevention of toxicity was nearly complete. At MTX concentrations of $10^{-6}$ and $10^{-5}$ M, leucovorin at 10-fold greater concentrations yielded colony counts of 60 to 80% of control value, and $10^{-4}$ M leucovorin protected cells from $10^{-6}$ M MTX resulting in colony growth of approximately 80% of control. The highest concentration of leucovorin used in these experiments was $10^{-4}$ M; at $10^{-4}$ M MTX, this was effective only in yielding a colony count of 40% of control.

**Reversal by dThd and Purine of MTX Cytotoxicity to L1210 Cells.** Initially, the ability of dThd and purines to block the MTX-mediated inhibition of colony formation was examined using equimolar concentrations of dThd and inosine in combination at varying concentrations of MTX (Chart 3). In the absence of MTX, at nucleoside concentrations of $10^{-4}$ and $10^{-3}$ M, no colonies $\geq 0.10$ mm diameter were formed. The addition of MTX alone resulted in colony counts less than 10% of control at drug concentrations of $10^{-7}$ M and higher; at $10^{-6}$ M the colony count was 30% of control. Chart 3 illustrates that, at $10^{-7}$ to $10^{-4}$ M MTX, MTX toxicity is partially prevented by dThd plus inosine at only $10^{-5}$ M concentrations. At $10^{-6}$ M MTX, prevention of toxicity by nucleosides was more substantial and also occurred at either $10^{-6}$ or $10^{-5}$ M concentrations of dThd and inosine. Chart 4 shows that reversal of MTX-mediated inhibition of L1210 colony growth is enhanced when a combination of $10^{-5}$ M dThd and $10^{-4}$ M inosine is present at MTX concentrations as high as $10^{-4}$ M. This reversal of toxicity is independent of the MTX concentration. A higher concentration of inosine ($10^{-3}$ M), combined with $10^{-5}$ M dThd, was less effective. Results with other purines, adenosine and hypoxanthine, were similar to those with inosine; i.e., a greater reversal of the MTX inhibitory effect on colony formation was observed with dThd at $10^{-5}$ M and a purine at concentrations greater than $10^{-5}$ M (Table 1). Combinations of $10^{-6}$ M dThd with different purines appeared to have no effect on MTX toxicity (data not shown).

**Reversal by dThd and Inosine of MTX Cytotoxicity to Bone Marrow Cells.** In order to compare the results of the malignant cells with results obtained with normal cells, the reversal of MTX ($10^{-6}$ M) cytotoxicity to mouse bone marrow cells by dThd and inosine was tested in a CFU-C assay. Although in this case the results were evaluated under a light microscope, the findings are in one respect similar to the results with the L1210 cells evaluated on the basis of diameter sizes, i.e., the amount of reduction of the number of colonies by MTX and the reversal of this toxicity by dThd and inosine mainly depend on the criterion used for the minimal size (in this case in number of cells) of a colony. With the cutoff point for colony size at 120 cells, the data represented in Chart 5 were obtained. Independent of dThd concentration, the optimal reversal always
Table 1
Ability of different purines in combination with dThd to reverse MTX cytotoxicity of L1210 cells grown in semisolid medium

<table>
<thead>
<tr>
<th>Purine</th>
<th>MTX (10^{-6} M)</th>
<th>dThd (10^{-5} M)</th>
<th>MTX (10^{-6} M) + dThd (10^{-5} M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>7</td>
<td>91</td>
<td>10</td>
</tr>
<tr>
<td>Inosine</td>
<td>10^{-6} M</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>10^{-5} M</td>
<td>4</td>
<td>89</td>
</tr>
<tr>
<td></td>
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<td>88</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>10^{-5} M</td>
<td>0</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>10^{-4} M</td>
<td>5</td>
<td>94</td>
</tr>
<tr>
<td>Adenosine</td>
<td>10^{-5} M</td>
<td>0</td>
<td>112</td>
</tr>
<tr>
<td></td>
<td>10^{-4} M</td>
<td>4</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>10^{-3} M</td>
<td>0</td>
<td>81</td>
</tr>
</tbody>
</table>

a Diameter size, >=0.1 mm.

was obtained at 10^{-4} M inosine, although the extent of cytotoxicity reversal varied with the dThd concentration. Only looking at points with equimolar dThd and inosine concentration can one see that 10^{-5} M gives the higher percentage of colonies. Indeed, the colony size did increase in all colonies, but only 25% of the colonies contained more than 120 cells. This was also observed at other MTX concentrations (data not shown).

DISCUSSION

The cytotoxic effect of MTX on L1210 mouse leukemia cells and its reversal were studied with cells grown in semisolid medium in order to provide a closer comparison of results with those of an earlier in vitro study on normal mouse bone marrow cells (15) and of studies of this kind presented in this paper. L1210 cells were found to form colonies in methylcellulose-based medium even in the presence of 10^{-4} M MTX. Resistance of these cells to MTX is limited to the initial phase of colony formation, since continued growth of the colonies is blocked by MTX. The delayed action of MTX on L1210 colony formation cannot be explained by an inherent form of resistance since the growth of these cells in liquid suspension culture is rapidly inhibited after the addition of MTX at concentrations as low as 10^{-6} M. No attempt was made at this time to determine the reasons for this discrepancy in sensitivity of L1210 cells to MTX when grown in liquid and semisolid media. Since MTX was found to block the further growth of initiated colonies, it was possible to monitor this inhibitory effect by analysis of colony size. With the use of an electronic image analyzer, L1210 colonies with diameters of at least 0.05 mm could be counted. Although electronic detection of colonies in semisolid medium was dependent on density or compactness as well as diameter, only the latter colony characteristic was examined in the present study. Detection of colonies was carried out at instrument settings for maximal sensitivity based on colony density. Therefore, no distinction was made between colonies of different densities. This may present a problem when different culture conditions cause changes in the compactness of colonies as well as their size. An increase in colony diameter size may reflect not only cell proliferation but also cell migration. It is not clear at this time whether these limitations have had any influence on the results of our experiments. Nevertheless, we decided that this way of colony detection gave satisfying results because we were interested in the reversal of the toxic effect of MTX which can be analyzed without exact knowledge of the effect of MTX on colony growth kinetics and diameter size distribution of colonies with diameters of <0.05 mm. Furthermore, counting of colonies >=0.10 mm gave a maximal control colony number and a substantially reduced colony number with MTX, permitting the evaluation of the ability of various dThd and purine concentrations to reverse MTX toxicity. In this respect, our L1210 colony studies resemble the microscopic counting of bone marrow colonies where there are artificial limits of 50 cells (4, 15) or 120 cells (this study). This limit depends on the size of control colonies, which is influenced by conditions such as growth factor and serum used and which may differ from laboratory to laboratory.

The reversal of MTX toxicity to L1210 cells by leucovorin was found to be similar to that observed with bone marrow cultures (15). At low MTX concentrations, less than 10^{-6} M, leucovorin is effective at equimolar concentrations; while at higher MTX concentrations, leucovorin must be at least 10 times in excess of MTX to achieve substantial prevention of toxicity. One notable difference is that the inhibitory effect of 10^{-4} M MTX to L1210 colony growth can be averted, at least partially, by 10^{-4} M leucovorin. Leucovorin at 10^{-4} or 10^{-3} M is ineffective in reversing toxicity to bone marrow cells in vitro at such a high MTX concentration. It appears that, in the myeloid precursor cells and in these leukemia cells, leucovorin acts in a competitive manner in reversing the inhibitory effects of MTX. However, a quantitative difference, albeit slight, does exist at high MTX concentrations which may be explained by differences in the competition between MTX and leucovorin for a common carrier in their transport into the cell (14).

Based on the findings of this study, reversal of MTX toxicity in vivo with dThd plus a purine could have advantages over using leucovorin. In the control of toxicity to normal host tissues, leucovorin would be effective only when MTX concentration is low. In addition, selective rescue of normal tissues with leucovorin appears doubtful. However, due to the culture
method used, our experiments were limited to concurrent exposure of cells to MTX and leucovorin. Goldin et al. (6) have demonstrated that leucovorin improves the therapeutic effectiveness of MTX in L1210 leukemia-bearing mice, with more marked improvement when leucovorin administration is delayed until 12 hr after MTX. An even longer delay of leucovorin administration (16 to 20 hr) in high-dose MTX treatment (400 mg/kg) of tumor-bearing mice has been shown recently to prevent toxicity and maintain antitumor activity (18).

Not only may leucovorin be ineffective in the prevention or alleviation of toxicity when given concurrently with or shortly after MTX, especially at high doses, but it may also reduce the potential antitumor activity of MTX. The results of the present study and earlier work support this concept at least to the point that the concurrent exposure of cells in vitro to MTX and leucovorin does not lead to differential toxicity to L1210 cells.

In both L1210 cells and bone marrow cells, the presence of dThd plus a purine reverses the inhibitory effects of MTX. The reversal of drug toxicity is independent of drug concentration and is therefore noncompetitive. MTX toxicity to L1210 cells is reversed by dThd at $10^{-5}$ m, while lower concentrations are ineffective and higher concentrations are toxic. This reversal cannot be obtained by dThd alone and requires the presence of purines. The optimal purine concentration for L1210 cells is $10^{-4}$ m. Reversal of MTX toxicity by dThd and purines has also been tested with mouse bone marrow cells. Since in our hands under control conditions the colonies contained more cells than in a previous study (15), the minimum limit for a colony had to be adapted. Since the MTX toxicity affects the size of the colonies (number of cells per colony) rather than the number of colonies just as has been reported here extensively for L1210 cells, the definition of the lower limit of a "colony" strongly influences the reduction of the number of "colonies" by certain concentrations of MTX and the effectiveness of reversal by dThd and purines. The results of the reversal studies with DBA/2 mouse bone marrow cells in the CFU-C assay are as follows. When equimolar concentrations of dThd and purines were tested, optimal reversal was obtained at $3 \times 10^{-8}$ m, as can, e.g., be deduced from Chart 5. This essentially also found in a previous study (15) that was limited to equimolar concentrations of dThd and purines. In contrast to that previous study, the reversal with $10^{-5}$ m dThd and inosine was not complete. However, reversal in our hand would have been higher if a lower limit for colonies had been used, since all cell clusters (also the ones containing less than 120 cells) increased in cell number by the addition of $10^{-9}$ m dThd and inosine to the MTX. Looking at nonequimolar concentrations, one can see that irrespective of the dThd concentration, $3 \times 10^{-4}$ m inosine gives higher reversal, while dThd at that concentration is toxic (Chart 5). In contrast to the previous study with C57BL mice (15), the present data with DBA/2 mice show that inosine alone is capable of reversing MTX toxicity to bone marrow cells. Since for L1210 cells a dThd concentration of $10^{-5}$ m is required for reversal of MTX toxicity (see "Results"), this means that a combination of $3 \times 10^{-4}$ m inosine with $10^{-6}$ m dThd would considerably reverse MTX toxicity towards bone marrow cells while having no effect on the toxicity towards L1210 cells.

We conclude that such combination is more useful than leucovorin as a protecting agent not only because of this possible selective effect (which still has to be proven in vivo) but also because leucovorin is effective in protecting normal host tissues only at low MTX concentrations.

The reversal of the cytotoxic effects of MTX by dThd and purines in cell culture has been studied by various investigators (1, 3, 7, 8, 20). The results obtained for a variety of cell lines, mostly of tumor origin, differ widely in degree of reversal and concentration requirements for dThd and purines. This variation suggests that the capacity for nucleotide synthesis via the salvage pathway differs from one cell type to another, which can lead to differential reversal of MTX toxicity by dThd and purines. The importance of purines and pyrimidines in determining MTX toxicity has been confirmed in animal studies. dThd administration to L1210 leukemia-bearing mice improved the therapeutic effectiveness of MTX (17, 19), but it was also shown that a combination of dThd and inosine can reduce both the cytotoxic and antitumor effect of MTX simultaneously (17). Conflicting results have been presented by Harrap et al. (9), indicating that dThd can be effective in rescuing MTX-treated mice only when purines are also administered. Moran et al. (13) suggested that the presence of dThd, purines, or folates alone or in combination may have different effects on the reversal of MTX toxicity both in vitro and in vivo. At this time, little is known about the availability to tissues of dThd and purine nucleosides and bases under different experimental conditions in vivo, whereas in cell culture experiments their concentrations can be well controlled. Moreover, there is a variability in regimens in the in vivo experiments making comparison of the in vivo results with the data presented impossible. The results of this study support the use in mice of constant infusions of high concentrations of purines and low concentrations of dThd concomitant with MTX to effect selective protection of myeloid precursor cells over L1210 cells.

REFERENCES


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