Reversal of Resistance to Methotrexate in L1210 Murine Leukemia by Uracil

By Harry Wallerstein, Lewis M. Slater, Ben Eng, and Neil Calman

BDF₁ mice bearing L1210 leukemia, when treated with a regimen of uracil and methotrexate, show an increase in survival time greater than when treated with methotrexate alone. The uracil and methotrexate regimen also delays the development of methotrexate resistance as L1210 leukemia is transferred with therapy through multiple generations of mice. When uracil is applied to multiple generations of mice bearing tumor resistant to methotrexate, there is a return to sensitivity to methotrexate. It is also noted that uracil blocks the responsiveness of L1210 leukemia to 6-mercaptopurine.

The responsiveness of these tumor lines to the addition of uracil in their regimens was monitored by in vitro tritiated thymidine incorporation into DNA and by dihydrofolate reductase activities. These data, when integrated with survival time data, suggest that in part, the reported results are mediated by a uracil-induced reduction in the availability of dihydrofolate reductase and phosphoribosylpyrophosphate.

A cute leukemia in humans can be effectively, though only temporarily, arrested by antifolic and antipurine analogs. It is evident that acquired cellular resistance to these agents constitutes a major obstacle to the prolonged control of the disease. This report describes a method for the reversal of resistance to methotrexate (MTX) in the management of L1210 murine leukemia, when uracil, a preformed pyrimidine base, is added to the MTX regimen.

Uracil is a normal nucleic acid metabolite having no antineoplastic activity, except in its substituted form as uracil mustard.

Materials and Methods

The L1210 transmissible mouse lymphoma (Goldin Strain) obtained from Dr. Joseph Burchenal, was maintained in the ascitic form in 4-6-wk-old Jackson Laboratory supplied DBA/2J and B6D2F1 mice. The B6D2F1 hybrid was used in all experimental procedures. The tumor was removed aseptically from two mice, pooled, and diluted with isotonic saline.
to 10 million cells/ml. Groups of 12 animals were inoculated intraperitoneally (i.p.) with one million cells each. Control animal lines were maintained untreated after tumor inoculation and the mean untreated survival time obtained. Experimental animals were treated 48 hrs after tumor inoculation. The methotrexate-uracil (MTX-Uc) group was injected with uracil 400 mg/kg i.p. on every other day for a total of three doses. Each uracil injection was followed in 1 hr by 3 mg/kg MTX i.p. The MTX group was treated in an identical manner without uracil. The uracil group was treated in an identical manner without MTX. When ascites developed, two animals from each group were sacrificed and tumor harvested for transfer. The remaining animals were observed until death and the mean survival time obtained.

Therapy was maintained through successive transfers in groups of ten or more host animals until each group's survival time approximated that of untreated tumor. Return to control survival time in any treated tumor line was taken as an indication of tumor resistance, and the resultant strains were maintained as established with treatment during continuous transfers.

A second series of experiments was carried out in a similar manner using a MTX resistant tumor (MTXR), (derived as outlined above) which was treated with uracil alone and MTX alone. The uracil treated tumor was carried through ten transfers with continuous uracil treatment (MTXR-Uc) and then rechallenged with MTX. The MTX treated tumor was carried through 12 transfers untreated and then rechallenged with MTX.

A third series of similar experiments was developed with uracil and 6-mercaptopurine (6MP). Animals bearing one million L1210 leukemia cells were treated with 6MP 37.5 mg/kg i.p. starting 24 hr after tumor implantation and daily for 7 days. Mean 6MP survival time was obtained. In another group each 6MP dose was preceded by 400 mg/kg uracil i.p. and the mean uracil-6MP survival time obtained. Another group of animals was treated with uracil alone and the subsequent transfer generation was treated with 6MP (Uracil-6MP). Mean uracil-6MP survival time was obtained.

**Tritiated Thymidine Incorporation into DNA**

The synthesis of DNA from deoxyuridine was measured by the following modifications of the method of Metz et al. L1210 tumor cells were aspirated in saline from the ascitic fluid of host animals. One part of tumor suspension was diluted with 3 parts distilled H$_2$O at 0-5$^\circ$ C for 30 sec. Isotonicity was restored with 3.5% saline. The tumor suspension was filtered through gauze and resuspended in Hanks' balanced salt solution. Cells were counted in a Model B Coulter Counter and the concentration adjusted to 1-1.5 million cells/ml in an incubation medium composed of 100 ml fetal calf serum, 200 ml Hanks' balanced salt solution and 5 ml standard penicillin streptomycin preparation. The pH was adjusted to 7.4 with 7.5% NaHCO$_3$. One mCi tritiated thymidine was added per milliliter of incubation media. One milliliter of this suspension was then pipetted into reagent tubes. Other reagents were added as outlined below in triplicate and the suspensions incubated 3 hr under 5% CO$_2$ and 95% O$_2$ at 37$^\circ$C. Tubes were then washed twice with saline at 0-4$^\circ$C and counted in Bray's solution with Tricarb liquid scintillation counter series 3000. The following experimental stock solutions were prepared: (1) 4.5 mg deoxyuridine (dU) per 20 ml Hanks' balanced salt solution (2) 44.8 mg uracil in a few drops 10 N NaOH dissolved in 50 ml Hanks' balanced salt solution, and (3) 4.5 mg MTX dissolved in 50 ml Hanks' balanced salt solution. An appropriate volume of these solutions were reacted with the tumor cell tritiated thymidine suspension either singly or in the combinations indicated in Table 1 to give final concentrations of 0.1 mM dU, 2 mM uracil and 2 mM MTX.

**Dihydrofolate Reductase Assays**

Tumor suspension was prepared as outlined above. It was washed with isotonic saline after isotonicity had been restored, resuspended in a small volume of phosphate buffered saline, and then extracted with cold (~20$^\circ$ C) acetone. The crude enzyme used for dihydrofolate reductase (DHFR) assay was extracted from the resulting acetone powder
Table 1.—Tritiated Thymidine Incorporation Into DNA of Various Tumor Lines

<table>
<thead>
<tr>
<th>Tumor Line</th>
<th>Additives (mM)</th>
<th>dU (0.1)</th>
<th>Uracil (2.0)</th>
<th>MTX (2.0)</th>
<th>dU and MTX</th>
<th>MTX and Uracil</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1210</td>
<td></td>
<td>21±3.1(8)</td>
<td>91±8.0(9)</td>
<td>179±24(6)</td>
<td>96±12 (8)</td>
<td>131±17 (9)</td>
</tr>
<tr>
<td>MTXR</td>
<td></td>
<td>27±2.1(12)</td>
<td>76±8.1(12)</td>
<td>134±15(11)</td>
<td>67± 9.1(12)</td>
<td>82± 9.7(8)</td>
</tr>
<tr>
<td>MTXR-Uc</td>
<td></td>
<td>26±2.8(8)</td>
<td>56±8.9(11)</td>
<td>184±28(7)</td>
<td>142±16 (9)</td>
<td>150±19 (7)</td>
</tr>
<tr>
<td>MTX-Uc</td>
<td></td>
<td>30±4.1(11)</td>
<td>83±7.2(11)</td>
<td>156±17(12)</td>
<td>82±14 (10)</td>
<td>137±11 (7)</td>
</tr>
</tbody>
</table>

* Figures are in per cent of counts per minute compared to uptake of thymidine without additives. Figures in parenthesis are number of determinations in triplicate.

with 0.05 M phosphate buffer containing 0.01 M mercaptoethanol and 0.001 M EDTA. The DHFR assays were performed on established tumor lines which had been carried for 10–15 transfer generations, as described by Friedkin et al.² using dihydrofolate substrate prepared by the method of Futterman.³

Results

Survival Time

The mean survival times (MST) of the various tumor lines, with standard deviations, given in days, are noted in Tables 2 and 3. The MST of untreated L1210 tumor is 8.2 days, and that of uracil treated tumor is 8.1 days. Treatment with MTX alone or with MTX and uracil results in a marked increase of MST. The lines treated with both agents (MTX and uracil) show a higher MST than those treated with MTX alone, 19.7 and 20.6 days compared to 17.5 and 17.4 days. The differences are significant at a p value of less than 0.05.

The MST of the MTX resistant line, Table 3, when treated with uracil is 9.8 days, and when treated with MTX 8.3 days. When this line is carried for more than ten transfers in the presence of continuous uracil challenge its MST is essentially unchanged from the control at 7.8 days. Upon rechallenge with MTX, however, an increase of the MST to 16.3 days is again noted. This difference is significant at a p value of less than 0.01. The MTX resistant line continuously transferred in the absence of uracil shows a MST unchanged from the control when rechallenged with MTX, 9.3 days compared to 8.8 days.

6-Mercaptopurine treatment increases the MST to 11.1 days. When 6MP is given with uracil or to the first transfer generation after uracil treatment

Table 2.—Mean Survival Time, in Days, of Various L1210 Tumor Lines

<table>
<thead>
<tr>
<th>Treatment Regimen</th>
<th>MST ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>8.2 ± 0.9</td>
</tr>
<tr>
<td>Uracil</td>
<td>8.1 ± 2.3</td>
</tr>
<tr>
<td>MTX</td>
<td>17.5 ± 1.2</td>
</tr>
<tr>
<td>MTX</td>
<td>17.4 ± 1.9</td>
</tr>
<tr>
<td>MTX and Uracil (MTX-Uc)</td>
<td>19.7 ± 1.0</td>
</tr>
<tr>
<td>MTX-Uc</td>
<td>20.6 ± 2.2</td>
</tr>
<tr>
<td>6MP</td>
<td>11.1 ± 1.0</td>
</tr>
<tr>
<td>6MP and Uracil</td>
<td>8.3 ± 0.9</td>
</tr>
<tr>
<td>Uracil followed by 6MP in the first transfer generation (Uracil-6MP)</td>
<td>7.9 ± 1.4</td>
</tr>
</tbody>
</table>

* Ten animals per group.
the MST remains at control level of 8.3 and 7.9 days. These differences are also significant at a p value of less than 0.01.

Figure 1 shows the development of resistance to MTX as two tumor lines are carried through multiple transfers. One line is carried with MTX alone and the other with MTX and uracil. The MST for the MTX-uracil treated tumor line is significantly greater at each comparable transfer generation. In the line treated with MTX alone the MST reaches control level by the sixth or seventh transfer, whereas the MTX-uracil treated line approaches baseline but does not yet reach it even by the ninth transfer.
Tritiated Thymidine Uptake Studies

Table 1 describes the results of tritiated thymidine incorporation into DNA (3H-Tdr incorporation) of four tumor lines, derived as above. The incorporation in counts per minute of tritiated thymidine into DNA of cells from each tumor line, incubated in media without additives was used as control. Results for the experimental additives are expressed as per cent counts per minute of the control. Five incubation experiments were done for each tumor line: (1) dU, (2) uracil, (3) MTX, (4) dU and MTX, (5) MTX and uracil. When untreated L1210 tumor is incubated with 0.1 mM dU there is an inhibition of thymidine uptake to 21 ± 3.1%. Incubation with 2mM uracil results in only a slight change in thymidine uptake to 91 ± 8.0%. MTX-2 mM increases 3H-TdR incorporation to 179 ± 24% through its inhibition of deoxyuridylate conversion to thymidylate. When tumor is incubated with similar concentrations of dU and MTX combined, results are again essentially at baseline value 96 ± 12%. This represents a partial escape of MTX inhibition of dU conversion. MTX combined with uracil results in an increase to 131 ± 17% 3H-TdR incorporation, which is less than the increase to 179 ± 24% produced by MTX alone.

The second tumor line, MTXR, is resistant to MTX as defined by mean survival times. It shows inhibition of 3H-TdR incorporation, 27 ± 2.1%, similar to that of the untreated L1210 tumor when incubated with dU. The MTX effect in this line is less intense, 134 ± 15%, when used alone and 67 ± 9.1% when used in combination with dU. When MTX is combined with uracil further apparent inhibition of MTX effect is noted, 82 ± 9.7% as compared to 131 ± 17%, in the untreated line.

Line MTXR-Uc is a MTX resistant line that has been carried with uracil for more than ten transfer generations. 3H-TdR incorporation inhibition by dU is unchanged at 26 ± 2.8% compared to that of the untreated L1210 tumor. MTX effect is in the same range as in nonresistant L1210 tumor, 184 ± 28% compared to 179 ± 24%, when MTX is the sole reagent and 150 ± 19% compared to 131 ± 17% when MTX is combined with uracil. However when MTX and dU are combined, thymidine incorporation is enhanced to 142 ± 16% as opposed to 96 ± 12% in the untreated L1210 line. Thus there is a greater MTX effect, when MTX is combined with dU in the resistant line than that seen in untreated tumor alone. The fourth tumor line, denoted as MTX-Uc, is an L1210 tumor that has been carried for more than 40 transfer generations with uracil and MTX. This line shows MTX effect 156 ± 17% similar to that seen in MTXR which is 134 ± 15%. When MTX and uracil are used, reactivity is in the same range as untreated tumor, 137 ± 11% and 131 ± 17%, respectively.

Dihydrofolate Reductase Activity

Table 4 lists DHFR activity in these tumor lines, given as millimicromoles dihydrofolate reduced per hour per milligram protein. Untreated tumor shows a low level of activity, whereas the MTXR line shows a level approximately 19 times as great. The lines treated by combination of MTX and uracil have a
Table 4

<table>
<thead>
<tr>
<th>Tumor Line</th>
<th>DHFR Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1210</td>
<td>114</td>
</tr>
<tr>
<td>MTXR</td>
<td>2120</td>
</tr>
<tr>
<td>MTXR-Uc</td>
<td>640</td>
</tr>
<tr>
<td>MTX-Uc</td>
<td>840</td>
</tr>
</tbody>
</table>

DHFR activity is given as millimicromoles dihydrofolate converted to tetrahydrofolate/hour/mg protein. The data for the MTXR, MTXR-Uc and MTX-Uc lines is pooled from several established lines.

level of activity midway between that of the untreated tumor and the resistant line.

DISCUSSION

It is well known that MTX binds the enzyme DHFR, which results in a decrease of dihydrofolate conversion to tetrahydrofolate, a depression of thymidylate synthesis, failure of de novo DNA-thymine synthesis and resultant arrest of cell division, Fig. 2. The phenomenon of MTX resistance is universally recognized as severely limiting the clinical efficacy of the drug. The mechanisms by which resistance to MTX develops are not fully understood, but it has been clearly demonstrated that dose modification of the drug can lead to increased response.

![Fig. 2.-The interrelationship of uracil in nucleic acid synthesis. The enzymes designated numerically are: (1) uridylic pyrophosphorylase; (2) uridine phosphorylase; (3) uridine kinase; (4) ribonucleotide reductase; (5) purine pyrophosphorylase; (6) pyrimidine trans-N-deoxyribosylase; (7) deoxyuridine kinase; (8) thymidylate synthetase; (9) thymidine kinase; and (10) dihydrofolate reductase. Substrate abbreviations are: UR, uridine; dU, deoxyuridine; UMP, uridine monophosphate; dUMP, deoxyuridine monophosphate; TdR, thymidine; hTFHA, N³N¹0-methylene tetrahydrofolate; FAH₂, dihydrofolate acid. Note that PRPP is involved in the conversion of uracil to UMP and in both de novo and salvage purine synthesis.](image-url)
The data presented in Table 2 show a statistically significant increase of MST of BDF$_1$ mice with L1210 leukemia when they are treated with a combination of MTX and uracil when compared with that of MTX alone. In addition, MTX rechallenge of animals bearing MTX resistant tumor which has been transferred under a continuous treatment of uracil results in a second MTX response. This response is of the same magnitude as that effected by the original MTX challenge. Figure 1 demonstrates the delay in development of MTX resistance effected when uracil is combined with MTX. It is of additional interest to note that the MST of each transfer generation is greater than that of the corresponding MTX treated generation.

Thus with appropriate modification of the uracil-MTX regimen it has been possible to show (1) increased MTX responsiveness, (2) delay in the development of MTX resistance, and (3) reversal of MTX resistance of L1210 leukemia in BDF$_1$ mice. It is of considerable interest however that this regimen blocks the 6MP effect on L1210 leukemia in BDF$_1$ mice.

Table 2 presents tritiated thymidine incorporation into DNA of four of these tumor lines. All the lines are capable of utilizing deoxyuridine to synthesize DNA, as demonstrated by a significant decrease in exogenous thymidine incorporation when deoxyuridine is added to the system. MTX inhibits the de novo pathway and causes increased $^3$H-TdR incorporation in all the tumor cell lines. This effect, as expected, is least intense in the resistant to MTX, 134%. However the $^3$H-TdR incorporation with MTX incubation remains in the range of the untreated control in MTXR-Uc, the line capable of a second response to MTX despite its prior resistance, 184 v. 179%. The MTX mediated inhibition of DHFR conversion of dihydrofolate to tetrahydrofolate is the same in both lines; reversal of the diminished MTX effect has occurred during passage of tumor through ten transfer generations in the presence of uracil, which indicates a return of sensitivity. In all the lines, uracil tends to stimulate de novo DNA synthesis. This may be mediated by uracil conversion to uridine via uridine phosphorylase, and subsequent transformation to deoxyuridine. Thus, addition of uracil to the in vitro incubation system simulates the addition of dU, Fig. 2. When MTX is added with dU, less MTX blockade of deoxyuridylate conversion to thymidylate is seen, which is expected from mass action considerations alone. However the MTXR-Uc line shows less inhibition of MTX blockade of dU conversion by dU incubation with MTX than do the other lines. Similarly, uracil incorporation with MTX tends to mimic the dU effect, again to a smaller extent, and once again probably via the conversion of uracil to dU. In contrast to the MTX resistant line, both MTXR-Uc and MTX-Uc tumor lines show MTX responsiveness of $^3$H-TdR incorporation in the range of control L1210 tumor when incubated in vitro with MTX and uracil.

When the results of thymidine incorporation are considered with DHFR activities as outlined in Table 4, a possible mechanism of the uracil effect suggests itself. DHFR activity is low in the untreated L1210 line, very high in the resistant line and at intermediate levels in both the MTXR-Uc and MTX-Uc lines. The capacity of MTX to shift a sensitive tumor cell population to one of high DHFR production and thereby MTX resistance has been altered by the addition of uracil. It has been previously shown in L1210 tumor cell
populations that the interference by MTX of de novo purine synthesis can result in a tumor population increasingly dependent on the formation of DNA from the salvage of preformed purines. In the presence of uracil this phenomenon may be prevented. Uracil is converted to uracil monophosphate as well as to uridine and dU. Phosphoribosylpyrophosphate (PRPP) is consumed in the conversion of uracil to uracil monophosphate. Under these circumstances, since de novo purine formation is blocked by MTX, the PRPP dependent purine salvage pathway, which would ordinarily tend to minimize dependence on de novo synthesis, might be simultaneously inhibited. This sequence would also explain the uracil inhibition of the mercaptopurine effect, as PRPP is necessary for the conversion of mercaptopurine to its lethal form. It is known that uridine can stimulate the incorporation of 14C formate into nucleic acid purines of Ehrlich ascites tumor cells. It may be that uracil, by stimulating a tendency toward increased de novo purine synthesis during which DHFR is bound, reduces the availability of that enzyme, which is needed to overcome the MTX block of deoxyuridine conversion to thymidylate. Thus uracil may have a dual effect by reducing the availability of both PRPP and DHFR. Preliminary studies in this area are being pursued.

Acknowledgment

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