Fate of Amethopterin-resistant Mutants in L1210 Mouse Leukemia Populations

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Summary

The disappearance and persistence of amethopterin-resistant L1210 mouse leukemic cells in a population of overtly sensitive cells have been followed. The response of the population depends entirely upon the presence or absence of therapy. Over-all, the quantitative assay methods are capable of detecting 5% or better resistant cells in a population. This sensitivity is not adequate for the detection of amethopterin-resistant transformants in a population.

Certain aspects of the biologic and biochemical nature of amethopterin-resistant cells are indicated.

Introduction

The fate of drug-resistant neoplastic cells in mixed populations in the presence or absence of a selective drug has been studied by Klein (9), Sköld et al. (13) and Glynn [reported by Venditti and Goldin (15)]. Klein (9) indicated that amethopterin-resistant L1210 cells admixed with sensitive L1210 cells showed no sign of disadvantage in the absence of amethopterin (methotrexate). Resistant cells persisted through 14 passages in the absence of treatment in populations made up of 1 resistant cell in 10⁶ sensitive cells.

Friedkin and Goldin (3) observed a decrease in dihydrofolate reductase activity in certain of their antifolate-resistant L1210 lines when maintained in untreated animals. This observation was examined further by Schrecker and Greenberg (11). They maintained 1 antifolate-resistant line of L1210 for 30-50 transfer generations in untreated mice and the line remained resistant to amethopterin in dichloromethotrexate (DCM) therapy. Up to the 30th untreated transfer generation it was possible to raise the reductase level to that characteristic of the treated line by only 1 course of treatment with DCM (80 mg/kg/day). We have also followed the loss of reductase in certain amethopterin-resistant sublines of the L1210 mouse leukemia (5, 6) and confirmed the above observations.

This paper, which describes the use of 3 methods for determining the relative numbers of antifolate-resistant cells in a population, provides information about the fate of a small number of resistant cells in a population of leukemic cells. A major problem exists in differentiating between the selection of an existing resistant cell and the induction of a mutation because of exposure of the cells to amethopterin.

Materials and Methods

Lines of Leukemia

The L1210 (V) mouse leukemia now maintained in DBA-2 and BDF₁ (C57BL 9 X DBA₂c) mice in the ascitic form was described previously (8). This line of L1210 is sensitive to amethopterin, 6-mercaptopurine (6-MP), and 5-fluorouracil (5-FU) (8). It possesses a subtelocentric marker chromosome (2) and its reductase activity is approximately 100 μmoles dihydrofolate reduced/mg protein/hr (1, 12).

L1210/A/MP/FU (XVI₂), a subline resistant to amethopterin, 6-MP and 5-FU, exhibits an 18-fold increase in reductase activity (2, 7, 12).

L27, which is sensitive to amethopterin, is a subline of L1210 (V) obtained by cloning from a population of cells which had been treated with homologous DNA (6).

The L27/A₁ line, resistant to amethopterin, was selected from the L27 population and possesses a 10-fold increase in reductase activity. Its karyotype is identical to those of L27 and L1210 (V) (1, 6).

In Vitro Mixture of Sensitive and Resistant Cells

Cells of each line of leukemia were withdrawn aseptically from the peritoneal cavities of mice on the 7th day of leukemic cell growth and suspended in physiologic saline containing 0.1% heparin. The suspended cells were counted and then each suspension was diluted with saline to 10⁶ cells/ml. Aliquots of the respective sensitive and resistant cells ranging from 100 to 0.0001% were mixed in vitro. BDF₁ mice were used and 10⁴ cells were implanted i.p. Aliquots were also used to determine the enzyme activity.

Total Cell Counts

The response of the mixed cell populations to amethopterin, 6-MP, and 5-FU was evaluated by a method of total cell count (TCC). Forty-eight hr after i.p. implantation of 10⁴ leukemic cells therapy was started. Groups of 5 mice each were treated

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2 Present address: Aichi Cancer Center, Nagoya, Japan.
3 The abbreviations used are: DCM, dichloromethotrexate; 6-MP, 6-mercaptopurine; 5-FU, 5-fluorouracil; TCC, total cell count; AST, average survival time.
4 Received for publication August 9, 1965; revised December 2, 1965.
i.p. daily with amethopterin (0.4 mg/kg), 6-MP (5.0 mg/kg), or 5-FU (3.0 mg/kg). On the 6th day all animals were weighed, injected i.p. with 1 ml of 3.8% sodium citrate, and sacrificed. An aliquot of the diluted ascitic fluid was removed and the cells were counted. After the peritoneal cavity was opened and the total ascitic fluid was collected the animals were weighed again. The volume of ascitic fluid was determined from the weight change. The specific gravity of the ascitic fluid was assumed to be 1.0. The TCC of the peritoneal cavity was then estimated by multiplying the cell count by the weight change. The effect of the drugs was calculated as the % change in TCC of treated and untreated animals.

Average Survival Time

The change in the average survival time (AST) of groups of 10 mice bearing the various mixtures of leukemic cells compared with untreated control groups of mice after treatment with amethopterin (3 mg/kg) was also used as an indicator of resistance.

Dihydrofolate Reductase Activity

The procedures for the preparation of extracts of leukemic cells and the quantitative assay of this enzyme have been described (1, 12). The enzyme assays were carried out on control (untreated) groups, 1 transfer generation removed from exposure to amethopterin.

Maintenance of Mixed Cell Population Lines

On the 7th day after implantation of the cell mixtures described above routine transfers were made (8). At selected transfer generations the resistance levels were evaluated by the 3 methods, TCC, AST, and reductase activity.

Selection of Resistant Cells from Mixed Populations

Twenty-four hr after implantation of the cell mixtures, therapy with amethopterin was initiated. Animals were treated with amethopterin (3 mg/kg) every other day for 6 injections. The AST was recorded.

Growth Curves

Fifty BDF1 mice were transplanted with 10⁴ cells of L1210 (V) and 50 BDF1 with L1210/A/MP/FU (XVI2). Beginning at the 3rd day postimplantation 5 mice of each group were sacrificed daily and the TCC was detected. The resulting average cell counts were calculated as described above.

Inoculum Size and Host Response

Leukemic cells were harvested aseptically from mice. Saline was added so that the suspension contained 10⁷ cells/ml. After making several 1:10 dilutions in saline, 0.1 ml was implanted either i.p. or s.c. into DBA₂ or BDF1 mice. The death of all animals was recorded and the AST calculated.

Results

The disappearance of cells of L1210/A/MP/FU (XVI2) from mixtures with L1210 (V) following transplantation in vivo in untreated animals is shown in Table 1.
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**TABLE 3**

<table>
<thead>
<tr>
<th>SIZE OF INOCULUM</th>
<th>NO. OF 60-DAY SURVIVORS</th>
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<tbody>
<tr>
<td></td>
<td>L1210 (V)</td>
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<tr>
<td></td>
<td>DBA₂ (i.p.) 0/10 0/40 0/20</td>
</tr>
<tr>
<td></td>
<td>BDF₁ (i.p.) 0/10 0/40 0/20</td>
</tr>
<tr>
<td></td>
<td>BDF₁ (s.c.) 0/10 0/40 0/20</td>
</tr>
<tr>
<td>10⁴</td>
<td>0/10 0/40 0/20 0/10 0/40 0/20</td>
</tr>
<tr>
<td>10³</td>
<td>0/10 0/40 0/20 0/10 0/40 0/20</td>
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<td>10²</td>
<td>0/10 0/40 0/20 0/10 0/40 0/20</td>
</tr>
<tr>
<td>10¹</td>
<td>0/10 0/40 0/20 0/10 0/40 0/20</td>
</tr>
</tbody>
</table>

**TABLE 4**

<table>
<thead>
<tr>
<th>% RESISTANT CELLS</th>
<th>AMETHOPTERIN (3 mg/kg)-TREATED TRANSFER GENERATIONS</th>
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<tr>
<td></td>
<td>Generation 1</td>
</tr>
<tr>
<td></td>
<td>AST (treated/control) Reductase activityb</td>
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<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>20/8.7 91</td>
</tr>
<tr>
<td>0.001</td>
<td>20/8.7 91</td>
</tr>
<tr>
<td>0.1</td>
<td>15/8.7 91</td>
</tr>
<tr>
<td>1.0</td>
<td>14/8.7 91</td>
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<td>10</td>
<td>13/8.7 91</td>
</tr>
<tr>
<td>100</td>
<td>12/13 1342</td>
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</table>

* AST, Average survival time.

**TABLE 5**

<table>
<thead>
<tr>
<th>% RESISTANT CELLS</th>
<th>TOTAL CELL COUNT (%TREATED/CONTROL)</th>
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</thead>
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<tr>
<td></td>
<td>0 1 2 3 4 5 6 7 9</td>
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<tr>
<td></td>
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<tr>
<td>0</td>
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<td>10</td>
<td>37</td>
</tr>
<tr>
<td>50</td>
<td>56</td>
</tr>
<tr>
<td>100</td>
<td>135</td>
</tr>
</tbody>
</table>

* Amethopterin, 0.4 mg/kg.

**Chart 1.**—Growth of mouse leukemia L1210 (V) and L1210/A/MP/FU (XVI₂) in the peritoneal cavities of BDF₁ mice.

The 2 L1210 lines, V and XVI₂, were examined for relative lethality in 2 different kinds of host mice. The i.p. and s.c. routes of inoculation were compared in the BDF₁ mouse. As is shown in Table 3, the DBA₂ mouse was slightly more susceptible to both leukemias than the BDF₁ mouse. There was little difference in lethality in the BDF₁ mouse following s.c. inoculation.

The growth rates of L1210 (V) and L1210 (XVI₂) (Chart 1) varied. The doubling time for L1210 (V) was 10.6 hr, and for L1210 (XVI₂) 16.2 hr. These dissimilarities in growth rates reflected differences in survival time (Table 4). L1210 (V) had an AST of 8.7 days, and XVI₂ 13 days. In the experiments detailed in Table 3 the AST with the 10⁴ inoculum for L1210 (V) in BDF₁ mice was 9.4 and for L1210 (XVI₂) 12.6.

Admixtures of sensitive and resistant cells, L1210 (V) and (XVI₂), were passed through amethopterin-treated mice. After 1 treatment passage the population that contained only 0.001% resistant cells was completely resistant as determined by AST, enzyme activity, and TCC (Table 4).

To determine the reproducibility of the sensitivity of this type of mixing experiment and to determine the mutation frequency to amethopterin resistance, 2 different cell lines were chosen: L27, amethopterin-sensitive, and L27/A₁, amethopterin-resistant, both of which have been described (6). The disappearance of L27/A₁ cells from the mixed population (Table 5) as determined by TCC method was not completely evident at the 9th untreated transfer generation. The enzyme level of the
50% mixture did not reflect the presence of resistant cells at the 7th transfer generation. From the plot of enzyme activity (Chart 2), approximately 5% resistant cells were present at the 5th transfer generation.

The selective action of amethopterin was also determined with lower percentages of resistant (L27/A) cells added to the L27 population (Table 6). After 1 treated generation 0.001% resistant cells appeared as a totally resistant population when quantitated by AST and TCC. According to the reductase level, the population appeared to be 25% L27/A1 cells (Chart 2). By the 4th treated generation the 0.0001% mixture was totally resistant by the AST and TCC methods of evaluation and contained at least 50% resistant cells by the reductase method (Chart 2). It is of importance to note that at the 4th transfer generation a resistant population had developed from the control L27 line which had a reductase level similar to the L27/A1 line (6) and was resistant also by the AST method but contained about 10% resistant cells by the TCC method (Table 5).

Discussion

The fate of amethopterin-resistant cells in populations of L1210 mouse leukemia cells has been followed. The presence or absence of the drug during the course of the experiments played a very important role in the consequences of the experiments. The studies with L1210 (V) and L1210/A/MP/FU (XVI2) presented in Table 1 show that in untreated animals the mixed population originally containing an equal number of sensitive and triply resistant cells appeared as a sensitive population by the 20th transfer generation. That the TCC method can detect between 5 and 10% resistant cells in a given population was borne out by the data on the 1st transplant generation. These results suggest that the population of XVI2 is homogeneous, i.e., each resistant cell carries or possesses the 3 drug-resistance markers.

The decrease of reductase activity as a result of passage of the mixed populations in the absence of amethopterin was also rapid. This assay procedure can detect approximately 5% high reductase cells in a population (see Chart 2).

The gradual disappearance of amethopterin-resistant cells was also observed in the mixture of 2 totally different cell lines, L27 and L27/A1 (Table 5). However, the TCC method of evaluation did not show that the 10% population was completely free of resistant cells at the 9th transfer generation: in fact, it appeared as approximately a 5% resistant population which, if made up of high reductase cells, such as L27/A1, would not be

### TABLE 6

**SELECTION OF AMETHOPTERIN-RESISTANT CELLS FROM MIXED POPULATIONS OF L27 AND L27/A1 CELLS**

<table>
<thead>
<tr>
<th>% RESISTANT CELLS</th>
<th>AMETHOPTERIN-TREATED TRANSFER GENERATION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>AST (T/C)*</td>
</tr>
<tr>
<td>0</td>
<td>19/8</td>
</tr>
<tr>
<td>0.0001</td>
<td>19/—*</td>
</tr>
<tr>
<td>0.001</td>
<td>18/—*</td>
</tr>
<tr>
<td>0.01</td>
<td>10/—*</td>
</tr>
<tr>
<td>0.1</td>
<td>11/9</td>
</tr>
<tr>
<td>1.0</td>
<td>10/—*</td>
</tr>
<tr>
<td>10</td>
<td>10/—*</td>
</tr>
<tr>
<td>50</td>
<td>10/10</td>
</tr>
</tbody>
</table>

*Amethopterin, 3 mg/kg; AST, average survival time in days; T/C, treated/control.

†Amethopterin, 0.4 mg/kg; TCC, total cell count.

dDihydrofolate, mumoles reduced/mg protein/hr.

*No control value.
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detectable by the reductase assay. The loss of reductase activity
in the L27/A1 population was noted in the 20–24 untreated
transfer generations based on AST (6).

In the previous study (6), after the 50th untreated transfer
generation the L27/A line was still resistant to amethopterin.
It was of interest then to make mixtures in vitro of L27 and
L27/A1 (Table 6). Results on the 2nd transfer generation showed
that the population which contained 0.999% resistant cells was
totally resistant to amethopterin. In 2 additional transfer gen-
erations the 0.00001% resistant population was resistant by the
3 criteria used, and at the same time a new population of ame-
thopterin-resistant cells had been selected from the sensitive
population (Table 6). These results indicate that the mutation
frequency to amethopterin resistance in this population is
in the order of 10⁻⁴.

The fate of resistant cells in a population may depend on factors
other than the presence or absence of a selective drug. Certainly
if the growth rate is markedly different the cells with the shorter
doubling time, as was the case of L1210 (Chart 1), will have an
advantage over those with a longer doubling time [L1210 (XVI)].
This difference in growth rate was also apparent in the differen-
ces in the survival times of the 2 lines (Table 4).

Another factor worth consideration is the difference in survi-
ors or lethality (Table 3). When the lethality of L1210 (V) was
compared with that of L1210 (XVI), each leukemia was equally
lethal and the number of cells required to kill the hybrid
mouse and the DBA₂ mouse was the same.

Sköld et al. (13) and Klein (9) emphasized the selective ad-
vantage of resistant cells in mixed populations during treatment.
It was possible (13) to obtain a totally resistant population of
5-FU-resistant Ehrlich cells after 1 treatment if the mixture was
made up of 1 resistant cell/1000 sensitive cells. With 1 resistant
cell/10⁴ cells, 2–3 passages were necessary to obtain a resistant
population. Our results are in agreement but are more extensive
in that the emergence of resistant populations from mixtures
containing fewer resistant cells has been determined. Our results
are also in accord with other data on L1210 (15). The mutation
rate to amethopterin resistance with high enzyme action of our
L1210 (V) and L27 population was between 10⁻⁴ and 10⁻⁵, the
same as the rate suggested by Klein (9), Law (10) and Friedkin
and Goldin (3).

The possibility that resistant cells are different “antigenically”
is suggested by the fact that as the population became com-
pletely resistant to amethopterin the TCC of the treated leukemic
animals was greater than the TCC of untreated mice (Tables
1 and 4). This in turn indicated increased growth of the leukemia
in the presence of amethopterin, which has a definite suppressive
effect on the antibody-forming mechanism (4, 14).

These experiments were designed originally to quantitate the
number of amethopterin-resistant cells in a population following
its exposure to DNA carrying a high level resistance marker
and an elevated dihydrofolate reductase activity. The present
studies reveal that at least 5% of the population must be resis-
tant and possess a high level of this enzyme before detection
is possible. This level of transformation in a situation in vitro is not
likely because of the inherent insensitivity of our test systems.
The need for more sensitive assays is obvious.

Acknowledgments
The excellent technical assistance of Mr. Dennis L. Robinson,
Miss Patricia O’Grady, and Mrs. Jane L. Palmer is acknowledged.

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