Perturbation of Generation Cycle of Human Leukemic Myeloblasts In Vivo by Methotrexate

By Peter Ernst and Sven-Aage Killmann

The cytokinetic effects of Methotrexate (MTX) on leukemic myeloblasts in vivo in man have been studied, utilizing in vitro and in vivo labeling with 3H-TDR, mitotic index, stathmokinetic index after VCR, measurements of single-cell DNA content, and in vitro labeling with 3H-UDR. It is demonstrated that MTX arrests cells in S for a period (about 20 hr) that corresponds to their DNA synthesis time. This S-phase arresting effect of MTX seems restricted to those cells that are in S at the time of MTX exposure. Cells in G1, G2, and M are not directly influenced by MTX. Some implications of these observations for combination drug therapy and for exploiting differences in the duration of cell cycle phases between leukemic blast cells and differentiating hemopoietic cells to obtain preferential killing of leukemic cells are briefly discussed. In one case, erythroblasts hardly incorporated exogenous TDR and UDR, although their mitotic activity was high. After MTX there was a transient but very pronounced increase in TDR and UDR utilization. It is suggested that these cells may have had a large intracellular pool of thymidine triphosphate that was temporarily reduced by the action of MTX. Whether these erythroblasts were of leukemic origin remains undecided.

With the rising number of drugs and drug combinations available for the treatment of acute leukemia, the problems of antileukemic chemotherapy become increasingly complex. Considerable therapeutic improvements have resulted from controlled clinical trials. We believe that therapeutic strategies may be further improved by detailed studies in man of the pharmacologic effects of cytostatic drugs. One such aspect that recently has attracted some attention is the in vivo effect of cytostatics on the generation cycle of leukemic blast cells in man. In a previous study we reported that MTX inhibits the passage of leukemic myeloblasts through DNA-synthesis phase (S) and possibly produces an extra recruitment of cells from G1 to S. In the present paper we confirm and extend these results. It will be

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Abbreviations used in text: G1, presynthetic phase; S, DNA-synthesis phase; G2, postsynthetic phase of cell cycle; M, mitosis; L, labeling index (percentage of blast cells labeled with 3H-TDR in vitro); I, mitotic index (percentage of blast cells in mitosis); 3H-TDR, 3H-thymidine; 3H-UDR, 3H-deoxyuridine; MTX, Methotrexate; VCR, vincristine; AML, acute myeloid leukemia; EL, erythroleukemia (di Guglielmo).
shown that with the doses used, MTX effectively inhibits the progression of cells through S phase but does not prevent the entry from G1 phase to S; as a result, cells pile up in early S, whereas G2 and mitosis (M) are depleted. This blocking effect lasts for a period that approximates S duration.

**Materials and Methods**

Ten patients with acute myeloid leukemia (AML) were studied; in one case three studies were performed. The patients are listed in Tables 1 and 2, together with some pertinent data. The patients were given a single intravenous dose of 15-40 mg MTX, except in one case (Y.A.) where 120 mg MTX was given intravenously four times at 6-hr intervals. Serial bone marrow samples were obtained before and during the treatment period. Part of the aspirate was smeared immediately and stained with May-Grünwald-Giemsa for determination of the mitotic index (I_m). The I_m counts are based on at least 3000 cells in each sample; mitotic cells include cells from late prophase to late telophase. The remainder of the marrow material was incubated for 1 hr with 3H-thymidine (H-TDR) (specific activity, 1.9 Ci/mM). In one case, uptake of 3H-deoxyuridine (3H-UDR) (specific activity, 5 Ci/mM) was also determined in an analogous way. The slides were then processed for radioautography and exposed for 7 days; more details have been given previously. The labeling index (I_l) of blast cells was determined by counting at least 1000 cells. In some samples radioautography was combined with ultramicrospectrophotometric determination of single-cell DNA content, using the Zeiss UMSP-I instrument, the procedure has been described in a previous paper.

One patient (F.P.) was labeled in vivo with 3H-TDR (0.1 mCi/kg body weight). The Kodak NTB-2 coated slides were exposed for 85 days; the percentage of labeled mitotic figures was determined by counting 50-100 mitoses at each time point. Cells with five grains or more were considered labeled. In this patient, in vitro 3H-TDR-uptake studies as described above were also carried out. The prior in vivo labeling did not interfere with the study, because the in vivo label alone did not produce grain counts after 7 days exposure, which was adequate for the development of grains after in vitro labeling.
The data on labeling and mitotic indices shown in Table 2 are consistent with an arrest of cells in S phase. In all cases, a rise in labeling index was observed after MTX. This increase becomes apparent 1–2 hr after the injection, and within 3–10 hr the \( I_L \) reaches a value that is about twice the pretreatment level. After this, the rise levels off, but \( I_L \), higher than the base-line value, has been observed up to 48 hr after a single dose of MTX. Two typical sequences are shown in Fig. 1. When MTX was given repeatedly (Table 2, Y.A.; and Fig. 2) the \( I_L \) continued to rise, although the slope was steepest after the first injection.

The mitotic index changes are also shown in Table 2. The \( I_M \) remained at pretreatment levels for 1–1½ hr after MTX and then declined rapidly and markedly. At 24 hr, the \( I_M \) was still below the base-line \( I_M \), albeit higher than the nadir value. A prolonged depression of the \( I_M \) was seen when MTX was given repeatedly (Fig. 2). When VCR was given 16–20 hr after MTX, (Table 2, A.E.M.-3 and A.M.T. and Fig. 3), \( I_M \) values exceeding the pretreatment level appeared within 4–12 hr, i.e., 20–28 hr after MTX. When VCR was given at the same time as MTX (Fig. 4, curve b), a transitory increase
of the IM was observed at 2 hr; this was followed by a decline that lasted until 20 hr.

It was of interest to know whether the mitoses that reappeared about 1 day after MTX were derived from cells that had been in S or in G1 at the time of drug exposure. Therefore, an 3H-TDR in vivo labeling study was carried out (Table 3). 3H-TDR was given at the same time as MTX. Mitotic figures were absent for many hours but began to reappear at 19 hr; around 30 hr there was an overshoot of mitotic figures, but at 40 hr this was no longer apparent. The percentage of labeled mitoses was quite low throughout; it should be noted that the wave of mitoses observed around 30 hr was predominantly unlabeled mitoses. At all times, except at 28 hr, the number of labeled mitotic figures per 100 cells was definitely lower than the number of mitotic

Fig. 3.—Effect of VCR on mitotic index (IM) when given 16 hr after MTX; note delayed rise in IM. IM = 3H-TDR labeling index.
PERTURBATION OF LEUKEMIC MYELOBLASTS

of Leukemic Bone Marrow Myeloblasts During Methotrexate Treatment

<table>
<thead>
<tr>
<th>Hours</th>
<th>18</th>
<th>19</th>
<th>20</th>
<th>22</th>
<th>24</th>
<th>25</th>
<th>26</th>
<th>28</th>
<th>31</th>
<th>38</th>
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<td>VCR given 16 hr after MTX</td>
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<td>Repeated MTX doses</td>
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<td>VCR given 16 hr after MTX</td>
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<td></td>
<td></td>
<td>VCR given together with MTX</td>
</tr>
</tbody>
</table>

figures per 100 cells prior to MTX. The I<sub>L</sub> of interphase cells did not rise during the period of observation. The fraction of cells with a G<sub>2</sub> content of DNA was determined by ultramicrospectrophotometry of 200 unlabeled cells before MTX and was found to be 3.5%. The serum uric acid concentration before MTX was 4.6 mg/100 ml; a moderate but constant rise started at 16 hr after MTX, and from 22 to 28 hr after MTX the concentration was stable at 5.4 mg/100 ml and then again declined slowly.

Figure 5 shows the nuclear DNA content of individual <sup>3</sup>H-TDR-labeled

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**Fig. 4.—Effect of MTX and VCR on mitotic index of bone marrow blasts in patient with AML. Curve a: VCR given alone. Curve b: VCR and MTX given simultaneously. Curve c: VCR given alone. Sequence of three observations was a-b-c.**
Table 3.—Labeling Data on Bone Marrow Myeloblasts in Patient F.P.
After In Vivo Labeling With ³H-TDR

<table>
<thead>
<tr>
<th>Hr after MTX and ³H-TDR labeling</th>
<th>0</th>
<th>8</th>
<th>16</th>
<th>19</th>
<th>22</th>
<th>25</th>
<th>28</th>
<th>31</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td>in vivo † in vivo</td>
<td>0.5</td>
<td>0</td>
<td>0</td>
<td>0.2</td>
<td>0.35 —*</td>
<td>2.0</td>
<td>2.0</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>Mitotic index (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% mitoses labeled with ³H-TDR</td>
<td>—</td>
<td>—</td>
<td>63</td>
<td>64</td>
<td>32</td>
<td>28</td>
<td>8</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>No. of unlabeled mitoses/100 interphase cells</td>
<td>0.5</td>
<td>0</td>
<td>0</td>
<td>0.07</td>
<td>0.13 —</td>
<td>1.44</td>
<td>1.84</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>No. of labeled mitoses/100 interphase cells</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.13</td>
<td>0.22 —</td>
<td>0.56</td>
<td>0.16</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>In vivo labeling index of interphase cells</td>
<td>7.8†</td>
<td>7.0</td>
<td>7.5</td>
<td>7.0</td>
<td>6.0 —</td>
<td>5.6</td>
<td>6.0</td>
<td>5.6</td>
<td></td>
</tr>
</tbody>
</table>

* Contaminated with peripheral blood.
† Determined from in vitro labeling.
† Methotrexate and ³H-TDR were given simultaneously.

blast cells before and after a single injection of MTX. Six hours after MTX there is an accumulation of cells in early S phase. Figure 6 gives the results of DNA measurements of ³H-TDR-labeled blast cells when repeated injections of MTX were given. Here a progressive accumulation of cells in early S phase is apparent. These data imply that MTX not only prevents the exit from S, but in large measure prevents the movement through S.

Figure 7 illustrates the effect of MTX and VCR on the myeloblasts and the early red cell precursors (basophilic + slightly polychromatic forms) in a case of AML. It is seen that mitotic figures reappear at an earlier time after MTX in the erythroid cells than in the myeloblasts. This difference in myeloblasts and erythroid precursors was also observed in patient V.K. (Table 4); here, significant erythroid mitotic activity was resumed between 6 and 16 hr after MTX, whereas myeloblastic mitotic activity did not get under way until between 16 and 24 hr. As discussed later, these observations strongly suggest that the duration of the S arrest is related to the DNA synthesis time of the particular cells.

To get another check on the duration of the MTX effect ³H-UDR-incorpora-
Fig. 6.—Distribution of in vitro $^3$H-TDR-labeled bone marrow myeloblasts with respect to nuclear DNA content. $2n$, $4n$: see legend Fig. 5. AU, arbitrary units. With repeated doses of MTX, cells in early S phase become increasingly predominant: from first (top) to last (bottom) sample, the $2n$–$3n$/$3n$–$4n$ ratio changes as follows: 41/59, 65/35, 65/35, 77/23.
tion studies were carried out in one case (Table 4). Before MTX, the Iₘ of myeloblasts with ³H-TDR and ³H-UDR, as expected, were very similar. Two and 6 hr after MTX, ³H-UDR incorporation was completely blocked; 16 hr after MTX, the Iₘ with ³H-UDR was about one half of the ³H-TDR-Iₘ, and by 24 hr the two labeling indices again were and subsequently remained identical. The erythroid cells showed a very different and unexpected pattern. In spite of high mitotic activity prior to MTX, both the ³H-TDR and ³H-UDR index was extremely low. After MTX, the ³H-TDR-Iₘ rose rapidly, reached a maximum at 16 hr, and then returned to the low pretreatment level. The ³H-UDR-Iₘ dropped to zero for a period of 6 hr or more, then rose markedly at 16 hr, and subsequently again decreased to base-line values.

**DISCUSSION**

The results of this study show that cells in S phase are the target of MTX.

**Table 4.—Data on ³H-TDR Labeling Index (³H-TDR-Iₘ), ³H-UDR Labeling Index (³H-UDR-Iₘ), and Mitotic Index (Iₘ) of Bone Marrow Myeloblasts and Erythroblasts in Patient V.K.**

<table>
<thead>
<tr>
<th>Hours after MTX</th>
<th>Myeloblasts</th>
<th>Erythroblasts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>³H-TDR-Iₘ</td>
<td>³H-UDR-Iₘ</td>
</tr>
<tr>
<td>0</td>
<td>8.5</td>
<td>10.8</td>
</tr>
<tr>
<td>2</td>
<td>10.6</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>14.4</td>
<td>0.5</td>
</tr>
<tr>
<td>16</td>
<td>15.7</td>
<td>7.9</td>
</tr>
<tr>
<td>24</td>
<td>16.4</td>
<td>17.5</td>
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<tr>
<td>31</td>
<td>16.6</td>
<td>16.4</td>
</tr>
<tr>
<td>38</td>
<td>19.7</td>
<td>20.2</td>
</tr>
</tbody>
</table>
PERTURBATION OF LEUKEMIC MYELOBLASTS

In contrast, cells in $G_1$ are quite resistant to the cytokinetic action of MTX. MTX arrests leukemic myeloblasts and erythroid precursors in S phase; the duration of this effect depends on the DNA synthesis time of the cells. As a result of the block in S, mitotic activity temporarily ceases. On the other hand, transition from $G_1$ into $S$ is not impaired and even appears to be accelerated. As a result, cells accumulate in S. In the following, the evidence for these conclusions will be considered.

**Effect of Methotrexate on Cell Cycle Phases**

If MTX arrested cells in S without directly influencing cells in $G_1$, $G_2$, or M, the following cytokinetic effects of MTX would be predicted: a decline in the $I_M$ after a time corresponding to the transit time through $G_2$; a rise in $^3$H-TDR $I_s$, since cells can enter but not leave S; an accumulation in early S of cells entering from $G_1$ into S during the time of MTX effect. The present data, detailed in the following, show that with the dosage used, MTX produces these effects in human leukemic myeloblasts. Hence, the main cytokinetic action of MTX is to arrest the cells in S.

After MTX, the $I_I$ remains approximately at the pretreatment level for about 1½ hr, whereas by 3 hr the $I_M$ has decreased markedly. Since in human leukemic cells the duration of mitosis is about 1 hr and $G_2$ time is 1–2 hr, the time course of the mitotic index shows that mitosis is not directly influenced by MTX and that the flux of cells from $G_2$ to mitosis is not impaired. This is supported by the data in patient L.N. (Fig. 4) where MTX and VCR were given simultaneously. Here, the $I_M$ increased by a factor of three after 2 hr and then again fell; it is to be noted in this context that the mitotic block produced by VCR takes effect almost instantaneously. The regular drop in $I_M$ after about 3 hr is explained from a depletion of the $G_2$ compartment that has no influx from the S compartment because of S-phase arrest. The assumed S-phase arrest should also manifest itself by an increase in the $^3$H-TDR $I_s$ after MTX, since exogenously supplied thymidine should circumvent the biochemical block introduced by MTX. Such an increase is apparent from the data shown in Table 2. This S-phase arrest is in accordance with tissue culture data and consistent with the major biochemical effect of MTX: by binding to folate reductase, MTX inhibits the conversion of deoxyuridine monophosphate to thymidine monophosphate. In this way the de novo synthesis of DNA is blocked, whereas exogenous thymidine, at least in some situations, is readily incorporated and utilized for DNA synthesis by means of the kinase system.

If MTX not only prevented the exit from S without impairing the entry into S but also arrested the progression of cells through S, one would anticipate an accumulation of cells in early S after MTX. More specifically, the total rise in $I_M$ should for some time be accounted for by a pile up of cells in early S. In patient F.V.S. (Fig. 5), the S population (before MTX) was quite evenly distributed between 2n and 4n, where 2n and 4n are the DNA content of $G_1$ and $G_2$ cells, respectively; 54% of the cells were situated between 2n and 3n, and 46% between 3n and 4n. Six hours after MTX, the $I_M$ had risen from
13.3 to 25.5; if all cells had been effectively arrested and all cells that entered S during the 6 hr period were located in early S, the predicted distribution would be 76% 2n-3n cells and 24% 3n-4n cells. The observed values were 73% and 27% respectively. These data show that the increase in IL after MTX can be fully accounted for by an accumulation of cells in early S and thus demonstrate that MTX effectively inhibits the progression of cells through S phase.

If one ignores the slight mitotic activity after MTX and assumes a pattern of “free, normal entry—no exit” with respect to the S phase, then the increment in IL after MTX could be used to estimate S duration. From Table 2 it is apparent that with a few exceptions, this would mean that S duration was about 8 hr. However, most direct studies show that DNA synthesis time of leukemic blast cells usually is 15–20 hr. To explain the unexpectedly large increment in IL, one might assume that MTX abolishes non-S cells; however, this is ruled out by the data showing that the rise in IL is fully accounted for by the accumulation of cells in early S. Another mechanism of the large increase in IL could be that the S-phase arrest elicits an extra recruitment of cells from late G1 into S, conceivably triggered by a feedback sensitive to a decreased flux through G2 or mitosis. This extra recruitment may involve a suddenly increased flux of (presumably late) G1 cells into S. The apparent recruitment alternatively may depend on the operational criteria that are used: here, S cells are defined as cells that incorporate exogenous thymidine. Among other things, this incorporation requires thymidine kinase activity; conceivably, cells in the very beginning of DNA synthesis may not have sufficient kinase to become labeled with 3H-TDR. In this way, the IL might somewhat underestimate the fraction of cells actually engaged in DNA synthesis. If MTX would call forth a higher thymidine kinase activity in such cells in very early S phase, the resulting increase in IL would simulate a true recruitment from G1 into S.

It is of interest to note that the rise in IL after MTX levels off after about 6–8 hr (Table 2, Fig. 1). The change in the slope of the IL curve cannot be accounted for by resumption of the normal progression out of S, since at this time the IL is still severely depressed. However, it might be the result of a beginning cell loss due to death of cells arrested in S. Another explanation for the leveling off of the IL would be that after a period of higher than normal flux from G1 to S, the flux now decreases to normal or even to below normal, which might be the case if the G1 pool, due to the supposed increased demand, has been depleted of cells ready to enter S phase. The response of the IL to repeated doses of MTX (Fig. 2) would appear to fit this notion that the rise after the second and third injection is less than after the first dose. The immediate effect of the fourth injection cannot be evaluated because of a long sampling interval. A possibility to keep in mind concerning the possibly reduced entry into S after the initial high influx is that the transition from G1 to S may conceivably be impaired by various late-appearing biochemical effects of MTX, e.g., on purine synthesis.
**Duration of Methotrexate-Induced S-Phase Arrest and Subsequent Fate of Arrested Cells**

MTX is cleared from the plasma with a half time of about 2 hr\(^{10,20}\) and rapidly and firmly taken up by cells.\(^{20-24}\)

The S-phase arrest by MTX sets in very rapidly, as shown by the rise in \(I_e\) that is present already after 1–1½ hr (Table 2). From 3 to 4½ hr on, very few cells pass through mitosis. Around 24 hr, \(I_M\) values begin to increase but are still lower than before MTX (Tables 2 and 3). These figures suggest that the S-phase block is rather effectively maintained for about 20 hr. This is also supported by the VCR data (Table 2, Figs. 3 and 4) that show the stathmokinetic action of VCR does not take effect until 20–28 hr after MTX. The best single estimate of the duration of the S-phase arrest was obtained in patient L.N. (Fig. 4, curve b) who received MTX and VCR simultaneously; apart from the early rise in \(I_e\) due to flux from G2, the \(I_M\) remained low until 20 hr and then rose in the 24 hr sample. This points to an S-phase arrest of approximately 20 hr. That the poor response to VCR was not due to drug resistance is shown by curves a and c in Fig. 4 demonstrating that both before and after the combined MTX and VCR medication, VCR alone had a very definite stathmokinetic effect. Summarizing, the data presented show that the S-phase arrest by MTX sets in very quickly and lasts for approximately 20 hr; during this period the block is rather effective, but a few cells succeed to pass through mitosis.

The mitotic cells seen around 24 hr when the cell flux through mitosis is being restored raise an important question. Were these cells in S at the time MTX was given and have they now resumed their progression through the cell cycle after many hours of arrest, or were these cells still in G1 when MTX took effect? Data bearing on this point were obtained in patient F.P. who was labeled in vivo with \(^3\)H-TDR and at the same time received MTX (Table 3). In the 8-hr and 16-hr samples, no mitotic figures were encountered among 3000 interphase cells; during a prolonged search of the slides, a few mitotic figures were seen, of which about two thirds were labeled. Measurable mitotic activity reappeared around 20 hr after MTX; at this time 63%–64% of the mitotic figures were labeled. Around 30 hr mitoses were more numerous than before MTX, but at the same time the proportion of labeled mitoses declined rapidly. These data show that at least some cells that were in S when MTX was given can make their way through cell division. To which extent these cells have been arrested in S is more difficult to evaluate. The labeled cells that passed through mitosis at 20 hr may have been temporarily held up but could also be cells that were in the very beginning of S when the drug was injected, in which case they could be expected to arrive at mitosis about 20 hr later. The labeled mitotic figures at 30 hr were quite probably cells that had been arrested. The immediate MTX effect on these cells would therefore appear to be merely cytotstatic; this action of the drug has also been pointed out by Hofer et al.\(^{25}\) in the L 1210 system. It is important to realize, however, that the data in Table 3 do not represent a normal, labeled mitotic figure curve that simply has been displaced to the right along the time axis.
The $I_L$ of mitotic figures reached a maximum of 64% and then declined, whereas curves of labeled mitotic figures in leukemic cells usually show a plateau at 80%–100% for many hours. When one considers the number of labeled mitotic figures per 100 cells and compares this with the $I_M$ before MTX, it is evident that at all time points, except 28 hr, this labeled mitotic index is definitely lower than the pretreatment mitotic index. Assuming an unchanged mitotic duration, this could mean either that many of the arrested labeled cells die during the observation period before they reach mitosis (cytocidal effect), or that the progression of the labeled cells through the cycle is so severely delayed that many labeled cells have not yet reached mitosis at the end of the observation period (cytostatic effect). That a cytostatic mechanism is operative has already been discussed. It is not likely, however, that this can fully explain the data. In the case of a purely cytostatic action of MTX, the reduced flux of labeled cells into mitosis should have been compensated for by a prolonged flux of labeled cells into mitosis. A rise in the interphase cell $I_L$ would also have been expected, although somewhat delayed as compared to the definite increase in the $I_M$ that is usually observed during the first day after in vivo $^3$H-TDR labeling in untreated acute leukemia. In contrast, the data show that labeled mitoses were no longer present in the last sample, and that the interphase $I_L$ declined slightly during the study. This is best explained by a cytotoxic action of MTX that is also supported by the moderate rise in serum uric acid concentration observed after MTX. Whatever the proportion between immediate cytotoxic and cytostatic action of MTX may be, it is of interest to note that fair numbers of labeled cells were still present 40 hr after MTX. As discussed, at least a fraction of these cells had gone through division. Whether they carried residual damage cannot be decided from the data but is likely in view of the clinical effectiveness of MTX.

**Relationship Between DNA Synthesis Time and Duration of MTX Effect**

Turning now to the interpretation of the unlabeled mitoses (F.P., Table 3) and considering the large size of the $C_2$ pool (3.5%, i.e., one-half of the S pool), it is surprising that mitotic activity could not be maintained for a considerable period of time as would have been expected if cells could pass freely from $C_2$ to $M$. As discussed previously, MTX does not appear to impair the flux of cells from $C_2$ to $M$. This must mean that in this patient the $C_2$ pool was not a simple pipeline transit compartment but was at least in part a cell cycle cul-de-sac. As seen from Table 3, the majority of the mitotic figures after the 22 hr sample were unlabeled. This demonstrates that the resumption of mitotic activity after MTX is mainly due to cells that were in $C_1$ at the time of MTX injection. Since S duration of leukemic blast cells ordinarily is 15–20 hr, the appearance of unlabeled cells in mitosis after 22–28 hr suggests that the passage of these cells through S has not been impaired. In terms of S-phase arrest it appears therefore that leukemic myeloblasts in $C_1$ are quite resistant to MTX. Although they have been exposed to the same extracellular MTX concentrations as S cells, they enter S and go through S in what appears to be an approximately normal S duration, whereas cells that already are in S at the time of MTX exposure are arrested. How the $G_1$ cells are protected...
against the effect of MTX, by lack of incorporation, catabolism, inactivation, or other mechanisms is not clear. In the foregoing it has been pointed out that MTX seems to have no direct effect on cells in G₂ and mitosis. From a cyto-
kine tic point of view it appears then that the effect of MTX (in the present dosage) on leukemic myeloblasts is restricted to those cells that are in S during the availability time of the drug. This is in good agreement with observations by Bruce et al.²⁶ who studied the killing effect of MTX and cytotoxic doses of ³H-TDR. The dose response curves of the two agents were very similar and both had a shoulder that was interpreted to be due to a cell cycle phase-dependent resistance of the cells.

From what has been said, the similarity between the ordinary DNA synthesis time of leukemic blast cells (15-20 hr) and the duration of the MTX S-phase arrest (about 20 hr) is not thought to be fortuitous. If it is correct that the main cytokinetic effect of MTX is to more or less cut out a segment of the cycling cell population corresponding to the length of S duration, the effect of MTX should be shorter lasting in cells with a shorter S duration, e.g., erythroid cells. Two of our patients had sufficient erythropoietic activity to allow a comparative study of the effect of MTX on the myeloblasts and on early erythroid precursors (Fig. 7 and Table 4). It will be noted that the response of the myeloblasts and of the erythroblasts is quite different. The erythroid precursors resume cell division definitely earlier than the myeloblasts. The S duration of erythroid cells is about 12-13 hr;²⁷ after MTX, erythroid mitoses reappear after about 13-16 hr. One is led to the conclusion that the time until mitotic activity recovers after MTX exposure is primarily determined by the S duration of the particular cells. The practical implications of this, if confirmed, for the proper spacing of MTX injections are obvious.

³H-UDR-Incorporation Data

Since MTX blocks the methylation of UDR to TDR,¹⁵,¹⁶ one would expect a temporary stop in ³H-UDR incorporation into DNA of S-cells after MTX. This has recently been observed in leukemic lymphoblasts.²⁷α We have studied ³H-UDR incorporation after MTX in one AML patient (Table 4). In the myeloblasts, the incorporation of UDR rapidly fell to zero, indicating an effective block of the conversion of deoxyuridylate to thymidylate. Sixteen hours after MTX, partial recovery of ³H-UDR utilization for DNA synthesis was apparent. The fraction of cells labeled with ³H-UDR was about one-half of the fraction labeled with ³H-TDR. At 24 hr, the ³H-UDR and ³H-TDR were identical and about twice the pretreatment values. These data pose the problem whether the resumption of utilization of ³H-UDR for DNA synthesis is restricted to cells that have entered S phase after the plasma level of MTX has fallen below a certain threshold, or whether cells that were in S when MTX was given may also begin to utilize ³H-UDR again after a period of blocked utilization. The fact that at 16 hr the ³H-UDR I₁ was only one-half of the ³H-TDR I₁ would be compatible with the assumption that in large measure ³H-UDR labeling is accounted for by cells that have entered S after MTX was given. If so, one would expect these ³H-UDR-labeled cells to be located rather early in S. Ultramicrospectrophotometry of the ³H-UDR-
labeled cells at 16 hr showed that of 53 cells, 45 were in the 2n–3n S-content
bracket and only eight contained between 3n and 4n DNA. This is compatible
with the notion that the majority of cells that at 16 hr could incorporate UDR
into DNA were in G1 at the time of drug administration.

The identity of the 3H-UDR IL and 3H-TDR IL at 24 hr shows that at this
time the block in the deoxyuridylate-thymidylate conversion had vanished. It
should also be noted that the 24 hr IL is about twice as high as the pretreatment
value. This persistence of the increase in IL above the base-line value was
also observed in several other cases (Table 2). This might represent some
synchronization, perhaps from the suggested extra G1 recruitment, that should
also manifest itself in the mitotic index. A mitotic overshoot was
observed in F.P. (Fig. 1) but was not seen in other cases (Table 2); however,
our data on mitotic indices without VCR at this time after MTX are few.
Therefore, at the present time the significance of the elevation of the 3H-TDR
IL at around 24 hr as compared to the pretreatment value is difficult to evaluate
completely, since this elevation may result from the interplay of several
factors: (1) changes in the proportions between cells in cycle (“proliferating”)
and out of cycle or in long cycle (“nonproliferating,” “quiescent”); in several
patients the blood blast count, which mainly comprises quiescent cells,7 had
decreased considerably at 24 hr; (2) the question whether all cells that 24 hr
after MTX label with 3H-TDR are able to proceed to mitosis, or whether
some of them are still arrested and perhaps finally will die. The identity be-
tween the 3H-TDR IL and the 3H-UDR IL at 24 hr in V.K., discussed above,
suggests the first possibility. In that case, a larger fraction of leukemic cells are
in effective S 24 hr after MTX than before the drug. It is possible, however,
that other effects of MTX, e.g., on purine synthesis,18 might be
less easily
overcome than the deoxyuridylate/thymidylate conversion block and that this
could hamper the cells’ progression through S.

The labeling characteristics of the erythroid cells in V.K. (Table 4) were
quite unusual. Before MTX, extremely few of the erythroid cells incorporated
3H-TDR and 3H-UDR in spite of marked proliferative activity (mitotic index =
5%); after MTX, the 3H-TDR IL increased markedly but returned to pretreat-
ment values at 24 hr. A tentative explanation is that the erythroid cells con-
tained a large intracellular pool of thymidine triphosphate (TTP) that would
dilute the label and that is also known28 to exert a negative feedback on thy-
midine kinase activity. By blocking the de novo thymidylate synthesis, MTX
reduced the intracellular TTP pool that in turn resulted in increased kinase
activity and hence utilization of exogenous TDR. When the MTX effect faded
away and intracellular TTP again increased, 3H-TDR incorporation returned
to pretreatment levels. The changes in 3H-UDR IL could be explained in the
same way,29 although modified by the MTX effect on its conversion to thy-
midylate. The interpretation of this erythroblast abnormality is open to ques-
tion. It may mean that the red cell precursors are leukemic or may reflect some
environmental effect on normal cells. The present observation does not appear
to be a unique phenomenon. We have recently noted the same discrepancy
between erythroblast labeling and mitotic index in several other patients with
AML and with chronic myelocytic leukemia.
Possible Implications For Planning of Chemotherapy

It is to be hoped that studies of the type of kinetic perturbation induced by various drugs, the duration of the flux arrest, and knowledge about the cytkinetic characteristics of leukemic cells will be helpful in designing schedules for combination drug therapy. Without entering a lengthy and perhaps premature discussion, a few examples may serve to illustrate this point. From a cytkinetic point of view one would expect that VCR would have little effect on leukemic cells when given simultaneously with MTX, because MTX would severely curtail the influx into the mitotic trap set by VCR. The validity of this prediction is illustrated by Fig. 4. VCR, given 24 hr after MTX, when the flux into mitosis has been resumed, would appear rational. Considerations of this sort evidently should not be restricted to leukemic cells but should also take into account the preservation of normal cells as much as possible. Another highly important aspect is to consider drug action at the stem cell level. Perhaps some information on this point may be gained from the culture of marrow cells in soft agar. An observation in patient V. K. suggests that the agar colony-forming cells may behave differently from the directly observable blast cells. Already 2 hr after MTX the colony count was reduced to 25% of the pretreatment value and remained at this low level for at least 24 hr. In contrast, the directly observable blast cells had recovered their ability to utilize \(^3\)H-UDR by 24 hr, when mitotic activity was also reappearing. Further comparative studies of this type may be useful; however, a present difficulty is that it is not known whether agar colony-forming cells from AML marrows are leukemic, normal, or both.

For the time being one must be satisfied to plan for a maximal conservation of differentiating hemopoietic cells. (From a nonradical point of view, this holds true although the differentiating cells in AML may in part be of leukemic origin as a case in point, destruction of nondifferentiating leukemic clones may open the way for differentiating leukemic clones). Some preferential chemotherapeutic effect on leukemic cells can conceivably be attained by exploiting the differences in cell cycle parameters between differentiating cells and leukemic cells of the blastic variety; this calls for more intensive study of the behavior of differentiating cells in leukemic marrows. A possible approach can be illustrated by an admittedly rigid example in which the following assumptions are made: all red cell precursors have an S duration of 10 hr and a cycle time of 20 hr; all leukemic blast cells have an S duration of 20 hr and a cycle time of 60 hr; MTX kills all cells in S at the time of drug injection but kills no cells in G1, G2, or M; the rate of progression of the surviving cells through the cell cycle is unchanged and follows a strict pipeline pattern. In this case, the first MTX injection will kill 10/20 of the erythroid precursors and 20/60 of the leukemic blast cells. The next MTX injection, given 20 hr later (= the cycle time of the erythroid precursors) will kill none of the remaining erythroblasts (because at this time cells that were in G1, G2, or M at the first MTX injection will again be in G1, G2, and M, respectively) but another 20/60 of the blast cells. A third MTX dose 20 hr after the second dose will kill none of the remaining erythroblasts but the last 20/60 of the proliferat-
ing blast cells. Thus, the stock after the third MTX dose is 10/20 of the original number of erythroid precursors and zero of the proliferating leukemic blast cells. This is obviously a constructed example. Several factors, among which are the spread in individual cell-cycle time, the plasma clearance of the drug, and possible changes in phase duration under the pressure of depopulation, would make it difficult to obtain such a degree of selectivity. Nevertheless, it appears worthwhile to explore this approach of cycle-dependent differential chemosensitivity further.

What is not taken into account in this approach is the problem of quiescent leukemic cells that resume proliferation; however, this is an obstacle common to any type of chemotherapeutic strategy.

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Perturbation of Generation Cycle of Human Leukemic Myeloblasts In Vivo by Methotrexate

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