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Methotrexate Cytotoxicity as Related to Irreversible S Phase Arrest in Mouse L1210 Leukemia Cells

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The association between cytotoxicity and cell cycle perturbation caused by methotrexate (MTX) was investigated in mouse L1210 leukemia cells by flow cytometric bromodeoxyuridine/DNA assay. In the range of concentrations of MTX from 10^{-7} *M* to 10^{-6} *M*, *in vitro* exposure to the drug for 6 h caused a dose-dependent suppression of clonal growth of the tumor cells and S phase arrest in the cycle progression, resulting in an accumulation of cells in early S phase, in which they showed no definite increase of DNA content above G1 levels. The surviving fraction of the clonogenic cells corresponded with the fraction of cells which recovered from the S phase arrest in MTX-free medium. In mice bearing L1210 ascites tumors, a bolus injection of MTX caused the S phase arrest of the tumor cells as shown in suspension cultures, and cytokinetic recovery was observed in parallel with the regrowth of the tumor. These results showed that irreversible S phase arrest is a critical cytokinetic event associated with the cytotoxicity of MTX.

Key words: Flow cytometry — L1210 leukemia — Methotrexate — S phase arrest

The recent progress in the clinical pharmacology of methotrexate (MTX) has enabled to use high doses of MTX against various human cancers.[1)] Although the possible advantage of this therapy has been discussed with respect to uptake of the drug or amount of the target enzyme dihydrofolate reductase (DHFR), the cytokinetic effects as related to the cytotoxicity of the therapy have not been elucidated and there are some apparent contradictions in available data on this subject. If a high dose of MTX, in fact, kills tumor cells only in the S phase and can stop the progression of the G1 phase cells into S phase, it will cause a paradoxical decrease of cell-killing action.[2,3)] Recently, we have reported that a high dose of MTX caused S phase arrest and resulted in a marked accumulation of tumor cells in early S phase, where they have G1 DNA content and are able to incorporate bromodeoxyuridine (BrdUrd) as a thymidine analogue.[4)] In the present study, we investigated the *in vitro* and *in vivo* cytokinetic recovery of L1210 cells after exposure to MTX and found a close correlation between the irreversible S phase arrest and the cytotoxicity of MTX.

MATERIALS AND METHODS

Growth of L1210 leukemia cells L1210 leukemia cells were supplied by the Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo. The cells were cultured *in vitro* in RPMI 1640 medium supplemented with L-glutamine and 5% FCS at 37°C in a humidified atmosphere of 95% air and 5% CO_2. All treatments were carried out on exponentially growing cell cultures. Cell viability was assessed by trypan blue exclusion. Durations of the entire cell cycle and of S phase, calculated from the fraction of labeled cells in the mid S-phase curve, were 11.5 h and 6.3 h, respectively.[4)] For the *in vivo* experiments 1×10^5 viable L1210 cells, which were maintained in DBA/2 mice, were transplanted intraperitoneally into BDF1 hybrid mice obtained from Shizuoka Animal Co., Ltd., Hamamatsu. Tumor growth was measured as increase in cell number after inoculation of L1210 cells. At designated intervals after inoculation mice were killed, and injected ip with 3 ml of saline. The abdominal cavity was opened and the cells were aspirated. A smear was made from each cell suspension to control the contribution of non-leukemic cells.

Treatment *In vitro*: BrdUrd was obtained from Sigma Chemical Co., St. Louis, MO. MTX was supplied by Lederle Japan Co. Cells were exposed to MTX of various concentrations for 6 h, washed three times and then continuously grown in MTX-free medium. For the analysis of the cytokinetic response to MTX, cells were pulse-labeled with 10 μM BrdUrd for 20 min in two labeling schedules: (A) cells were labeled with BrdUrd and then incubated in BrdUrd-free medium with/without MTX (pulse chase method); (B) cells were incubated with/without MTX for designated intervals and then labeled with BrdUrd (pulse labeling method). The cytotoxicity of MTX was assessed in terms of colony-forming activity as described previously.[4)]

In vivo: MTX (10 mg/kg or 50 mg/kg) was injected ip into mice 4 days after inoculation of L1210 cells. Groups of four mice each were killed at intervals during

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96 h after MTX injection. BrdUrd of 50 mg/kg was given ip 30 min before killing the mice.

Staining Simultaneous staining for BrdUrd/DNA was performed as described previously.[4] In brief, BrdUrd incorporated into DNA was stained with a monoclonal anti-BrdUrd preparation (Beckton Dickinson Co.) by an indirect immunofluorescence method and then DNA was stained with 50 μg/ml of propidium iodide (PI, Calbiochem-Behringer Co.). Control cells without BrdUrd labeling were prepared by the same procedure.

Flow cytometry Fluorescence-stained cells were analyzed on a FACS IV flow cytometer (Becton Dickinson Co.). Cells were excited at 488 nm with an argon laser. Red fluorescence from PI was collected through a 590-nm long-pass filter and recorded as a measure of total DNA content. Green fluorescence from fluorescein was collected through a 530-nm band-pass filter and recorded after logarithmic amplification as a measure of the amount of incorporated BrdUrd. Routinely, 30,000 cells were measured for each distribution at a flow rate of less than 300 cells/s. A univariate 256-channel distribution and bivariate 64×64 channel distribution showing the distribution of DNA on the X axis and BrdUrd on the Y axis were generated by software supplied by Fujisawa Pharmaceutical Co., Osaka. The values for cell cycle distributions on DNA histograms were obtained by using the DNA histogram analyzer developed at Dr. Takahashi's laboratory.[5]

RESULTS

Typical BrdUrd/DNA distributions and the lines circumscribing the G1, S and G2M phases cells are shown in Fig. 1 for L1210 cells labeled for 20 min with BrdUrd. In our previous study, we set a window for the G1-S boundary in the S phase region in the BrdUrd/DNA distribution. The cells at the G1-S boundary corresponded to BrdUrd-labeled cells with G1 DNA content.[4] However, we omitted the area of the G1-S boundary from the bivariate distributions in this study. Therefore, cells at the G1-S boundary are considered to be early S phase cells in this study. The reasons for omitting the G1-S area are as follows. (1) It is reasonable physiologically that the incorporation of BrdUrd precedes the first increase of DNA content above G1 levels, and increases in parallel with production of DHFR, the target enzyme of MTX[6] (2) The term "cells at the G1-S boundary" causes semantical difficulties in using the terms "G1-S block" and "S phase arrest" which are commonly used to describe the drug-induced perturbation of the cell cycle, because these cells are not in either G1 or S phase.

***In vitro* cultures** Growth of L1210 cells after treatment with MTX for 6 h was assessed in both suspension and

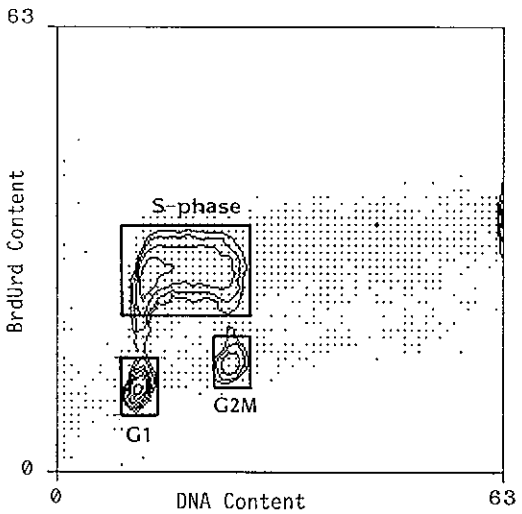

Fig. 1. This figure shows a contour plot of BrdUrd/DNA distribution for L1210 cells labeled for 20 min with BrdUrd. Also shown are regions describing the G1-, S-, and G2M populations. The Y-axis is log green fluorescence of fluorescein (anti BrdUrd) and the X-axis is linear intensity of red fluorescence of PI (DNA content).

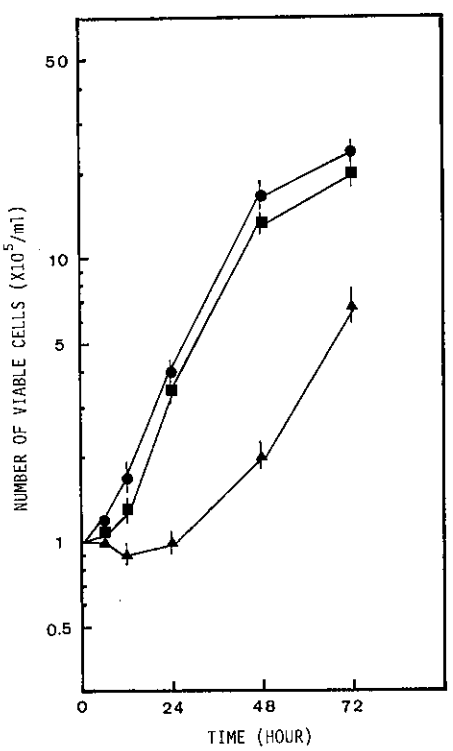

Fig. 2. Survival curves for L1210 cells treated with 10^{-7} *M* (■) and 10^{-6} *M* (▲) MTX for 6 h. Untreated control culture (●). Mean of 4 cultures; bars, SD. MTX was added to the cultures at 0 h.

Table I. Effects of MTX on the Colony-forming Activity of L1210 Cells[a]

MTX concentration during 6 h incubation (M)	% of control[b]
1×10^{-8}	98
1×10^{-7}	83
2.5×10^{-7}	60
5×10^{-7}	41
7.5×10^{-7}	17
1×10^{-6}	9
1×10^{-5}	4

a) Colony-forming activity is defined as the percentage plating efficiency of treated cells/plating efficiency of untreated cells. *b*) Each number represents the mean result of three experiments.

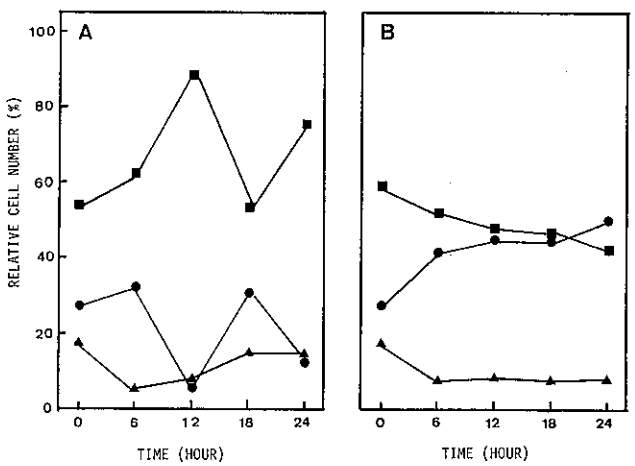

Fig. 3. Changes in distribution of L1210 cells in the G1 phase (●), S phase (■) and G2M phase (▲) of the DNA content as a function of time after exposure to MTX for 6 h. After incubation with MTX for 6 h, cells were washed, resuspended in MTX-free medium and at selected intervals thereafter were labeled with BrdUrd. They were collected 20 min later for FCM analysis. A representative one of three independent experiments is shown. A, 10^{-7} M; B, 10^{-6} M. The DNA histograms, in which the value of relative cell number in each phase was determined, correspond to the bivariate BrdUrd/DNA distributions shown in Fig. 4.

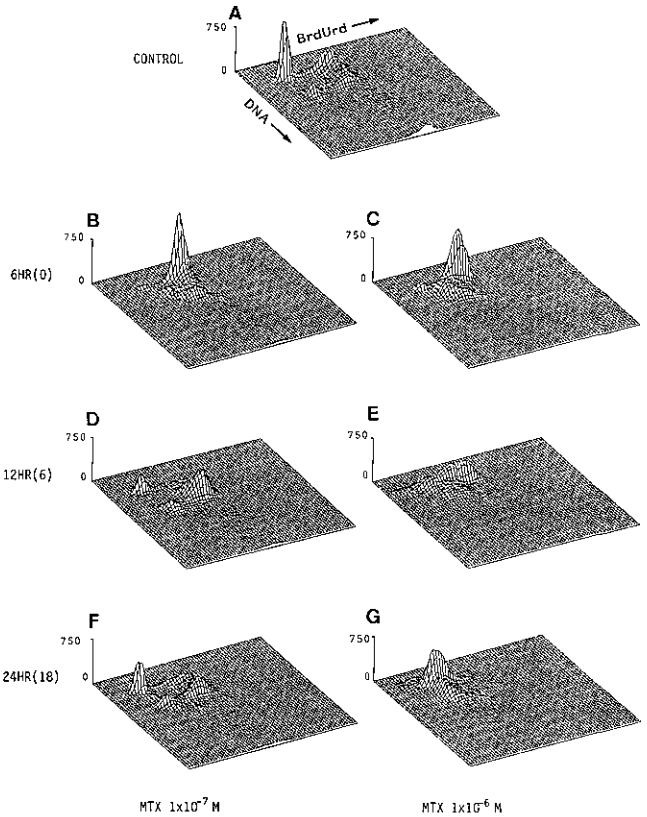

Fig. 4. Three-dimensional histograms for sequential bivariate BrdUrd/DNA distributions of L1210 cells treated with MTX for 6 h. Cells were cultured as described in Fig. 3. Numbers in parentheses indicate the time of the culture in MTX-free medium. Pretreatment control (A); 10^{-7} M (B, D, F); 10^{-6} M (C, E, G). Ordinate, number of cells.

semi solid cultures. In the suspension cultures, 10^{-7} M MTX slightly inhibited cell growth only for the initial 12 h and no decrease in viability of cells was observed throughout the culture period. MTX at 10^{-6} M greatly inhibited cell growth. The cells stopped growing during the first 24 h. The percentages of dead cells at 12 and 24 h were $7.5\pm3.2\%$ and $9.2\pm2.6\%$, respectively. Thereafter, the cells began to proliferate (Fig. 2). Colony-forming activity of L1210 cells was dose-dependently inhibited in the range of concentrations from 10^{-7} M to 10^{-6} M (Table I).

To study the cytokinetic behavior of cells in the presence of MTX and in the period following drug removal, we labeled cells with BrdUrd by method B. MTX at 10^{-7} M caused an accumulation of cells near the G1-S boundary and a decrease of G2M on the DNA histogram at the end of treatment (Fig. 3A). However, most of the cells near the G1-S boundary were actually in early S phase in the bivariate BrdUrd/DNA distribution (Fig. 4B). During the first 6 h of the culture in MTX-free medium the cohort of perturbed cells progressed through S phase in a parasynchronous fashion and some of them entered the G2M and G1 phases (Fig. 4D). At 24 h all the cells had recovered to the pretreatment status, but there was a small population of cells without the capability to incorporate BrdUrd in the region of S phase DNA content

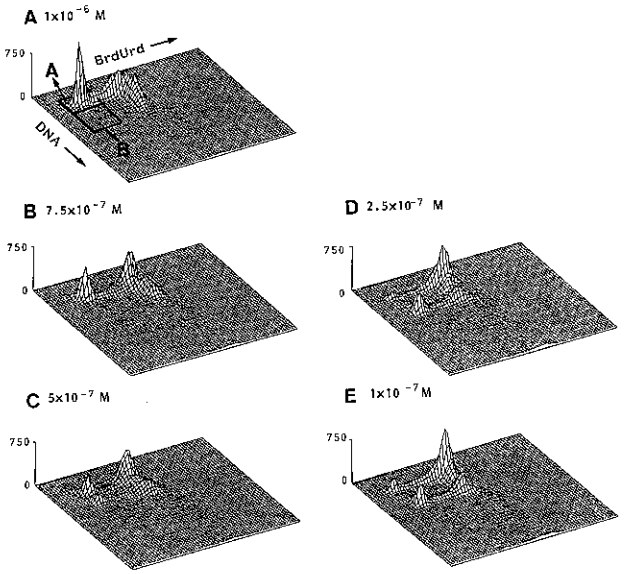

Fig. 5. Dose-related recovery of L1210 cells in the bivariate BrdUrd/DNA content distributions after treatment with MTX. Cells were labeled with BrdUrd for 20 min, washed and exposed to MTX at various concentrations between 10^{-7} *M* and 10^{-6} *M* for 6 h. After treatment they were resuspended in MTX-free medium and collected 6 h later for flow cytometric analysis. Two windows were set in the non-green population (BrdUrd-unlabeled cells): region A represents cells which contain G1 DNA content and region B represent cells which contain DNA content above the G1 level (see Table II). A representative one of three independent experiments is shown. Ordinate, number of cells.

Table II. Proportion of Recovered Cells in Region **B** to Arrested Cells in Region A in the BrdUrd-unlabeled Population after MTX Treatment

MTX concentration during 6 h incubation (*M*)	Phase fractions (%)	
	A	B
1×10^{-6}	92.6	7.4
7.5×10^{-7}	88.8	11.2
5×10^{-7}	65.0	35.0
2.5×10^{-7}	22.8	77.2
1×10^{-7}	34.2	65.8

Regions A and B are shown in Fig. 5. Relative cell number in each region was determined in the bivariate BrdUrd/DNA distributions 6 h after treatment with MTX at various concentrations.

(Fig. 4F). MTX at 10^{-6} *M* showed an apparent G1-S block on the DNA histogram at the end of treatment (Fig. 3B), whereas, in the bivariate distribution, many of the cells with G1 DNA content accumulated in early S phase (Fig. 4C). The cytokinetic recovery of cells in MTX-free medium was not observed in either DNA histograms or bivariate distributions: the perturbed cells were not able to progress through S phase and then lost the capacity to incorporate BrdUrd at 24 h (Fig. 4E, G). Next, to study the dose-related recovery of the S phase-arrested cells, we labeled cells with BrdUrd by method A. In this pulse chase experiment, the cytokinetic recovery of BrdUrd-unlabeled cells was shown separately from that of BrdUrd-labeled cells. Fig. 5 shows the bivariate BrdUrd/DNA distributions of L1210 cells at 6 h after treatment with MTX at various concentrations between 10^{-7} *M* and 10^{-6} *M*. In the three-dimensional histogram at 10^{-6} *M* MTX, there was a large G1 peak, of which many cells appeared to be like the early S phase cells in the three-dimensional histogram obtained by the pulse labeling method (Figs. 4C and 5A). This means that the G1 population, which was in G1 or G2M phase at the start of treatment, progressed to early S phase during MTX treatment, but progression across S phase was still prevented in MTX-free medium. As the concentration of MTX was decreased from 10^{-6} *M* to 2.5×10^{-7} *M*, the fraction of cells leaving the G1 population increased (Fig. 5 A–D). This is quantitatively shown as the ratio of the number of cells with G1 DNA content (region A) to the number of cells with DNA content above the G1 level (region B) in the BrdUrd-negative cells (Table II). At 10^{-7} *M* some cells moved back into G1 through G2M due to the rapid release from the S phase arrest (Fig. 5E, Table II).

***In vivo* cultures** MTX at two doses (10 mg/kg and 50 mg/kg) resulted in a rapid fall in the number of viable tumor cells in the peritoneal fluid. At 24 h after treatment the number of cells was decreased to $66.6\pm10.5\%$ of the pretreatment value at the low dose of MTX and decreased to $25.3\pm8.7\%$ at the high dose of MTX. Thereafter the number of tumor cells began to increase on days 2 and 3, respectively (data not shown).

The recovery kinetics of tumor cells was studied by the pulse labeling method with BrdUrd at various intervals after treatment (Fig. 6). The proliferating S phase cells in the nonperturbed control cells with the capability to incorporate BrdUrd clearly display elevated levels of BrdUrd-linked antibody fluorescence although the proportion of S phase population (about 50%) is less than that (about 65%) of cells in cultures (Fig. 6A). The low dose of MTX caused S phase arrest with a decrease of DNA synthesis and resulted in an accumulation of cells in early S phase within 6 h after treatment (Fig. 6B). At 24 h the perturbed cells were released from the S phase arrest and the cohort of cells capable of incorporating

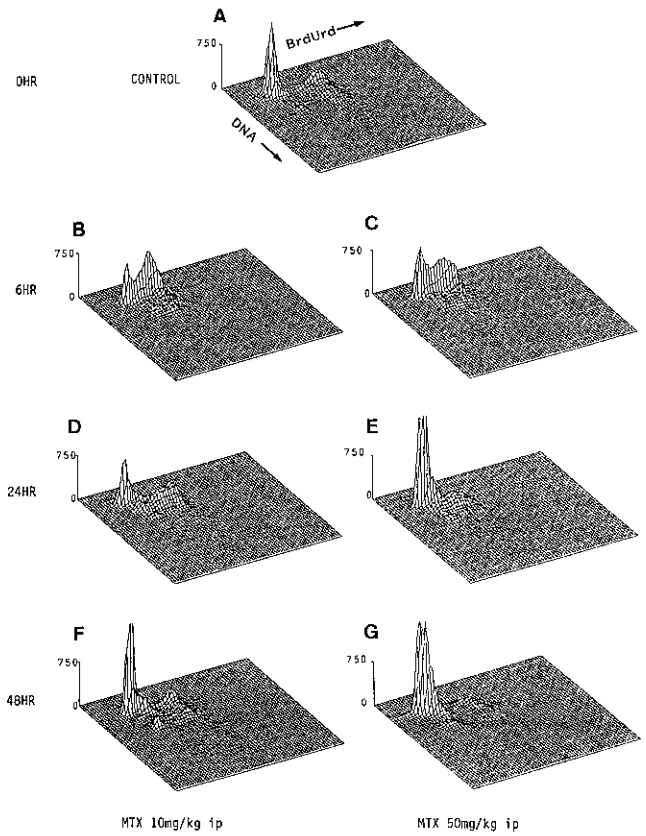

Fig. 6. Sequential bivariate BrdUrd/DNA distributions of ascitic L1210 cells treated with MTX (10 mg/kg or 50 mg/kg). Mice were injected with MTX at time 0 and at selected intervals thereafter were injected with BrdUrd (50 mg/kg). They were killed 30 min later for flow cytometric analysis.

BrdUrd progressed parasynchronously through S phase (Fig. 6D), and back into S phase at 48 h (Fig. 6F). The cells killed by MTX were visible as the subpopulation with S phase DNA content, but without the capability to incorporate BrdUrd at 24 h. The fraction of cells in this drug-killed subpopulation seemed to decrease during the next 24 h due to cell loss and to division of the viable cells. The high dose of MTX also caused S phase arrest at 6 h after treatment (Fig. 6C). In contrast to the small dose of MTX, the release of cells from the S phase arrest was not apparent at 24 h and there was a large peak of G1 population (Fig. 6E). In the next 24 h, although this G1 peak still remained unchanged, a small population of cells capable of incorporating BrdUrd appeared in the S phase region (Fig. 6G). The recovery of cells to the pretreatment status was apparent in the bivariate distributions at 72 h (data not shown). The great increase in the number of cells in the G1 region at 24 h strongly suggests that the majority of cells arrested in early S phase at 6 h were killed by the high dose of MTX. This increase of G1 population also suggests contamination with normal diploid cells, because it was impossible to discriminate normal diploid cells from the tumor cells based on their DNA contents (when the DNA content of normal human diploid cells was defined as 1.0, DNA indices of mouse peripheral lymphocytes and L1210 cells were 0.87 and 0.89, respectively). However, the contamination of normal diploid cells such as mononuclear phagocytes or neutrophils in the degenerated tumor cell population was less than 10% in the cytospin preparations obtained 24 and 48 h after treatment with the high dose of MTX.

DISCUSSION

Many flow cytometric studies of the cytokinetic effects of MTX have revealed that continuous exposure to a high dose of MTX caused a prolonged S phase arrest and/or a complete cessation of the cell cycle,[3, 4, 7-10] but there have been very few studies concerning the cytokinetic recovery of the perturbed cells after the drug removal. In the present study of short-term exposure to MTX, we found a good correlation between the colony-forming activity and cytokinetic behavior of L1210 cells in the period following drug removal. When the dose of MTX during treatment was reduced from 10^{-6} *M* to 10^{-7} *M*, the fraction of cells which recovered from the S phase arrest increased in parallel with the survival fraction of the clonogenic cells. These results showed that the cells accumulated in early S phase lost their clonogenicity when the MTX-induced S phase arrest was prolonged and that the irreversible S phase arrest was a critical cytokinetic event for cytostatic action of MTX. Since the cytokinetic behavior of cells at the end of treatment was almost uniform, it is likely that the dose-related effects of MTX on the tumor clonogenicity can be attributed to efflux of MTX or its metabolites.[11, 12]

The *in vivo* cytokinetic effects of MTX on the ascitic tumor cells were similar to these of *in vitro* cultures. Within 6 h after injection of MTX, many cells accumulated in early S phase and the number of cells in G1 and G2M was decreased. These results showed that MTX prevented S phase progression, but not G1-S transition. Goncharova and Frankfurt examined the cell kinetic effects of MTX on ascites L1210 leukemia by ^{3}H-TdR labeling and concluded that MTX causes G1-S block.[13] On the other hand, Ernst and Killman examined the cell kinetic effects of MTX on human leukemic myeloblasts by ^{3}H-TdR labeling and concluded that transition from G1 to S is not impaired.[14] Our results sup-

ported the latter conclution. In the mice treated with a low dose of MTX, the recovery from S phase arrest was apparently observed within 24 h after treatment, but not for the mice treated with a high dose of MTX. These results are reminiscent of the marrow cell kinetics in patients treated with a high dose MTX and citrovorum factor. The hematopoietic cells recovered from S phase arrest soon after the rescue therapy, but not for the patients who received no rescue.[15-17] In the mice treated with a high dose of MTX, there was a great reduction of tumor cell numbers from 24 to 48 h after MTX treatment. During this period most of the tumor cells were arrested in early S phase and they could not incorporate BrdUrd. These results strongly suggested that the cells arrested in early S phase are committed to die.

In conclusion, our studies showed that MTX prevented the progression of the S phase cells, but G1-S transition was not impaired. As a result, cells accumulated in early S phase, in which they could not initiate DNA replication, and they degenerated when the MTX-induced S phase arrest was prolonged.

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