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EFFECT OF METHIONINE DEPRIVATION ON L5178Y MURINE LEUKEMIA CELLS IN CULTURE. INTERFERENCE WITH THE ANTINEOPLASTIC EFFECT OF METHOTREXATE*

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Abstract—L5178Y cells in culture have a requirement for L-methionine which cannot be satisfied by supplying the components necessary for *de novo* biosynthesis [1]. Methionine deprivation produced a rapid and progressive loss of cell viability (30% by 6 hr; 90% by 24 hr). Cells which remained viable after being deprived of methionine could be rescued by L-methionine supplementation.

L5178Y cells in culture were also highly sensitive to the folate antagonist, methotrexate. Exposure to a concentration of 10^{-6} M for 6 hr resulted in a 95–97% loss of viability. However, if cells were deprived of methionine for 6 hr before exposure to methotrexate, the methotrexate effect was reduced. The cell-killing effect of methotrexate was blocked by longer intervals of methionine deprivation. If the deprived cells were resupplied with the amino acid, the effect of methotrexate was still reduced for at least 12 hr following the methionine resupplementation.

While studying the requirements of L5178Y cells in culture for 5-methyltetrahydrofolate, we made the observation that these cells had an absolute requirement for preformed L-methionine which could not be circumvented by supplying 5-methyltetrahydrofolate, DL-homocysteine, vitamin B_{12}, and transcobalamin II, components necessary for the *de novo* biosynthesis of methionine [1]. Although this finding was in contrast to the earlier work of Mangum *et al.* [2] which showed that the methionine requirement of HeLa and HEp-2 cells in culture could be satisfied by homocysteine and vitamin B_{12}, recent work by Halpern *et al.* [3] and Ashe *et al.* [4] demonstrated that J111, L1210, and Walker 256 cells in culture also had an absolute requirement for preformed L-methionine.

The present investigation was designed to study the effect of methionine deprivation on cell viability, as well as to determine the effect of such a deprivation on methotrexate cell kill. This paper presents the results of these investigations, a preliminary report of which has already been published [5].

MATERIALS AND METHODS

Fischer's medium (10 × concentrate) [6], Fischer's medium lacking L-methionine (10 × concentrate), and horse serum were purchased from the Grand Island Biological Company, Grand Island, New York. L-methionine, methotrexate (amethopterin: 2,4-diamino-10-methyl-pteroylglutamic acid), and Noble agar were purchased from Schwarz–Mann, Orangeburg, New York, Lederle Laboratories, Pearl River, New York, and Difco, Detroit, Michigan, respectively.

Both types of Fischer's medium were diluted, and the horse serum dialyzed as previously described [1]. Solutions of L-methionine and of methotrexate were prepared in resin-distilled water and sterile filtered through 0.22 μm Swinnex (Millipore) filters before use. L5178Y stocks were passaged in complete Fisher's medium, 10% with respect to non-dialyzed horse serum. For experimental purposes, cells in the logarithmic stage of growth were harvested, washed twice with methionine-free Fischer's medium, 10% with respect to dialyzed horse serum, and resuspended in the same medium. The final cell density varied according to the requirements of the experiment. After being dispensed into 15-ml sterile culture tubes, 5 ml per tube, the cells were placed in a 37° warm room and L-methionine and/or methotrexate added as the experiment required. At designated times, duplicate tubes were removed from the warm room, the cell clumps disrupted by pipetting, and the cell count taken with a Model B Coulter Counter (Coulter Electronics, Hialeah, Florida). The duplicate cell counts, which agreed within 10%, were averaged, and the means used to plot the cell growth patterns. In order to determine viability, cells were counted, washed twice with complete Fischer's medium, 10% with respect to non-dialyzed horse serum, recounted, diluted to the proper density with complete Fischer's medium, 15% with respect to nondialyzed horse serum, and implanted in soft agar according to the procedure of Chu and Fischer [7].

RESULTS

Previous work from this laboratory demonstrated that L5178Y cells suspended in Fischer's medium (10% with respect to dialyzed horse serum) containing an optimal concentration of either folic acid, or a

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reduced folate form, but no methionine, did not increase in number during the first 24 hr of the experiment, and underwent a progressive loss of cell number during the subsequent 48 hr period. Since the decrease in cell number in the absence of methionine indicated cell lysis, experiments were designed to determine when cell death began to occur.

The problem was approached by resuspending L5178Y cells harvested while in the logarithmic stage of growth in methionine-free Fischer's medium (10% with respect to dialyzed horse serum), and supplementing the medium with sterile L-methionine (100 mg/l final concentration). The results are shown in Fig. 1. In the absence of methionine, there was a progressive decrease in cell number after 18 hr. Cells supplemented with L-methionine at zero time underwent 6 divisions, with an average division time of 12 hr. Cells supplemented with L-methionine after 12 hr of methionine deprivation immediately entered a 12-hr division cycle. Supplementation at 18 hr appeared to 'rescue' the cells, and permit a slow resumption of normal growth, i.e., the first cell doubling required 28 hr and the second 18 hr, before a 12 hr division time was again evident. L-Methionine at 24 hr only stabilized the cell number, inasmuch as no substantial increase or decrease was evident during the remainder of the experiment. Later additions of L-methionine (30 hr: not shown; 36 hr: shown) failed to prevent cell loss.

Although these data indicated that methionine deprivation for 24 hr was lethal to L5178Y cells, they did not show whether there was cell death prior to 24 hr. To answer this, cells were deprived of L-methionine and cell viability determined every 6 hr by soft agar cloning. The results are shown in Fig. 2. Also

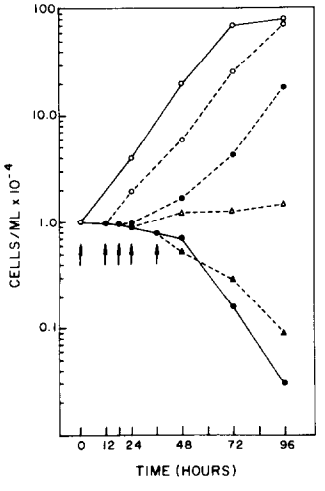

Fig. 1. Growth of L5187Y cells in methionine-free and methionine-supplemented medium. L5178Y cells were harvested, washed, and resuspended in methionine-free Fischer's medium containing 10% dialyzed horse serum. Cells received L-methionine (100 mg/l) at the times indicated by the arrows. Increase or decrease in cell number was measured for 96 hr: cells in methionine-free medium (●——●); cells supplemented with L-methionine at 0 time (○——○); cells supplemented with L-methionine at 12 hr (○- - -○); cells supplemented with L-methionine at 18 hr (●- - -●); cells supplemented with L-methionine at 24 hr (△- - -△); cells supplemented with L-methionine at 36 hr (▲ ▲).

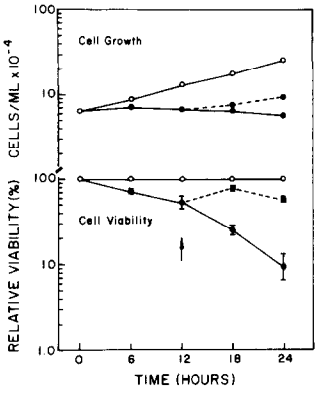

Fig. 2. Cell-growth and cell viability in methionine-free and methionine-supplemented medium. L5178Y cells were harvested, washed, and resuspended in methionine-free Fischer's medium, 10% with respect to dialyzed horse serum. Certain of the cells received L-methionine (100 mg/l) at 0 time or at 12 hr. The top shows a representative growth profile reproduced in a series of three experiments. The bottom shows cell viability relative to a control cloning efficiency of 70–80% and corrected to 100%. Vertical bars indicate standard errors of the geometric means, calculated from a series of three experiments. Cells in methionine-free medium (●——●). Cells supplemented with L-methionine at 0 time (○- - -○). Cells supplemented with L-methionine after 12 hr in methionine-free medium (●- - -●).

shown in Fig. 2 is the cell viability observed when cells were supplemented with L-methionine after 12 hr deprivation. The cloning efficiency of control cells (L-methionine supplementation at zero time) varied between 70 and 80%. This was in agreement with the cloning efficiency of exponentially growing L5178Y cells reported by Hryniuk *et al.* [8]. Taking this control efficiency as 100%, all experimental data were corrected and expressed as relative cloning efficiency. Methionine deprivation produced a significant loss of viability by 6 hr (70% relative viability: $P < 0.05$). Loss of cell viability was progressive, with only 10% of the cells in the methionine-free medium remaining viable at 24 hr ($P < 0.001$). Methionine supplementation at 12 hr prevented a further loss of viability. The difference in viability between 12-hr-supplemented cells and cells remaining in methionine-free medium was significant ($P < 0.05$ at 18 hr; $P < 0.001$ at 24 hr).

Exponentially growing L5178Y cells have been shown to be highly responsive to the lethal effect of methotrexate [8–10], but this effect is antagonized by asparagine deprivation [11]. In order to determine whether methionine deprivation would antagonize methotrexate cell kill, a series of experiments were performed. Fig. 3 shows that L5178Y cells in the logarithmic stage of growth (Fischer's medium, 10% dialyzed horse serum, 100 mg/l L-methionine) were highly sensitive to methotrexate. A 6-hr exposure to 10^{-6} M resulted in a 95–97% cell kill ($P < 0.001$). However, if the cells were deprived of methionine and then exposed to methotrexate, the results shown in Fig. 4 were obtained.

Exposure of cells at zero time to both methionine deprivation and 10^{-6} M methotrexate resulted in a cell kill (98%) identical to that produced by a 6-hr

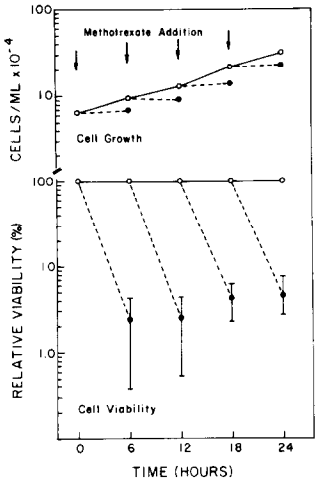

Fig. 3. Sensitivity of L5178Y cells to methotrexate. L5178Y cells were harvested, washed, and resuspended in methionine-free Fischer's medium, 10% with respect to dialyzed horse serum. At 0 time, the medium was supplemented with 100 mg/l L-methionine. At 0 time, or at 6-hr intervals, cells were exposed to 10^{-6} M methotrexate for 6 hr. After methotrexate, cells were counted, washed, and cloned to determine remaining viability. Cells not exposed to methotrexate were also cloned at 6-hr intervals. The top shows a representative growth profile reproduced in a series of three experiments: cell growth without methotrexate (○——○); cell growth with methotrexate (●- - -●). Arrows indicate the addition of methotrexate. The bottom shows cell viability after 10^{-6} M methotrexate for 6 hr (●) relative to an untreated cell cloning efficiency of 70–80% corrected to 100% (○). For illustrative purposes, each point which represents viability after methotrexate is connected by a dashed line to the respective point representing viability before drug. The dashed lines do not mean that the loss of viability was necessarily linear. The vertical bars indicate the standard errors of the geometric means, calculated from a series of 3 experiments.

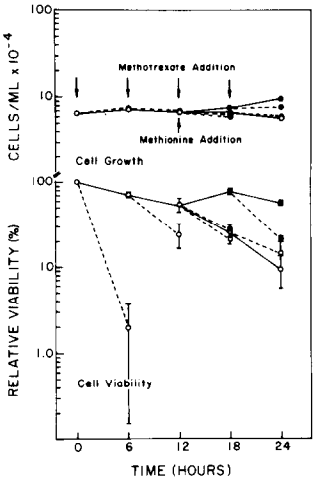

Fig. 4. Sensitivity of L5178Y cells to methotrexate after growth in methionine-free and methionine-supplemented Fischer's medium. L5178Y cells were harvested, washed, and resuspended in methionine-free Fischer's medium, 10% with respect to dialyzed horse serum. At 0 time, and at 6-hr intervals, certain of the cells were exposed to 10^{-6} M methotrexate for 6 hr. At 12 hr, a portion of the cells not yet exposed to methotrexate was supplemented with sterile L-methionine (100 mg/l). Certain of the supplemented cells were exposed to methotrexate at the time of methionine supplementation, or 6 hr later. After methotrexate, cells were counted, washed, and cloned to determine viability. Cells in methionine-free or 12-hr-supplemented medium not exposed to methotrexate were also cloned at 6-hr intervals. The top shows a representative growth profile reproduced in a series of three experiments. Cell growth in the absence of L-methionine (○——○); growth after L-methionine addition at 12 hr (indicated by the lower arrow) (●——●). Addition of methotrexate is indicated by the upper arrows. Cell growth in methionine-free medium during a 6-hr exposure to 10^{-6} M methotrexate (○ ○); cell growth in medium supplemented with L-methionine at 12 hr during a 6-hr exposure to 10^{-6} M methotrexate (●- - -●). The bottom shows cell viability relative to a control (cells in methionine-replete medium) cloning efficiency of 70–80%, corrected to 100% (shown in Figs 2 and 3, and discussed in Results). Loss of cell viability during methionine deprivation (○——○). Change in cell viability after methionine supplementation (●——●). For illustrative purposes, cell viability after exposure of cells in methionine-free (○) or 12-hr methionine-supplemented (●) medium to 10^{-6} M methotrexate for 6 hr is indicated by points connected through dashed lines to the respective points representing viability before drug. The dashed lines do not mean that the loss of viability was necessarily linear. The vertical bars indicate the standard errors of the geometric means, calculated from a series of 3 experiments.

exposure to methotrexate in the presence of methionine (97%; see Fig. 3). However, if cells were deprived of methionine for 6 hr (0–6 hr) before being exposed to methotrexate (from 6–12 hr in the continuing absence of methionine), the methotrexate cell kill at 12 hr was significantly reduced from 97% observed in control cells (see Fig. 3) to 75% ($P < 0.05$). If cells were deprived of methionine for 12 or 18 hr before being exposed to 10^{-6} M methotrexate for 6 hr (12–18 hr or 18–24 hr in the continuing absence of the amino acid), the cell kill from the combination of methotrexate and methionine deprivation observed at 18 hr (78%) or 24 hr (85%) was not significantly different ($P > 0.1$) from that produced at 18 hr or 24 hr by methionine deprivation alone (75% and 90%, respectively). Therefore, preexposure of L5178Y cells to methionine deprivation significantly reduced or blocked the cell-killing effect of methotrexate.

Sensitivity of L5178Y cells to methotrexate after resupplementation with L-methionine is also shown in Fig. 4. When cells deprived of methionine for 12 hr were resupplied with the amino acid and simultaneously exposed to methotrexate (from 12 to 18 hr), the cell kill produced by the drug at 18 hr (72%) was not significantly different from that produced in cells still in the methionine-deficient state (78%). However, the viability of the cells resupplemented with L-methionine but not exposed to methotrexate was significantly increased (76%), as compared to non-supplemented cells (25%: $P < 0.05$). If the resupplemented cells were allowed 6 hr in the presence of L-methionine (from 12 to 18 hr) before being exposed to methotrexate (from 18 to 24 hr), the methotrexate cell kill at 24 hr still remained at 75%. Apparently, methionine deprivation for 6–12 hr resulted in approximately 20–25% of the L5178Y cells becoming resistant to the toxic effects of methotrexate. This resistance remained evident for at least 6–12 hr after the cells were resupplied with the amino acid.

DISCUSSION

Halpern *et al.* [3], working with J111 human monocytic leukemia cells, L1210 murine lymphatic leukemia cells and Walker 256 rat-breast carcinoma cells in culture demonstrated that in the absence of preformed L-methionine there was no cell growth, and that viability of L1210 cells was lost by 20 days. The L5178Y line of murine leukemia cells in culture also showed a lack of growth in the absence of the amino acid, but was much more sensitive to the deprivation. Cell death, as indicated by clonability, commenced within 6 hr after exponentially growing L5178Y cells were resuspended in methionine-free medium, was progressive, and could be reversed. The mechanism of cell death resulting from the methionine deprivation is as yet unknown, but the reason for the requirement for L-methionine may reside in the findings of Ashe *et al.* [4]. These investigators showed that certain neoplastic cells in culture had a requirement for L-methionine which could not be satisfied by supplying the cells with DL-homocysteine, vitamin B_{12}, and folic acid. This was in contrast to normal adult rat and human fibroblasts which thrived under these same conditions [3]. The neoplastic cells were found to have a lower percentage of the 5-methyltetrahydrofolate–homocysteine transmethylase enzyme as active holoenzyme than did the fibroblast cells. In addition, certain of the tumor cells may also have had less total amount of enzyme than did the normal cells.

These data encourage the use of methionine deprivation as a possible chemotherapeutic approach. In relation to L5178Y cells, methionine deprivation produced a more rapid cell kill than when they were grown in the absence of L-asparagine, but a less rapid cell kill than when they were treated with L-asparaginase [11]. L1210 cells in culture, although insensitive to L-asparaginase [12], show a dependence on the presence of preformed L-methionine [3]. P815 cells in culture are sensitive to L-methionine-α-deamino-γ-mercaptomethanelyase (L-methioninase) from *C. sporogenes*, and some regression of the Walker 256 tumor in rats was obtained through use of the same enzyme [13, 14]. Another finding that may make methionine deprivation therapy useful is the finding of Kamely *et al.* [15]. These investigators found that methionine deprivation and homocysteine supplementation derepressed the synthesis of the 5-methyltetrahydrofolate–homocysteine transmethylase in normal human fibroblasts in culture. If neoplastic cells do in fact have a defect in either the synthesis of this enzyme and/or its conversion to holoenzyme, a form of rescue of normal cells may be readily available. These data on growth requirements, viability, and enzymatic parameters, however, are from cells in culture, and further study is required to determine whether malignant cells have a methionine requirement *in vivo* and whether a biochemical difference does exist between malignant and normal tissue, especially since the predominant folate form in serum is considered to be 5-methyltetrahydrofolate [18].

The L5178Y cell is extremely sensitive to the folate antagonist, methotrexate, suffering both a purineless and a thymineless death [9, 10, 16, 17]. The reason for the interference of methotrexate cell kill by methionine deprivation is not known. Concommitant exposure of the cells to both methionine deprivation and methotrexate produced a cell death comparable to that seen with methotrexate alone. However, if the cells were preincubated in the absence of L-methionine, the cytotoxic effect of methotrexate was reduced until cell kill was no different from that produced by the methionine deprivation alone. Also, sensitivity to methotrexate remained diminished for 6–12 hr after the cells were resupplemented with L-methionine. A possible explanation for the reduced effect is an altered uptake of methotrexate. Gawthorne and Smith [19] showed that liver slices from sheep subjected to prolonged methionine deficiency had a lowered ability to transport methotrexate. In addition, recent work by Nahas and Capizzi [12] demonstrated that L-asparaginase treatment of L5178Y cells decreased the uptake of methotrexate. Another possibility is that methionine deprivation, like asparagine deprivation, decreases protein synthesis, thus decreasing the purineless and thymineless death produced by methotrexate in these cells [10, 16, 17].

Based on these data, a chemotherapeutic approach employing concommitant use of methotrexate and methionine deprivation probably would not be useful. However, methionine deprivation at an appropriate interval after methotrexate treatment similar to the methotrexate–asparaginase sequence employed by Capizzi [20] may also result in an improved therapeutic index, and deserves further investigation.

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