Role of Amino Acid Depletion in Combined Treatment of Neoplastic Cells with Methotrexate and L-Asparaginase

Bożena Chłopkiewicz and Jadwiga Koziorowska
Department of Vitamins, Institute for Drug Research and Control, 30/34 Chelmska, Warsaw, Poland

SUMMARY

The effect of pretreatment with L-asparaginase on the cytotoxicity of methotrexate (MTX) was studied in L5178Y murine leukemic cells and L-929 cells grown in vitro. The results were correlated with the effects of L-asparaginase on DNA synthesis. At low concentrations of the enzyme, pretreatment of cells enhanced the effect of MTX; at higher concentrations, it prevented the effect of MTX. Elimination of the MTX action was demonstrated also in L5178Y cells growing in the medium lacking L-asparagine. L-Asparaginase readdition resulted in enhancement of MTX cytotoxicity. Pulse-labeling experiments demonstrated that in every case elimination of MTX action was related to a diminution of the fraction of cells in the phase of DNA synthesis. In contrast, enhancement of the effect of MTX was related to an increase in the fraction of cells in the DNA synthesis phase. A similar parallelism of effects occurred in L-929 cells when pretreatment with L-asparaginase was followed by washing the cells and culturing them in medium without the enzyme.

INTRODUCTION

Combined treatment with 2 drugs has often been found to have therapeutic advantages in clinical use. The efficacy of MTX as a single drug is disappointing, except in treatment of choriocarcinoma. Several experimental attempts were made to potentiate its efficacy with other chemotherapeutic agents (11), among them L-asparaginase. The results with L-asparaginase are controversial. Capizzi et al. (7) observed that, when L5178Y leukemic mice were treated with MTX and L-asparaginase, the therapeutic effect was synergism or antagonism, depending upon the sequence of drug administration. Connors and Jones (8) showed that administration of both drugs simultaneously to mice bearing R1 lymphoma was less effective than when one drug was followed by the other. Synergism or antagonism between these 2 drugs followed their use in the treatment of BALB/c mice that had P1798 lymphosarcoma (14). The parameters of schedule dependency in chemotherapy with L-asparaginase and MTX were investigated in more detail by Vadlamudi et al. (23). Some of their results are contradictory to those of Connors and Jones (8).

The mechanisms of diverse interactions between these drugs have not been explained satisfactorily. The possibility that L-asparaginase acts as a transport inhibitor (16) seems to be excluded (25). The antagonism between L-asparaginase and MTX is thought by some authors to be the result of inhibition of protein synthesis by the enzyme (6, 25).

The current experiments were conducted on the assumption that a better understanding of the mechanism involved might aid in establishing the schedule of administration of these drugs in combination therapy. MTX is known to affect cells in G phase (3). It was therefore hypothesized that synergism and antagonism between MTX and L-asparaginase result from changes in the number of cells passing in a time unit through the drug-sensitive phase.

MATERIALS AND METHODS

L5178Y cells were grown in vitro in suspension in Fischer's medium supplemented with 8% bovine serum and maintained in the logarithmic growth phase by periodic dilution. Cultures used for drug studies were started with approximately 18,000 cells/ml and allowed to grow to a population density of 10^6 cells/ml. In some experiments Fischer's medium lacking L-asparagine was used. In those cases the medium was supplemented with 2% bovine serum to diminish the possible contribution of L-asparaginase from the serum. For cell counts aliquots were drawn and cells were counted with a hemocytometer. Viability of cells was established by the trypan blue dye exclusion test.

L-929 cells were grown in flasks as monolayers in growth medium consisting of 75% Medium 199, 15% Hanks' balanced salt solution supplemented with 0.5% lactalbumin hydrolysate, and 10% pooled calf serum. The cells were passaged by trypsinization. L-Asparaginase was not included in the growth medium. For drug studies cultures were grown in tubes; for cell counts the tubes were treated with 0.25% trypsin.

L-Asparaginase from Escherichia coli (Bayer, Leverkusen, Federal Republic of Germany) and sodium MTX (Lederle Laboratories Division of Cyanamid Company, Wayne, N. J.) were used in all experiments.

Autoradiographic studies included pulse-labeling experiments. Pulse labeling was effected by exposing cells to [3H]thymidine for 15 min. The radioactive compound was purchased from the Radiochemical Centre, Amersham, Buckinghamshire, England. Cultures received [3H]thymidine dissolved in 0.5 ml of the growth medium to a final concentration of 0.5 μCi/ml. After exposure to the labeled precursor, the L5178Y cells were centrifuged at 800 rpm, washed twice with 0.8% NaCl solution, fixed in...
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ethanol:acetic acid (3:1), and air dried on glass slides. The slides were then coated with Kodak NTB 3 emulsion, exposed, developed, and stained with hematoxylin and eosin. From the L-929 cultures the medium containing [3H]thymidine was removed, and the cells on coverslips in Leighton tubes were gently washed twice and fixed in situ. The coverslips were then attached to a microscope slide, and the cultures were run as in the case of L5178Y cells. The percentage of labeled cells was estimated by counting about 1000 nuclei on each autoradiogram. In some experiments the number of grains over nuclei was counted in 50 cells and averaged to 10-cell units.

RESULTS

Killing of Cells Pretreated with L-Asparaginase. L5178Y cells in the exponential phase of growth were exposed to L-asparaginase alone (0.1 or 0.03 IU/ml) or to varying concentrations of MTX given alone or after L-asparaginase. In the combination treatment the cells were pretreated with L-asparaginase for 4 hr and then exposed to MTX in the presence of L-asparaginase for 44 hr. The findings of these experiments were interpreted as showing the effect of L-asparaginase on the action of MTX. They showed that proliferating L5178Y cell populations exposed successively to L-asparaginase and MTX exhibited 3 distinct types of response. Depending on the concentration of the enzyme, the pretreatment of cells enhanced, eliminated, or did not influence the effect of MTX. The results of these experiments are presented in Chart 1. L-Asparaginase in the concentration of 0.03 IU/ml either enhanced or did not influence the action of MTX. In contrast, when the cells were preincubated with the lower concentration of L-asparaginase (0.1 IU/ml) the effect of L-asparaginase was quite lost, since the cell count was either the same as that observed in cultures treated with L-asparaginase alone or the same as that in cultures treated with MTX alone (Chart 2).

In an attempt to determine whether these effects are related to the depletion of L-asparagine by the action of L-asparaginase, cells in the exponential phase of growth were spun down, washed once with 0.8% NaCl solution, and then resuspended in Fischer's medium supplemented with 2% serum and lacking L-asparagine. Four hr later these cells were exposed to MTX. At 44 hr after the addition of MTX to the cultures, aliquots were withdrawn and assayed for viable cells per ml. At a $10^{-8}$ M concentration this drug had no effect (Chart 3). Thus, under these experimental conditions depletion of L-asparagine resulted in antagonism. Similar results were obtained with L-asparaginase at the concentration of 0.1 IU/ml (cf. Chart 2).

In parallel experiments the effect of asparagine readdition was studied. For this purpose log L5178Y cells were processed as described above and fed either asparagine-free medium or medium containing L-asparaginase (0.1 IU/ml) for 4 hr. After this time MTX with L-asparagine (final L-asparagine concentration of 2.4 mg/100 ml) was introduced into the media of both kinds of cultures. As shown in Chart 3, the addition of L-asparagine made the cytotoxicity of MTX possible.

Autoradiography. In several experimental conditions pulse-labeling experiments with tritiated thymidine were performed to evaluate the influence of L-asparaginase treatment and of L-asparagine depletion and readdition on DNA synthesis of logarithmically growing L5178Y cells. The results of these experiments are presented in Charts 4 and 5.

As seen in Chart 4 the fraction of DNA-synthesizing cells in cultures treated with L-asparaginase in a concentration of 0.1 IU/ml decreased gradually, reaching after 4 hr about 50% of the mean control value, and steadily continued to decrease for several hr. It should be kept in mind that under such conditions (cf. Chart 2) the effect of MTX used in lower concentrations was eliminated. When L-asparaginase was added to parallel cultures treated for 4 hr with L-asparaginase in the same concentration, the fraction of DNA-synthesizing cells rose rapidly. Asparagine addition together with MTX made the killing action of the latter possible (cf. Chart 3). As shown in the same chart, after 4 hr of treatment with L-asparaginase the labeling index was in a

![Chart 1. Effect of pretreatment with L-asparaginase on the efficacy of cell killing by MTX. L5178Y cells in the exponential phase of growth were pretreated for 4 hr with L-asparaginase (0.03 IU/ml). MTX was added and the cultures were incubated at 37° C for 44 hr. Cell inhibition was determined by counting viable cells, and all data were normalized to 100% of the control. Bars, mean values of 3 experiments with 3 replicates: lines connecting bars, statistically significant difference between these values (p < 0.01); L-ase, L-asparaginase.](chart1)
Chart 2. Effect of pretreatment with L-asparaginase on the efficacy of cell killing by MTX. L5178Y cells in the exponential phase of growth were pretreated for 4 hr with L-asparaginase (0.1 IU/ml). MTX was added and the cultures were incubated at 37° for 44 hr. Cell inhibition was determined by counting viable cells, and all data were normalized to 100% of the control. Bars, mean values of 3 experiments with 3 replicates; L-ase, L-asparaginase.

Chart 3. Effect of L-asparaginase deprivation and readdition on the killing efficacy of MTX. Logarithmically growing L5178Y cells were processed as described in the text. The data are normalized to 100% of the control (Fischer's medium containing 8% serum). Bars connected by lines, values differing significantly (p < 0.01); L-ase, L-asparaginase.

concentration of 0.03 IU/ml higher than in the control. The grain count over the nuclei of cultures treated with L-asparaginase in this concentration gradually diminished (from about 300 grains at the beginning of the experiment to about 150 grains/10-cell unit after 4 hr of treatment). Since the reduced number of grains under identical conditions of pulse labeling and time of exposure may indicate slowing down of the rate of DNA synthesis, the increased pool of cells in the S phase might be the expression of inhibition of DNA synthesis.

In other experimental combinations pulse labeling with tritiated thymidine was used in order to study changes in the fraction of nuclei in S phase during asparagine deprivation and after addition of this amino acid. When logarithmically growing cultures were exposed to L-asparaginase (0.1 or 0.03 IU/ml), and pulse-labeling experiments were performed as described in “Materials and Methods.” At 4 hr L-asparaginase was added to some cultures growing with L-asparaginase (0.1 IU/ml) to a final L-asparaginase concentration of 2.4 mg/100 ml. Points, mean values of 3 different experiments.

Chart 4. Effect of L-asparaginase at different concentrations and of L-asparaginase addition on DNA synthesis of L5178Y cells. Logarithmically growing cultures were exposed to L-asparaginase (0.1 or 0.03 IU/ml), and pulse-labeling experiments were performed as described in “Materials and Methods.” At 4 hr L-asparaginase was added to some cultures growing with L-asparaginase (0.1 IU/ml) to a final L-asparaginase concentration of 2.4 mg/100 ml. Points, mean values of 3 different experiments.
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concentrations of the enzyme. In this context we considered it worthwhile to determine whether L-asparaginase influences the action of MTX on these cells the same way that it does the action of MTX on sensitive L5178Y cells.

The treatment of L-929 cells with both drugs was performed at different time intervals after seeding and was scheduled as given for L5178Y cells. The pertinent data of these experiments are presented in Chart 6. The pretreatment of L-929 cells with L-asparaginase 4 hr after seeding, when most of the cells are in the G1 phase (1), resulted in either enhancement or elimination of the effect of MTX, depending on the concentration of the enzyme. The results of pulse-labeling experiments are presented in Chart 7. The curve of DNA-synthesizing cells began to rise in control cells 4 hr after seeding and reached a maximum after 14 hr. The peak of labeled cells in cultures treated 4 hr post-seeding with L-asparaginase (1 IU/ml) was about 20% above the maximum value for control cultures, indicating a greater degree of synchrony than in the control.

In contrast, in the course of 14 hr after seeding, only a few cells treated with an L-asparaginase concentration of 100 IU/ml had incorporated [3H]thymidine, probably owing to the block of most of the cells of the population in G1 phase.

As shown in Chart 8 removing the medium containing L-asparaginase and supplying fresh medium to the cells resulted in unblocking the inhibition of DNA synthesis. Since the growth medium of L-929 cells does not contain L-asparagine, these results give indirect evidence that the effects on DNA synthesis in these cells are due to factors other than depletion of L-asparagine.

DISCUSSION

The effects described in this paper were synergism and antagonism between L-asparaginase and MTX, when treat-growing cells in medium containing 2% serum and lacking L-asparagine were exposed to a 15-min pulse of tritiated thymidine, about 50% of the nuclei became labeled. In asparagine-deficient medium containing 2% serum, the fraction of S-phase cells decreased as compared with control cells, so that in the time interval between the 3rd and the 5th hr the fraction of DNA-synthesizing cells was about 50% of the control. After the addition of L-asparagine, the fraction of labeled cells rose rapidly reaching a level above the range of values for the control cultures (Chart 5).

In each of the experimental conditions, therefore, the lowered labeling index, due to L-asparaginase action or to L-asparagine depletion, corresponded to an elimination of MTX cytotoxicity in lower concentrations. Enhancement of the effect of MTX by L-asparaginase at low concentration or by L-asparagine readdition to the asparagine-depleted medium, notwithstanding its cause, was related to the increase in the pool of cells in the phase of DNA synthesis.

Interaction of L-Asparaginase and MTX in L-929 Cells. In preliminary experiments it was established that growth of L-929 cells is arrested by L-asparaginase in very high concentrations of the enzyme. In this context we considered it worthwhile to determine whether L-asparaginase influences the action of MTX on these cells the same way that it does the action of MTX on sensitive L5178Y cells.

The treatment of L-929 cells with both drugs was performed at different time intervals after seeding and was scheduled as given for L5178Y cells. The pertinent data of these experiments are presented in Chart 6. The pretreatment of L-929 cells with L-asparaginase 4 hr after seeding, when most of the cells are in the G1 phase (1), resulted in either enhancement or elimination of the effect of MTX, depending on the concentration of the enzyme. The results of pulse-labeling experiments are presented in Chart 7. The curve of DNA-synthesizing cells began to rise in control cells 4 hr after seeding and reached a maximum after 14 hr. The peak of labeled cells in cultures treated 4 hr post-seeding with L-asparaginase (1 IU/ml) was about 20% above the maximum value for control cultures, indicating a greater degree of synchrony than in the control.

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DISCUSSION

The effects described in this paper were synergism and antagonism between L-asparaginase and MTX, when treat-
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Chart 7. Incorporation of tritiated thymidine into DNA during L-asparaginase treatment. L-929 cultures were exposed to various concentrations of L-asparaginase (1 and 100 IU/ml) and processed as described in "Materials and Methods." Cells were exposed to L-asparaginase for 10 hr.

A possible interpretation of all these types of action is proposed. MTX has been found to kill cells selectively in the S phase (3). The action of L-asparaginase on sensitive cells is inhibition primarily of protein synthesis and secondarily of DNA synthesis (4, 10). It was demonstrated in the present experiments that the inhibition of DNA synthesis might be expressed, depending on enzyme concentration, as a decrease or increase of the number of cells in DNA phase. An increase, resulting probably from the prolongation of the S phase, enhances the cytotoxicity of MTX. In contrast, a decrease in the fraction of S-phase cells eliminates the cytotoxicity of MTX. These effects in L5178Y cells seem to result from L-asparaginase depletion.

It is known that the principal action of MTX is inhibition of dihydrofolate conversion to tetrahydrofolate, catalyzed by dihydrofolate reductase (17, 20). The synthesis of thymidylate is particularly sensitive to inhibition by MTX (19). However, MTX interferes with the biosynthesis of dTMP and purines (2, 24) and various other anabolic and catabolic processes (12). Maximum population reduction by higher concentrations of MTX may be equally due to activity other than inhibition of thymidylate synthesis. This other mode of action of MTX in high concentrations may be independent of the fraction of the cells in S phase and therefore independent of the action of L-asparaginase.

It has been reported that in cells starved of essential amino acids, inhibition of DNA synthesis occurs (5, 13). L5178Y cells are auxotrophic for L-asparagine. As shown previously for other sensitive cells (22), it was demonstrated in the present experiments that depletion and readdition of L-asparagine are similarly regulatory mechanisms of DNA synthesis for L5178Y cells. Synchronization of cells resulting from DNA synthesis stimulation after L-asparagine addition is therefore the mechanism responsible for the enhancement of the MTX action.

Although similar dose-dependent antagonism and synergism were observed for L-929 cells, the mechanism of the antagonistic effect of L-asparaginase might be a depletion of L-glutamine. Bacterial preparations of L-asparaginase exhibit activity with L-glutamine as substrate (15, 18). L-Asparagine is not required by L-cells thus far studied in culture (9). However, these cells cause a marked decrease in the glutamine content of the medium (21). The regulatory function of L-glutamine may be expressed by inhibition and induction of DNA synthesis (13).

The results of this study suggest that some of the divergent findings in the literature are due not only to different schedules of administration of L-asparaginase and MTX but also to differences in drug doses and in the regulating role of L-asparagine and eventually L-glutamine in DNA synthesis.

There is a good general correlation between the mode of action of antineoplastic drugs on cells grown in vitro, in animals, and in human neoplasia. It seems, therefore, worth calling attention to the results with L5178Y cells, sensitive to L-asparaginase in vivo and in vitro, which indicate that synergism was demonstrated exclusively at low concentrations of the enzyme and of antifolate.
REFERENCES

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