Reversal of the in Vitro Methotrexate Suppression of Cell-mediated Immune Response by Folinic Acid and Thymidine plus Hypoxanthine

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ABSTRACT

The development of a primary complement-independent cellular cytotoxic immune response in culture by C57BL/6J spleen cells stimulated with X-irradiated allogeneic P815 tumor cells was inhibited more than 50% in the presence of 1.5 \times 10^{-9} \text{ m} \text{ methotrexate}. This immunosuppression by methotrexate was time and dose dependent. Equimolar folic acid administered at either -4, 0, +4, or +24 hr relative to 1.5 \times 10^{-9} \text{ m} \text{ methotrexate reversed immunosuppression by more than 50%}. Increased folic acid concentration (5 to 10-fold) completely restored the immune response only if added 4 hr prior to methotrexate. Thymidine plus hypoxanthine (100 \text{ \mu M} each) when present throughout the 4-day culture period gave total reversal of immunosuppression. The reversal was also obtained with hypoxanthine alone and was dose dependent. These results indicate that reversal of the methotrexate-induced impairment of cellular immune function depends on several parameters including the concentration of methotrexate and of the reversing agents as well as the time of exposure of relevant target cells to these agents.

INTRODUCTION

The use of MTX\(^4\) as an anticancer agent has been limited by the toxicity of this drug towards certain proliferating tissues. MTX also has immunosuppressive activity (1, 15).

The observation by Goldin et al. (6) that delayed administration of CF reduces aminopterin toxicity and favors host recovery led to investigations (2, 5, 14, 22) of CF rescue of MTX toxicity aimed at achieving higher selectivity of drug action. Studies by Hakala and Taylor (8, 9) showed that dThd and a source of preformed purine reverse the toxicity of MTX to certain cell lines in culture. The report by Tattersall et al. (23) that dThd could produce reversal of MTX toxicity in vivo while maintaining antitumor effectiveness was followed by further investigations of the ability of this agent to increase the therapeutic efficacy of MTX (20, 24).

As shown herein, the reversal of MTX-induced immunosuppression in culture is time and dose dependent. Both types of reversing treatments, namely, CF and dThd plus Hx, are effective in restoring the development of spleen cytokotoxic effector cells against allogeneic P815 mastocytes. The characteristics of the reversal of MTX effects by these agents are described.

MATERIALS AND METHODS

Mice

C57BL/6J mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. DBA/2J mice were obtained from the Roswell Park Memorial Institute breeding colony. All mice were females between the ages of 9 and 12 weeks.

Tumors

The P815 mastocytoma was maintained in the ascites form by i.p. passage of 5 \times 10^6 cells every 4 days into syngeneic DBA/2J mice. Mice were killed by cervical dislocation, and ascitic cells were washed 3 times in serum-free Roswell Park Memorial Institute (RPMI) Medium 1640. Cells were subsequently used for in vitro stimulation or as targets in the cytotoxicity assay at appropriate concentrations.

Other Materials

RPMI Medium 1640, cell culture nutrients, antibiotics, dialyzed fetal calf serum, and CF were obtained from Grand Island Biological Co., Grand Island, N. Y. MTX was obtained from ICN Nutritional Biochemicals Div., Cleveland, Ohio, and purified by column chromatography according to the method of Zakrzewski and Sansone (26). MTX was dissolved in RPMI Medium 1640 with a small amount of ammonium acetate just before use. dThd, Hx, adenosine, deoxyadenosine, and allo-purinol were obtained from Sigma Chemical Co., St. Louis, Mo. Compounds were added to the initial tissue culture which was maintained for 4 days except as otherwise indicated. Since the CF used as a DL mixture, concentrations were doubled to correct for the content of the active form.

Primary Sensitization In Vitro

The in vitro primary sensitization of murine spleen lymphocytes was carried out by the method of Cerottini et al. (4) as modified in this laboratory (16). All reagents and conditions were as reported (16) except that 2 \times 10^7 C57BL/6J spleen cells in 1 ml of "set up" medium were incubated with 2 \times 10^5 X-irradiated P815 tumor cells. For washout studies, the cells were collected at the indicated times, washed 3 times by centrifugation, and then returned in 1 ml of "set up" medium to fresh plastic Petri dishes, and cell culture was continued for the remainder of the 4-day period. Each treatment group represents 2 sets of triplicate culture plates.

CMC

\(^{51}\text{Cr} Labeling of P815 Cells. Approximately 2 \times 10^7 P815
cells obtained 4 days after i.p. tumor implantation and washed 3 times as indicated above were incubated in 0.1 ml RPMI Medium 1640 for 30 min at 37° with 50 μl of a fresh batch of 51Cr (Amersham-Searle Corp., Arlington Heights, Ill.; 1 mCi Na251CrO4, and 306 μg chromium per ml) in a 50-ml glass round-bottom centrifuge tube. The incubation was stopped by adding 40 ml of cold RPMI Medium 1640, and the cells were washed and resuspended in RPMI Medium 1640 containing 5% fetal calf serum. Viability was determined by trypan blue exclusion, and the cell number was adjusted to 2 x 10⁵/ml.

**Effector Cells.** Cultured cells were harvested by gently scraping with a rubber policeman, washed twice with RPMI Medium 1640, counted with viability measured by trypan blue dye exclusion, and adjusted to the desired effector cell concentration in RPMI Medium 1640 supplemented with 5% fetal calf serum. Viability was determined by trypan blue dye exclusion, and the cell number was adjusted to 2 x 10⁵/ml.

**Test Conditions.** Specified numbers of sensitized spleen cells ("effectors") were pipetted in 0.1-ml aliquots into 12 x 75-mm plastic Falcon tubes. Duplicate samples of pooled triplicates were assayed. To these were added with mixing 2 x 10⁴ ⁵¹Cr-labeled P815 cells ("targets") in 0.1 ml. These cell suspensions were incubated for 4 hr at 37° in a humid atmosphere containing 10% CO₂. The reaction was stopped by adding 2 ml of ice-cold RPMI Medium 1640, and the suspensions were centrifuged for 5 min at 2000 rpm in a refrigerated (4°) International PR-2 centrifuge. The supernatant was decanted to 12 x 75-mm disposable glass tubes. Pellets and supernatants were counted separately in a Packard Model 3375 γ spectrometer. The results were calculated as follows:

\[
\text{% of release} = \frac{\text{Supernatant cpm}}{\text{Pellet cpm} + \text{supernatant cpm}} \times 100
\]

Percentage of specific release represents the percentage of release with effector cells minus the percentage of release with nonstimulated spleen cells. All results shown with bars represent S.D. values. Data shown without bars indicate representative results from experiments that have been duplicated using different animals as the source of stimulator and responder cell populations.

**RESULTS**

**Immunosuppression by MTX and Its Reversal by CF.** As shown in a representative experiment (Chart 1), the development of the CMC response of spleen cells was reduced by more than 50% when 12 nM MTX was added to the cultures on Day 0. The ratio of 20 effector cells to one target cell was used in order to measure the CMC activity in a nonplateau region of the cytolytic response curve obtained at different effector to target cell ratios. Additions of equimolar CF to this system produced a substantial, but not a complete, reversal of the MTX effect, and there was no detectable time restriction with respect to CF addition between 4 hr before and 24 hr after MTX addition.

To test whether an increase in the molar ratio of CF to MTX would produce a complete reversal of immunosuppression, CF was added to cells 4 hr prior to MTX. Under these conditions, molar ratios of 5- and 10-fold produced complete reversal. However, when these same levels of CF were added to cultures 4 hr after MTX, 5- or 10-fold molar excess of CF did not provide better reversal than did equimolar CF (i.e., 70% reversal; data not shown).

The dose dependence of CF reversal was also investigated at several levels of MTX (Chart 2). At none of the levels tested (≈ 10⁻⁶ to 10⁻⁶ M) was MTX cytotoxic to the effector cells as judged by comparable yields of viable cells on Day 4 of culture in the presence or absence of the drug. The pattern of CF reversal appears to be competitive. However, while 10-fold higher CF concentrations reversed the effects of lower MTX, approximately 100-fold higher CF concentrations were needed to reverse the immunosuppression caused by 1 μM MTX. These dose-response patterns have been repeatedly observed. They

**Chart 1.** Reversal of MTX effects as a function of the timing of CF addition. Spleen cells (2 x 10⁷) from C57BL/6J mice were incubated with 2 x 10⁵ X-irradiated P815 mastocytoma cells in 1 ml of RPMI Medium 1640 supplemented with 5% dialyzed heat-inactivated fetal calf serum, L-glutamine (2 mM), sodium pyruvate (1 mM), 100-fold nonessential amino acids (0.01 ml/ml), penicillin (100 units/ml), and streptomycin (100 μg/ml). Cultures were maintained at 37° in sealed plexiglass chambers in an atmosphere of 83% nitrogen, 7% oxygen, and 10% carbon dioxide for 4 days and fed once daily with 0.1 ml of a feeding mixture containing 30 ml RPMI Medium 1640, 50-fold essential amino acids (5 ml), 100-fold nonessential amino acids (2.5 ml), L-glutamine (17 mM), sodium bicarbonate (200 mM), and glucose (100 mM). An equal volume of dialyzed heat-inactivated fetal calf serum was added just before use. Drugs were made up in RPMI Medium 1640 and added during the setup of the culture plates or at the times indicated. Three plates were pooled and used as the source of sensitized effectors for the Day 4 assays. CF was added at twice the concentration of MTX to give an equimolar level of the active isomer. The level of CMC activity of the cells sensitized to P815 cells (B) and the effect of MTX (12 nM) on the development of this activity (C) are indicated. Differences of the effect of MTX (12 nM) by CF (12 nM) when it was added at the indicated times as compared to MTX at 0 time.

**Chart 2.** Dose-response of CF reversal. Cultures were set up with addition of MTX at AM (B), 100 nM (A), or 1 μM (X) concentration. At the same time, CF was added to the cultures at the indicated concentrations. Cultures were then carried out for 4 days as described for Chart 1.

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resemble closely the dose response seen for CF reversal of MTX toxicity in the bone marrow colony formation system (18).

Timing of MTX Addition. Studies were undertaken to determine at what time during the 4-day culture period the presence of MTX was critical for immunosuppression. As shown in Chart 3, addition of 15 nM MTX after the first 24 hr of culture was ineffective. However, the addition of 100 nM MTX could be delayed for 48 hr and still result in significant immunosuppression while addition at 72 hr of 100 nM MTX consistently failed to have any effect (data not shown).

Since it is probable that both the concentration of MTX and the duration of MTX exposure are necessary for depletion of cellular folate pools, washout studies with MTX were carried out. Both 15 and 100 nM MTX could be removed from cultures after the first 24-hr exposure without significant CMC suppression (Chart 4) while 1 μM MTX produced prolonged suppression if it was present for 24 hr. As MTX concentration increased, its obligatory exposure time decreased.

Since RPMI Medium 1640 contains 2 μM folate, the 24-hr washout studies were repeated in folate-free RPMI Medium 1640. The results indicate that there was no significant difference using this media, and thus the small amount of folate in regular RPMI Medium 1640 was not contributing to the effects seen.

The reversal of MTX effect by metabolites will depend on

Reversal Studies with dThd plus Hx. Initial studies with dThd and Hx (100 μM each) added to cultures only at Time 0 showed no reversal of MTX immunosuppression. Additional feeding with dThd plus Hx preferably daily produced reversal (data not shown). While dThd alone at concentrations up to 100 μM produced no reversal (data not shown), a dose-dependent reversal of MTX suppression was seen with Hx alone (Chart 5). To further confirm that the reversal was due to purine, a suboptimal concentration (3 × 10⁻⁵ M) of Hx was added with an equimolar concentration of the xanthine oxidase inhibitor allopurinol. This combination gave complete reversal of MTX suppression. Deoxyadenosine at a concentration as high as 10⁻⁴ M produced much less reversal than did Hx (Chart 6) indicating that the Hx limitation may reflect metabolic requirements different from those fulfilled by deoxyadenosine. However, the fate of deoxyadenosine within this system should be clarified since deamination may need to occur before deoxyadenosine can exert its sparing effects.

DISCUSSION

The reversal of MTX effect by metabolites will depend on
how this antimitabolite has affected a number of biochemical parameters by the time the reversing agents are provided. These parameters include the pools of reduced folates, the remaining activity of dihydrofolate reductase, the activity of thymidylate synthetase, and the pools of purine nucleotides and thymidylate. These last 2 pool sizes can be affected by extracellular levels of purine and dThd. MTX sensitivity in different cell lines has been correlated with the rate of MTX uptake and efflux (13, 22), with the dissociation constant (K,) of dihydrofolate reductase-MTX complex (12), and with the intracellular level of dihydrofolate reductase (10).

The work reported herein indicates that a specific cellular immune response can be severely inhibited by MTX and that this inhibition can be reversed by either CF or Hx. The timing of CF addition is not as critical in this tissue culture system as has been reported for the reversal of immunosuppression in anti-sheep RBC (14) or anti-typhoid, paratyphoid A, and paratyphoid B vaccine (1) responses in mice. The duration of exposure to MTX needed to produce irreversible suppression is concentration dependent and shows a dose-time relation similar to that reported for MTX toxicity to nucleated bone marrow cells (25). Delayed addition studies showed that high concentrations of MTX can produce suppression when applied at time points just before the time after which cultured cells have passed the peak of their proliferative wave and have become relatively insensitive to MTX. At these high MTX levels, the time necessary to deplete cellular folate pools and initiate the metabolic block may be shorter. A purineless state has been postulated to develop which is more rapidly toxic to target cells than is a thymineless state (25). Levels of free intracellular MTX are achieved more readily and persist longer at high MTX concentrations, a necessary condition for MTX toxicity (21).

The reversal of MTX toxicity by dThd and/or purines has been shown in many systems but with different dependencies. Hakala (8) was the first to demonstrate the need for dThd, Hx, and glycine to support the growth of Sarcoma 180, HeLa, and J-111 cells in the presence of growth-inhibitory levels of MTX. Purine alone provided partial protection against MTX toxicity in some cell lines (9, 11) while in other systems, dThd alone was able to reverse the effect of MTX (3, 10). It is, of course, necessary when one evaluates in vivo data to consider other parameters. For instance, mouse serum contains low levels of Hx which correlates with high levels of xanthine oxidase (17, 19), and this may affect the reversibility of MTX toxicities by Hx or dThd (20).

Under the conditions of this investigation, Hx did not seem to persist in culture of these cells, and consequently, the addition of it had to be repeated to produce reversal. This was overcome by the addition of the xanthine oxidase inhibitor allopurinol which lowered the requirement for added Hx. Xanthine oxidase activity in these cultures was not measured. The medium from 4-day cultures was analyzed for extracellular dThd by a sensitive enzyme assay using herpes simplex dThd kinase and was found in one instance to contain 10 to 20 μM dThd. Based on the published dose relationships (7, 9) needed to produce MTX reversal by dThd and Hx, this amount of dThd would be sufficient to cooperate with the 50 μM Hx in reversing the effects of MTX. Since the media and the dialyzed fetal calf serum used were dThd free, the source of this end product was probably via cell death and degradation of DNA. Exogenously added, dThd had no further effects in this system.

These data suggest that with regard to immunosuppression both Hx and dThd are required for reversal of MTX effects. In comparing the requirements of Hx for reversal in this system with those reported by others in tumor cell culture system (7, 9), it becomes apparent that reversal of the inhibition of the development of the immune effectors requires substantially higher concentrations of this source of purine nucleotides.

These studies point out the need for a precise characterization of the levels of cellular metabolite pools and interrelationships of metabolic pathways in each cell type whose function is affected by MTX before the conditions of antimitabolite effect and reversal may be adequately understood.

REFERENCES


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Y-C. Cheng, unpublished results.


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