Methotrexate Therapy of Psoriasis: Differential Sensitivity of Proliferating Lymphoid and Epithelial Cells to the Cytotoxic and Growth-Inhibitory Effects of Methotrexate


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Although methotrexate (MTX) is one of the most clinically effective therapies employed to treat psoriasis, the mechanism by which low-dose MTX acts to modulate the hyperplasia of psoriasis, leading to the restoration of clinically normal skin, is only partially understood. MTX has been considered a cytotoxic agent that mediates its effect primarily on proliferating or cycling epidermal cells. Recently, proliferating lymphoid cells have been identified in psoriatic lesions, raising the possibility that proliferating lymphoid cells could be another target cell that is killed by MTX. In this study, we examined the growth-inhibitory and cytotoxic effects of MTX on proliferating lymphoid cells [THP-1 (macrophage), and MOLT-4 (T cell)], epithelial cells (HeLa, and HaCat), and normal human keratinocytes (NHK) in vitro. The proliferating cells were exposed to MTX for 24 h, and placed in fresh media to mimic the transient MTX blood levels that result from once-weekly therapy. THP-1 and MOLT-4 were found to be 10–100 times more sensitive to the cytotoxic effects of MTX than were HeLa and HaCat, and more than 1000 times more sensitive than primary human keratinocytes. At MTX concentrations that would be expected to occur in vivo during once-weekly therapy, a large percentage (>95%) of proliferating lymphoid targets would be killed, and only a small percentage (<10%) of proliferating epidermal cells would be affected. This in vitro data suggests that in psoriasis proliferating lymphoid cells are more likely than epithelial cells to be a major cellular target of MTX in vivo. Key words: lymphocyte/keratinocyte. J Invest Dermatol 104:183–188, 1995

Methotrexate (MTX) is one of the most effective systemic therapies of psoriasis [1,2]. Previous studies have suggested that the hyperproliferative keratinocyte is the cellular target of MTX therapy [3–5]. Clinical experience, however, demonstrates that MTX, when administered once weekly, is effective in clearing psoriasis without clinical evidence of skin necrosis [1,2]. With the advent of cyclosporine therapy of psoriasis, recent investigations suggest that activated proliferating lymphocytes are important in the pathogenesis of psoriasis [6–9]. Similarly, MTX may be mediating its therapeutic effect in psoriasis, to some extent, through its action on activated proliferating lymphoid cells [9,10].

In this study we evaluate the hypothesis that proliferating activated lymphoid cells may be an important therapeutic target of systemic “low-dose” MTX therapy in psoriasis. In the experiments being reported, we examine the relative sensitivity of proliferating lymphoid and proliferating epithelial cells to MTX administered over 24 h (which mimics the blood levels that occur in vivo after a single MTX dose) [11–13]. These experiments evaluate whether lymphoid cells and/or epithelial cells are killed or growth inhibited at MTX concentrations achieved during weekly MTX therapy that may result in long-term cumulative improvement in psoriasis.

MATERIALS AND METHODS

Cell Culture and Target-Cell Selection The human epidermal keratinocyte cell line, HaCaT, and HeLa (D98) were obtained from E. Stanbridge (Irvine, CA) and maintained in Dulbecco’s modified Eagle’s medium (MEM) supplemented with 10% bovine calf serum (BCS). MOLT 4 (T cell) and THP-1 (monocyte) were obtained from G.A. Granger (Irvine, CA) and maintained in RPMI 1640 supplemented with 10% BCS. Primary cultures of normal human keratinocytes (NHK) were passaged into modified complete MCDB 153 medium containing the standard growth factors, hormones, supplements [epidermal growth factor (EGF), insulin, and BPE] and nutrients, and NHK were cultivated as previously described [14]. These cell lines were chosen to mimic the rapid proliferation seen in activated proliferating lymphoid cells and hyperproliferative epidermis found in psoriasis.
Exposure of Lymphoid Cells to MTX

Cell Viability and Viable Cell Counts After Exposure to MTX: In each experiment, six 10-ml cultures of MOLT 4 or THP-1 were established in RPMI 1640 (10% BCS) at a density of 0.3 × 10^6/ml in T25 tissue-culture flasks and were incubated at 37°C. MTX was added to five cultures such that the final concentrations were 10^-8 M to 10^-5 M and the sixth culture was employed as an untreated control culture. In one set of experiments MTX was left in the media during the entire incubation. In a second set of experiments all six cultures were incubated at 37°C for 24 h, after which the cells in each flask were spun down, washed, and resuspended in 5 ml of fresh RPMI 1640 (10%) tissue-culture media. These cultures were incubated at 37°C for up to 6 d after MTX exposure. Viable cell number was assessed each day employing a hemocytometer and trypan blue dye exclusion.

Cloning Efficiency After MTX Exposure: The number of THP-1 cells surviving exposure to MTX was assessed by measuring the ability of THP-1 cells to form colonies in soft agar after a 24-h exposure to MTX. THP-1 cells were set up in 24-well plates at 0.3 × 10^6/ml in RPMI 1640 10% fetal bovine serum and exposed to MTX at concentrations ranging from 10^-10 M to 10^-5 M for 24 h. After the exposure the cells were washed, plated in 0.5% soft agar in petri dishes, and grown at 37°C in a humidified CO2 incubator for 12 d. The number of colonies that were measured at the beginning of the MTX exposure was defined as 100%, and the numbers of colonies after exposure to different concentrations of MTX were expressed as the percent of starting cell number.

Exposure of HaCaT and HeLa Epithelial Cells to MTX

Cell Viability and Viable Cell Counts After MTX Exposure: Tissue-culture plates were seeded with HaLa or HaCaT cells and incubated overnight. MTX was added to each well in concentrations ranging from 10^-7 M to 10^-5 M, and the culture was incubated for 24 h at 37°C. After removing the MTX-containing medium, the cells were washed two times, and then fresh medium added to the well. Individual cultures were incubated an additional 1 to 6 d before assessing viable cell number per well in 6- or 24-well tissue-culture plates. Viable cell number per well was assessed by trypan staining the cells and counting viable cell number employing trypan blue and a hemocytometer.

Cloning Efficiency After MTX Exposure: The ability of HaLa and HaCaT to form colonies after exposure to MTX was assessed. HeLa and HaCaT were plated in 6-well plates at a density of 200-1000 cells per plate and incubated overnight to allow the cells to attach and recover from the trypsin treatment. MTX was added to each plate at concentrations ranging from 10^-5 M to 10^-1 M for 24 h. The MTX-containing media was removed, the plates washed, fresh media added, and the plates incubated for 6-12 d before counting the number of colonies.

Exposure of NHK to MTX

Cell Viability and Viable Cell Counts After MTX Exposure: NHK cultures growing in MCDB 153 medium containing 0.2 μM thymidine (low-thymidine medium) were exposed to MTX at the designated MTX concentration for 24 h. Cultures were thoroughly washed and re-fed with complete, low-thymidine culture medium every other day. Cell density was determined by trypsinization and direct cell enumeration of duplicate cultures as previously reported [14]. Cell viability was assessed by propidium iodide exclusion using a previously described method [15].

Cloning Efficiency After MTX Exposure: Following the 24-h MTX exposure, and extensive washing to remove the MTX, the colony-forming efficiency of MTX-treated cultures was examined in a clonal growth assay. Five hundred cells were seeded into 60-mm dishes containing low-thymidine medium, EGF, insulin, and supplements. Ten days later, the dishes were fixed and stained, and the colonies were enumerated using a macroscopic video imaging camera and IBAS image-analysis system (Kontron Elektronik, Munich, Germany) as previously described [14].

RESULTS

An In Vitro Model of “Low-Dose” MTX Therapy of Psoriasis

To design an in vitro model that mimics low-dose MTX therapy in vivo, the pharmacokinetic data in the literature was examined to estimate the duration that MTX is present in the blood in levels capable of inhibiting the growth of or killing sensitive proliferating target cells. To simplify this analysis, we chose to evaluate the pharmacokinetics of a single dose of MTX administered at weekly intervals. This clinical regimen is an effective therapy employed to treat psoriasis when administered at doses of 12.5-25 mg/week [1,2]. In vitro studies examining proliferating target cells have demonstrated that MTX has negligible biochemical, cytotoxic, or growth-inhibitory effects on proliferating cells in vivo or in vitro below 0.01 μg/ml ([2.2 × 10^-9] M); most cytotoxic effects observed in vitro occur above 0.1 μg/ml ([2.2 × 10^-7] M [3,16-18]). Thus, the duration for which MTX blood levels are above 0.01 μg/ml provides a measure of the total duration for which MTX is capable of mediating growth-inhibitory effects in vivo, and the duration above 0.1 μg/ml provides an estimate of the duration for which cytotoxic levels of MTX are present in vivo.

The MTX blood levels that occur after a single dose of MTX in patients with psoriasis and rheumatoid arthritis have been reported by a number of investigators [11-13]. After a single 25-mg oral dose of MTX the blood levels remained above 0.1 μg/ml ([2.2 × 10^-7] M) for 8 h, and above 0.01 μg/ml ([2.2 × 10^-5] M) for 24 h [12]. Thus, MTX blood levels exceed minimum cytotoxic or growth-inhibitory levels for, at most, 24 h after a single oral 25 mg MTX dose. This compares well with in vivo studies, demonstrating that decreased epidermal proliferation, as measured by decreased thymidine (thymidine) incorporation in psoriatic lesions, can be demonstrated for only 18-24 h after a single 25-50 mg dose of MTX [3].

When MTX is administered weekly to treat psoriatic patients, MTX is present for only the first 24 h, and not for the remaining 6 d. Thus, to a first approximation, MTX therapy can be modeled in vitro by exposing cellular targets to varying concentrations of MTX for 24 h, washing out the MTX, and then observing the cells’ viability and proliferative capacity while being maintained in fresh tissue culture medium. In the following experiments, proliferating lymphoid and epithelial cell lines were exposed to varying concentrations of MTX for 24 h and viability and viable cell number were assessed before, during, and for 3-12 d after MTX exposure.

Correlation of In Vitro MTX Dose and In Vivo MTX Dose

To determine the relevant in vitro MTX concentrations for these studies, the area under the concentration versus time curve (AUC) in vivo of clinically effective MTX doses (2.5-25 mg) was calculated. The AUC in vivo can be used to determine a comparable MTX concentration in vitro to be administered over 24 h to generate the same AUC. The effect of this MTX concentration in vitro on lymphoid and epithelial cells will presumably reflect the growth-inhibitory and cytotoxic effects of MTX in vivo.

The MTX blood levels that occur during low-dose MTX therapy, and the corresponding AUC, have to be estimated because no studies to date have reported the blood levels of MTX given in increasing doses (2.5-25 mg) to individual patients. The observed blood levels of MTX administered orally to psoriatic patients follow first-order kinetics after peaking at 1-2 h [11-13]. Thus, if the peak concentration achieved at any oral dose can be measured and the kinetic decay parameters determined, the MTX blood levels versus time curve for patients can be calculated. Sinnet et al provided the best kinetic half-life data for patients receiving 7.5 mg MTX orally, and demonstrated a t 1/2 = 4.45 h (range 3.56-5.34 h) [13]. Halprin et al provided an analysis of the peak MTX concentration in patients as a function of administered MTX dose and found that [peak MTX (μg/ml)] = [dose (mg/kg)] × [2.76] + 0.21 [11]. Thus, MTX blood levels as a function of time [c(t)] can be calculated: [c(t) = [peak MTX] exp(-kt); (k = ln2/MTX blood half life), after reaching peak concentration at 2 h.

MTX blood levels have been estimated using the pharmacokinetic information presented above for a 70-kg patient taking a single oral MTX dose in the range 2.5-25 mg MTX. In this model, the MTX concentration increases linearly and reaches a peak blood level at 2 h, and then decay: exponentially with a half life of 4.45 h. The correlation of in vivo therapeutic MTX blood levels to concentrations for the in vitro model of psoriasis therapy are given in Table I. Based on this analysis, the relevant range of MTX concentrations in our in vitro model system that mimics the clinically employed 2.5 to 25 mg MTX oral doses is a 24-h exposure of 1.5-6.42 × 10^-7 M MTX.
Effect of MTX on the Growth and Viability of Lymphoid Cells  

The first experiments examine the sensitivity of proliferating THP-1 (monocyte) cells to MTX-mediated growth inhibition and cytotoxicity. The effect of a 24-h exposure of various concentrations of MTX on growth and viability of THP-1 is presented in Fig 1. A 24-h exposure to $10^{-7}$ M or higher MTX concentration leads to a sustained net decrease in viable cell number up to 168 h (1 week), whereas exposure to $5 \times 10^{-8}$ M MTX for 24 h does not produce a net decrease in cell number below the starting cell number.

MOLT-4 cells (T cells) were treated with various concentrations of MTX for 24 h, as already described, and viable cell number was assessed. MTX concentrations of $5 \times 10^{-8}$ M or greater produced a decrease in viable cell number below starting cell number (Fig 2). The period below the starting cell number was transient for 5 to 6 d. As with THP-1 and MOLT-4, the decrease in viability is apparent after about 40 h (16 h after the MTX is removed from the culture). Comparing the data in Fig 3 with the data in Figs 1 and 2 demonstrate that MOLT-4 cells require approximately 10 times the dose of MTX to obtain growth curves similar to those obtained with THP-1 and MOLT-4.

Effect of MTX on HeLa (Epithelial) And HaCaT (Epidermal) Cells  
The cytotoxic and anti-proliferative effects of a 24-h exposure of HeLa cells to MTX were examined. A 24-h exposure to $1 \times 10^{-7}$ M or $2 \times 10^{-7}$ M MTX decreases viable HeLa cell number only a small amount (Fig 3). MTX concentrations of $1 \times 10^{-6}$ M and greater are required to substantially decrease viable cell number particularly after 5 to 6 d. As with THP-1 and MOLT-4, the decrease in viability is apparent after about 40 h (16 h after the MTX is removed from the culture). Comparing the data in Fig 3 with the data in Figs 1 and 2 demonstrate that HeLa cells require approximately 10 times the dose of MTX to obtain growth curves similar to those obtained with THP-1 and MOLT-4.

The cytotoxic and growth-inhibitory effect of a 24-h MTX exposure on HaCaT epidermal cells was next examined. The data in Fig 4 demonstrate that a concentration greater than or equal to $1 \times 10^{-6}$ M MTX results in a sustained decrease in viable cell number. A 24-h treatment with $10^{-7}$ M MTX leads to only a transient decrease in viable cell number. Treatment of these cells with weekly MTX in the clinically relevant range ($1 \times 10^{-7}$ M) in this model would not be expected to decrease cell number at the end of one week. Because a net decrease in proliferating cells is not produced at clinically relevant MTX concentrations, clinical improvement (i.e., net decrease in proliferating lesional keratinocytes) would not be expected to occur with weekly MTX therapy.

Effect of MTX on Normal Human Keratinocytes (NHK)  
The cytotoxic and growth-inhibitory effects of a 24-h MTX exposure on normal human keratinocytes was next examined. To
mimic the in vivo conditions, MCDB 153 culture medium containing thymidine at a concentration of 0.2 μM was prepared. This thymidine concentration approximates the observed in vivo serum concentrations of thymidine (0.33 ± 0.16 μM) in normal individuals [19], and also approximates the in vitro concentrations of thymidine in tissue-culture media containing 10% bovine serum that was employed to grow the other cell lines [20]. The data in Fig 5 demonstrates that only growth inhibition was obtained with MTX exposures as high as 10^{-5} M for 24 h. No evidence of a decrease in cell number, cell death, or differentiation at all MTX concentrations (including 10^{-5} M MTX) was seen. At an MTX concentration of 10^{-7} M only transient growth inhibition was noted. Other studies with thymidine free media demonstrated only growth inhibition when evaluated 48 h after MTX exposure (data not shown). This growth inhibition was overcome with medium containing normal (high) levels of thymidine. There was no evidence that the growth inhibition produced by MTX exposure had induced the cells to irreversibly growth arrest and terminally differentiate.

Cloning Efficiency of THP-1, HeLa, HaCaT, and NHK Cells After a 24-h MTX Exposure The growth curves of MTX-treated cells, presented thus far, assess the combined measures of both cell death and cell growth as a function of time. Therefore, specific studies were performed to examine the cytotoxic effects of MTX using a cloning efficiency assay. In the cloning assays the confounding effects of delayed cellular death and slowed growth rate of the cell line under test is eliminated. The ability of THP-1, HeLa, HaCaT, and NHK to grow into colonies after a 24-h exposure to 10^{-6} M to 10^{-5} M MTX is shown in Fig 6. In these studies, THP-1 cells are the most sensitive cellular target to MTX-mediated cytotoxicity, whereas NHK is the most resistant. THP-1 plating efficiency is reduced by more than 95% when exposed to 10^{-7} M MTX for as little as 8 h (data not shown). No
significant decrease in plating efficiency of NHK was seen after a 24-h exposure to $10^{-5}$ M MTX.

Comparing the relative sensitivity of the various cells at the concentration at which 50% plating efficiency occurs using THP-1 as a baseline of 1, HeLa are 12 times, HaCaT are 97 times, and NHK are more than 1563 times more resistant to MTX than are THP. At the expected concentrations achieved during low-dose MTX therapy ($1 - 5 \times 10^{-7}$ M), 95–99% of THP cells would be expected to die, whereas only a small percentage (<10%) of epidermal keratinocytes would be killed.

**DISCUSSION**

The mechanism(s) by which low-dose MTX administered weekly acts to modulate the epidermal hyperplasia seen in psoriasis leading to the restoration of clinically normal skin is still only partially understood. MTX and other antimetabolites used to treat psoriasis have been considered cytotoxic agents that mediate their effects primarily on proliferating or cycling epidermal cells [3-5]. In psoriasis, both local (intralional) and systemic MTX inhibit *de novo* epidermal DNA synthesis [*H-deoxyuridine (UDR) incorporation*], decrease mitotic activity and produce histologically “damaged” epidermal cells [3-5]. On the basis of these observations it has been assumed that local cytotoxic effects on keratinocytes are responsible for the therapeutic activity of MTX in psoriasis. However, MTX administered weekly to treat psoriasis does not cause clinically evident necrosis of psoriasis plaques [2,3], and rarely affects other rapidly proliferating epithelial cells or other tissues (e.g., hair, bone marrow, or gastrointestinal epithelium). Biopsies of psoriatic skin after MTX therapy reveal that only a very small percentage of keratinocytes [less than 100 keratinocytes per 4-mm section of psoriatic epidermis (<1%) are “damaged”] [3,5]. This suggests that pathways other than direct cytotoxic effects on keratinocytes may be involved in MTX’s action in psoriasis.

Another less well-known observation is that locally administered (intralional or topical) MTX, which provides adequate tissue delivery comparable to that obtained with low dose MTX therapy [21-24], does not produce significant clinical improvement of psoriatic skin lesions [25-28]. This finding suggests that direct effects of MTX on psoriatic epidermal cells are not the primary mechanism of action by which MTX exerts its activity in psoriasis. Because systemically administered MTX is clinically effective, and locally administered MTX is not effective, we postulate that MTX may mediate some of its therapeutic effects at sites other than the skin. Because many studies suggest a role for the immune system in psoriasis, we hypothesize that activated lymphoid cells (i.e., proliferating lymphoid cells) in various tissue compartments (e.g., lymph nodes, spleen, blood, skin) may be a major target of systemically administered MTX in psoriasis.

The available data suggest that MTX mediates its effects on cellular proliferation of keratinocytes while the drug is present during the first 24 h following a therapeutic administration of a single dose of MTX [3]. Inhibition of DNA synthesis in keratinocytes and “damaged” keratinocytes are only seen for 18-24 h after administering MTX. Serum MTX levels, after a single MTX dose, are not biochemically significant by 18-24 h [11,12]. This data also demonstrates that hyperproliferative growth of epidermal cells indistinguishable from pretreatment occurs *in vivo* by 18-24 h after a single MTX dose [3]. This last observation suggests that there is no cumulative growth-inhibitory effect on keratinocytes after weekly doses of MTX *in vivo*.

In the model system developed in this study, proliferating lymphoid and epithelial cells were exposed to MTX for 24 h *in vitro* to mimic the transient MTX blood levels that occur during weekly MTX therapy *in vivo*. The cytotoxic and growth-inhibitory effects were examined *in vitro* over the first 24 h of MTX treatment and the subsequent 4–6 d, when MTX was no longer present in the cultures. These experiments demonstrate that lymphoid target cells (THP-1, MOLT-4) are very sensitive to MTX-induced cytotoxicity when compared to epithelial cells. At MTX concentrations easily reached in patients ($1 - 5 \times 10^{-7}$ M MTX), the lymphoid cell lines are effectively killed (>95%) by a 24-h MTX exposure *in vitro*. It takes a 10-100-times higher MTX concentration to obtain approximately equivalent killing with HeLa and HaCat compared with lymphoid cells. Furthermore, normal human keratinocytes were not killed, even at concentrations of $10^{-5}$ M MTX, and were more than 1000 times more resistant to the cytotoxic effects of MTX than were THP-1 cells. These data strongly suggest that there is a differential sensitivity of lymphoid cells and keratinocytes to MTX, and that proliferating activated lymphoid cells *in vivo* may be a major cellular target of MTX therapy in psoriasis.

We hypothesize that for MTX to have a sustained beneficial effect on psoriasis via a cytotoxic and/or growth-inhibitory mechanism, the number of proliferating cells at the end of a week (i.e., just prior to the next MTX dose) must be less than the number with which one started. In the model system presented, a 24-h exposure to MTX results in a significant amount of lymphoid cell death but only a minimal amount of psoriatic keratinocyte death. The small number of lymphoid cells surviving the MTX exposure continue to proliferate. At clinically relevant MTX concentrations in this model the proliferating lymphoid cells final number does not exceed the starting cell number when the next dose of MTX would be administered. Thus, each subsequent dose of MTX should result in a progressive loss of proliferating lymphoid cells, and this may be ultimately expressed as improvement in the psoriatic plaque. Epidermal cells, in contrast, easily replace all the cells killed by MTX during the 6-d interval before the next MTX dose. Thus, no net decrease in epidermal cells would occur with each weekly dose of MTX.

Although we have been examining the mechanism of action of MTX in psoriasis in the current experiments, the *in vitro* studies examining the effect of MTX on proliferating lymphoid cells are relevant to the mechanism of MTX in other diseases. MTX is effective treatment for several immunologically-mediated autoimmune diseases that include psoriasis [2,29,30], psoriatic arthritis [31], rheumatoid arthritis (RA) [32], primary biliary cirrhosis [33], and adjuvant arthritis [34]. The mechanism by which MTX modulates the activity of these diseases is unknown. MTX could be working 1) locally in the target organ (skin in psoriasis, joints in RA and psoriatic arthritis, and liver in primary biliary cirrhosis), or 2) at sites distant to the target organ (e.g., lymph nodes, spleen, etc.), or 3) at both sites. Because MTX has an effect in all these autoimmune diseases, this suggests that the primary site at which MTX operates is not at the target organ alone. MTX could be working at both the target end organ and at sites other than the end organ, or alternatively, primarily at non-target end organ sites (e.g., lymph nodes, spleen, etc.). Clinical observations in RA have demonstrated that MTX injections into affected joints do not improve the joint disease [35,36]. This evidence again suggests that MTX may mediate its effects at sites other than the target organ that is affected by the immune system.

By combining the *in vitro* experiments presented in this study with the clinical responses observed in psoriasis, RA, and primary biliary cirrhosis to MTX, a plausible mechanism of MTX action common to all these diseases can be discerned. MTX is not a cure in each of these diseases. It controls the active disease while being administered, and the disease returns when it is discontinued. Thus, it appears that MTX does not permanently alter the fundamental pathologic processes generating the autoreactive cells. Because MTX is a cytotoxic agent that specifically affects proliferating cells, it is reasonable to hypothesize that the target cell(s) are proliferating. In RA, the number of activated proliferating lymphocytes have been demonstrated to be elevated in both the blood and synovium [37-40], and to decrease after low-dose MTX therapy [37-40]. We hypothesize that MTX may be killing or altering the number of activated proliferating effector lymphoid cells involved in mediating these autoimmune diseases. The relative contribution of MTX’s therapeutic effect on circulating activated proliferating lymphoid cells and those proliferating cells in the target end organ tissues remains to be determined.
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