Immunosuppressive Properties of Methotrexate: Apoptosis and Clonal Deletion of Activated Peripheral T Cells

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Abstract

The folate antagonist methotrexate (MTX) is extensively used in graft-versus-host disease, rheumatoid arthritis, and other chronic inflammatory disorders. In addition to its antiinflammatory activity associated with increased release of adenosine, MTX exerts antiproliferative properties by inhibition of dihydrofolate reductase and other folate-dependent enzymes. However, the mechanisms of immunosuppressive properties associated with low-dose MTX treatments are still elusive. We report here that MTX (0.1–10 μM) induces apoptosis of in vitro activated T cells from human peripheral blood. PBL exposed to MTX for 8 h, then activated in drug-free medium, underwent apoptosis, which was completely abrogated by addition of folic acid or thymidine. Apoptosis of activated T cells did not require interaction between CD95 (Fas, APO-1) and its ligand, and adenosine release accounted for only a small part of this MTX activity. Apoptosis required progression of activated T cells to the S phase of the cell cycle, as it was prevented by drugs or antibodies that interfere with IL-2 synthesis or signaling pathways. MTX achieved clonal deletion of activated T cells in mixed lymphocyte reactions. Finally, in vitro activation of PBL from rheumatoid arthritis patients after MTX injection resulted in apoptosis. Altogether, the data demonstrate that MTX can selectively delete activated peripheral blood T cells by a CD95-independent pathway. This property could be used as a new pharmacological end point to optimize dosage and timing of MTX administration. It may account for the immunosuppressive effects of low-dose MTX treatments. (J. Clin. Invest. 1998. 102:322–328.) Key words: methotrexate • apoptosis • T lymphocytes • thymidine synthase • rheumatoid arthritis

Introduction

Methotrexate (MTX) is a folate antagonist first developed for the treatment of malignancies (1) and, subsequently, used in nonneoplastic diseases as an antiinflammatory and/or immunosuppressive drug. MTX is currently the most commonly used treatment of rheumatoid arthritis (2, 3), and other chronic inflammatory disorders. MTX is also effective in the prophylaxis of acute graft-versus-host disease either alone or in association with cyclosporin A (CsA) and/or prednisone (4–6) or FK506 (7). MTX has also been used as an adjunct therapy for persistent mild cardiac allograft rejection (8). Most pharmacological studies have addressed the use of MTX in cancer chemotherapy, where doses could be escalated up to 30 g/m² by administration of the antidote leucovorin (folinic acid, citrovorum factor). In autoimmune diseases and allografts, however, MTX dosage is usually in a range of 7–15 mg per week, given orally or by intramuscular injections.

Biochemical pharmacology studies of MTX in tumor cell lines by J. Jolivet, B.A. Chabner, and co-workers have shown that MTX, like physiological folates, is converted to polyglutamate forms that are not readily transported across the cell membrane (9–11). Those polyglutamated derivatives not only inhibit dihydrofolate reductase (DHFR), the major MTX target, but also have markedly increased affinity for certain folate-dependent enzymes such as thymidylate synthase (TS), 5-aminoimidazol-4-carboxamide ribonucleotide transformylase, and the triple complex of enzymes that interconvert various forms of reduced folate (11, 12). The enzyme responsible for MTX conversion, polyglutamyl synthetase, catalyzes the addition of γ-linked glutamate groups to the end carboxyl group of the neighboring folyl glutamate, using ATP as its energy source. The activity of this enzyme, first demonstrated in erythrocytes and then in human liver, was shown to vary among tumor cell lines. Surprisingly, the polyglutamation of MTX in normal tissues, including the lymphoid system, has received little attention so far, and the sensitivity of resting or activated peripheral T cells to growth inhibition and, eventually, apoptosis in the presence of MTX concentrations, achieved during low-dose treatment, has not been investigated.

The present study addressed the in vitro activity of MTX on human PBL. We report here that MTX selectively induces apoptosis of activated but not resting lymphocytes, even after short term exposure to MTX and subsequent activation in drug-free medium, thus, providing the first evidence for an immunosuppressive activity of low-dose intermittent MTX administration. Selective susceptibility of activated T cells in the S/G2 phase of the cell cycle may result in clonal deletion of T cells that are activated by antigen at the time of MTX administration.
Methods

Reagents and monoclonal antibodies. MTX, folic acid, folinic acid, adenosine, thymidine, adenosine deaminase (ADA), α,β-methylene adenosine-5′-diphosphate (ACPD), PHA, PMA, ionomycin, concanavalin A, and Staphylococcus aureus enterotoxin B were obtained from Sigma Chemical Co. (St. Louis, MO). Rapamycin (RPM) and FK506 were a gift from A. Altman (La Jolla Institute for Allergy and Immunology, La Jolla, CA), and CsA was supplied by Sandoz Pharmaceutical Division (Novartis, Paris, France). The CD3 mAb OKT3 was from Cilag Laboratories (Levallois-Perret, France). The CD25 mAb ARIL-2 (IgG1) and anti-thymocyte globulins were a gift from 7 Alberici (Pasteur-Merieux, Lyon, France). The agonist (CH11, IgM) anti-human CD39 mAb was from Coulter-Immunotech (Marseille, France). The antagonist anti-human CD95 mAb ZB4 (IgG1) was from Kamiya Biomedical (Thousand Oaks, CA). The CD25, CD69, and CD95 mAbs (fluorescein isothiocyanate conjugates) were obtained from Becton Dickinson (Mountain View, CA) and Immunotech (Marseille, France), respectively.

Cell preparation and culture. PBL were collected from healthy donors in the presence of sodium citrate. Blood was defibrinated, and bated in RPMI medium for 12 h with MTX, and DNA preparations previously described (14). For DNA fragmentation assay, cells were incubated for 1–24 h in the presence of MTX, and then activated with PHA for 24 to 72 h, or MTX and PHA were administered concomitantly to the PBL. PBL were either incubated for 1–24 h in the presence of MTX, and then activated with PHA for 24 to 72 h, or MTX and PHA were added together at the onset of the culture. Cell death was evaluated by a fluorescence microscopy after staining with Hoechst 33342 (Sigma Chemical Co.) at 10 μg/ml after previously described methods (14). Apoptosis was also measured by flow cytometry after addition of biotinylated annexin V (Boehringer Mannheim, Indianapolis, IN) and by TdT-mediated dUTP–FITC nick end labeling (TUNEL), as previously described (16), using reagents from Boehringer Mannheim. Samples were analyzed by flow cytometry on a FACScan® (Becton Dickinson). Nuclear fragmentation and/or marked condensation of the chromatin with reduction of nuclear size were considered as typical features of apoptotic cells. Based on these measurements, results were expressed as percentage of apoptotic cells or percentage of specific apoptosis according to the following formula:

\[ \text{specific apoptosis} = (T - C)/(100 - C) \]

where T stands for % of apoptotic-treated cells and C for % of apoptotic control cells.

The morphological features of the cells after MTX treatment were also observed by transmission electronic microscopy, as previously described (14). For DNA fragmentation assay, cells were incubated in RPMI medium for 12 h with MTX, and DNA preparations were obtained and processed for electrophoresis in 2% agarose gel after previously described methods (17).

Immunofluorescence assays. After 1 d of culture, cells were washed with isotonic NaCl/Pi buffer containing 1% BSA and 0.2% NaCl (PBS/BSA/azide), and then incubated with 10 μl of fluorescein isothiocyanate conjugated CD25, CD69, or CD95 mAbs per 5 × 10^6 cells for 30 min at 4°C. After two washes in PBS/BSA/azide, cell were fixed with 1% formaldehyde in PBS/BSA/azide. Lymphocytes were identified by cell size (small angle light diffraction) and this fraction was gated for analysis, thereby excluding dead cells but including small lymphocytes and blasts. To evaluate the mitochondrial transmembrane potential (ΔΨm), cells (5 × 10^5/ml) were incubated with 3,3′-dihexyloxocarbocyanine (DiOC_6(3)) 40 nM in PBS (Molecular Probes, Inc., Eugene, OR) for 15 min at 37°C, followed by flow cytometry analysis (λ. Ex. Max., 488 nm; λ. Em. Max., 525 nm). The decrease in ΔΨm is a characteristic of apoptotic cells (18).

CD95-ligand mRNA quantification was carried out following methods described in detail in another report (19).

Results

MTX induces apoptosis of mitogen-activated but not resting peripheral T cells. PBL were activated by PHA for 3 d, and then dead cells were removed by centrifugation, and viable cells were incubated for 15 h with MTX (1 μM). Apoptosis was demonstrated by internucleosomal fragmentation resulting in a typical “ladder” of 180-bp fragments and multiples thereof in agarose gel electrophoresis, whereas such fragments were not detected in nonactivated PBL (Fig. 1 A). Apoptosis of PHA-activated cells, but not resting PBL was confirmed by typical condensation or fragmentation of cell nuclei, as revealed by Hoechst staining in fluorescence microscopy (Fig. 1 B), electron microscopy (Fig. 1 C), by the decrease of mitochondrial transmembrane potential (ΔΨm) (Fig. 1 D), and the presence of DNA breaks revealed by the TUNEL assay, (Fig. 1 D).

The kinetics of apoptosis showed a progressive increase up to 16 h (10 μM) or 28 h (0.1 μM) (data not shown). Similar observations were made with PBL activated by PMA plus ionomycin, concanavalin A, the CD3 mAb OKT3, polyclonal rabbit anti-thymocyte globulins and S. aureus enterotoxin B, but not by PMA alone (data not shown). We concluded that MTX (1 μM) induced apoptosis of activated T cells. A dose range of MTX concentrations was tested, demonstrating that apoptosis occurred at 0.1–10 μM, but not at 100 μM (Fig. 2 A). Counts of viable cells for 4 d after removal of PHA showed a progressive increase in controls (without MTX), a borderline decrease in the presence of MTX at 0.01 and 100 μM, and a considerable loss of viable cells with MTX at either 1 or 10 μM (Fig. 2 B). When compared with measurements of apoptosis, these data indicate that MTX at low (0.01 μM) and high (100 μM) concentrations inhibits cell proliferation without inducing apoptosis, whereas at intermediate concentrations (from 0.1 to 10 μM), the decrease of viable cells is mostly accounted for by apoptosis. Addition of MTX at the onset of PHA activation resulted in a marked progressive decrease of viable cell counts in the presence of MTX (from 0.1 to 10 μM), whereas at low (0.01 μM) and high (100 μM) concentrations, MTX inhibited

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cell proliferation but induced only a moderate and/or delayed loss of viable cells (data not shown).

PBL incubated with MTX undergo apoptosis upon subsequent activation in drug-free medium. Preincubation of PBL with MTX (from 0.1 to 50 μM) resulted in apoptosis on subsequent PHA activation for 24 h (Fig. 3A). To determine the half-life of MTX apoptosis–inducing activity, PBL were incubated with MTX (1 μM) and, subsequently, cultured in drug-free medium for various time intervals before activation by PHA. Results indicate a progressive linear decrease of the percentage of apoptotic cells with an average half-life of 3 d (Fig. 3B). Counts of viable cells after PHA activation in drug-free medium demonstrated that MTX inhibits PHA-induced cell proliferation at 0.01 and 100 μM, and induced a transient decrease of viable cell numbers at intermediate (0.1–10 μM) concentrations (data not shown).

MTX-induced apoptosis of activated T cells results from DHFR and TS inhibition. Knowing that MTX and its polyglutamated derivatives can interfere with several folate-dependent enzymes, we determined whether blockade of the two major targets of MTX activity, DHFR and TS, could account for MTX-induced apoptosis of activated T cells. To this end, 3-d PHA–activated cells were incubated with MTX (1 μM) together with folic acid, folinic acid, or thymidine. Folinic acid showed dose-dependent inhibition of MTX activity, with >80% decrease at 1 μM (Fig. 4A). Folic acid was >10–50 times less effective on a molar basis, and complete inhibition required 100 μM. Increasing the extracellular concentration of

Figure 1. MTX induces apoptosis of activated but not resting T cells. PBL were incubated 3 d with medium or PHA (5 μg/ml). Dead cells were removed and viable cells were treated for 15 h with MTX (10−6 M). (A) DNA fragmentation was evaluated by electrophoresis on 2% agarose gel (Medium and PHA, without (−) or with (+) MTX). (B and C) Morphology of activated PBL after Hoescht 33342 staining and by electron microscopy, respectively (without MTX (left) or with MTX (right)). (D) Alteration of the mitochondrial transmembrane potential (ΔΨm) measured by staining with DiOC6(3) and DNA strand breaks detection by the TUNEL assay as described in Methods. Numbers in each histogram refer to the percentage of cells below (DiOC6(3)) or above (TUNEL) the threshold of normal controls.

Figure 2. Characteristics of MTX-induced apoptosis. (A) Dose response: resting T cells (■) or cells activated by PHA (5 μg/ml) during 3 d (□) were treated with a dose range of MTX. Apoptotic cells were evaluated after 24 h. Values are the mean±SEM of three independent experiments. (B) Effect of MTX on viable cell recovery. PBL were stimulated with PHA (5 μg/ml) without or with MTX (10−6 M to 10−4 M) added at day 3. Viable cell number was determined by trypan blue exclusion at the indicated times. Values are the mean±SEM of three independent experiments.

Figure 3. Apoptosis induced by MTX pretreatment: dose response (A) and half-life of intracellular MTX (B). PBL were incubated with a dose range of MTX (A) or MTX (10−6 M) for 24 h (B). After removing the drug by two washes in HBSS, cells were immediately activated by PHA (5 μg/ml) (A) or maintained between 1 and 5 d in medium before PHA activation (B). Percent apoptotic cells were evaluated at 24 h in control cell suspensions incubated in medium alone (closed symbols) or in cells cultured with PHA (5 μg/ml) (open symbols). Results are expressed as percent-specific MTX apoptosis. Values are the mean±SEM of four individual experiments.
subset of PHA-activated cells, accounting for sensitivity to adenosine-mediated apoptosis was restricted to a only marginally decreased (Fig. 4
rogated by adenosine deaminase, whereas that of MTX was
The apoptotic activity of adenosine (1
crease of apoptotic PHA–activated cells did not increase. The apoptotic activity of adenosine (1 μM) was completely abrogated by adenosine deaminase, whereas that of MTX was only marginally decreased (Fig. 4 C). It was concluded that sensitivity to adenosine-mediated apoptosis was restricted to a subset of PHA-activated cells, accounting for ∼15–20% of cells susceptible to MTX.

MTX-induced apoptosis requires progression to the S phase of the cell cycle. The apoptotic signal triggered by MTX is likely to be initiated at the time of DNA synthesis, during the S phase of the cell cycle. Supporting this hypothesis, the percentage of blasts and the cell surface expression of CD69, CD25 (ε chain of the IL-2 receptor), and CD95 (Fas, APO-1), that are typical markers of activated T cells in the G1 phase of the cell cycle were not decreased by MTX (Fig. 5 A). Furthermore, inhibition of IL-2 synthesis by CsA or FK506, interference with IL-2 receptor signaling by rapamycin, and competition with IL-2 binding to its receptor by a CD25 mAb, all markedly decreased [3H]TdR incorporation and reduced MTX-induced apoptosis in parallel (Fig. 5 B). None of the inhibitors used to interfere with the IL-2 pathway increased the basal level of apoptosis in the absence of MTX.

MTX-induced apoptosis does not involve CD95L/CD95 interaction. Activated T and B lymphocytes express the death-signaling receptor CD95 (Fas, APO-1), which mediates apoptosis upon oligomerization. Because MTX was reported to stimulate CD95 ligand (CD95L) mRNA expression in human leukemia T cell lines (22), we studied the possible contribution of CD95L/CD95 interaction in the apoptotic effect of MTX. Expression of CD95L mRNA by 3-d PHA-activated cells could be readily induced by the association of a phorbol ester with a calcium ionophore, whereas MTX (10 and 200 μM) triggered only borderline CD95L gene transcription (Fig. 6 A).

Apoptosis induced by MTX was not altered by addition of the CD95 antagonist antibody ZB4, which completely inhibits apoptosis induced by CD95L or by the CD95 agonist antibody CH11 (Fig. 6 B). PBL rapidly express CD95 upon activation, but their sensitivity to CD95-dependent apoptosis progressively develops from day 3 to day 6 of mitogenic activation, and requires IL-2 (23). After 24 h of mitogenic activation, PBL were fully susceptible to MTX-induced apoptosis (Figs. 3 B and 6 C), yet resistant to the CD95 agonist antibody CH11. Finally, PBL from a patient with normal CD95 expression but defective CD95 signaling pathway, as shown by their resistance to CH11-induced apoptosis, were fully susceptible to MTX (Fig. 6 D). It was concluded that MTX-induced apoptosis of mitogen-activated cells occurred through a CD95-independent pathway.

MTX induces clonal deletion of alloreactive T cells. Alloreactive T cells undergo activation and clonal expansion when cocultured with cells that express different MHC class II molecules, a reaction termed MLR (mixed lymphocyte reaction) (24). Subsequent culture with the same stimulator cells results in accelerated proliferation, as shown by a peak of [3H]TdR incorporation and CD25 expression at 2–3 d, whereas the response to third party stimulator cells follows primary kinetics with maximal cell proliferation occurring after 5–6 d (13). Such differences indicate that the two types of stimulator cells are

Figure 4. Effect of folic acid, folinic acid, or thymidine (A); adenosine deaminase and ACPD on apoptosis induced by adenosine or MTX (C). Resting T cells (closed symbols) or cells activated by PHA (5 μg/ml) in serum-free medium for 3 d (open symbols) were treated with MTX (10−4 M) plus a dose range of folic acid, folinic acid, or thymidine (A) or with adenosine in serum-free medium. Identical values were observed in the presence of ACPD (10 μM) (B). 3-d PHA–activated cells were incubated with ADA (2 U), or ACPD (10 μM), and treated 24 h with MTX (10−4 M) or adenosine (10−4 M) (C). Apoptotic cells were evaluated after 24 h, and results are expressed as percent-specific apoptosis as described in Methods. Values are the mean±SEM of four independent experiments.

Figure 5. MTX-induced apoptosis and progression of activated PBL in the S phase of the cell cycle. PBL were activated by PHA (5 μg/ml) with or without MTX. (A) Percentage of cells expressing G1 phase markers (CD69, CD25, CD95) among PHA-activated PBL after 24 h in the presence (gray bands) or absence (white bands) of MTX (10−4 M). (B) Effect of inhibition of the G1 to S phase transition on MTX-induced apoptosis. PBL were activated by PHA (5 μg/ml) with or without CsA (250 ng/ml), FK506 (10 nM), CD25 mAb (10 μg/ml), and RPM (60 nM). Results are expressed as the specific MTX apoptosis, as described in Methods. Values are the mean±SEM of four independent experiments. [3H]TdR incorporation was determined during the last 8 h of culture with PHA: mean dpm (stimulation index in brackets). Nonactivated PBL: 300, PHA alone: 5,250 (17.5), PHA + CsA: 1,280 (4.2), PHA + FK506: 1,420 (4.7), PHA + CD25 mAb: 2,050 (6.8), PHA + RPM: 1,300 (4.3).

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recognized by distinct T cell clones, although cross-reactivity and/or HLA allele sharing between stimulator cells may often result in intermediate kinetics. To investigate whether a brief exposure to MTX could selectively delete activated T cells without impairing the functional capacities of other T cells in the same culture, we added MTX (1 μM) during the last 16 h of a primary MLR. Cells were then incubated in fresh medium for 4 d to allow the decrease of intracellular MTX in accord with the kinetics shown in Fig. 3 C, and the viable cells were then cocultured with either the same or a different allogeneic B cell line. In these conditions, the secondary response to identical stimulator cells was nearly completely abrogated, whereas the response to a third party cell line was not affected (Fig. 7), indicating that the effect of MTX was clonally restricted to the T cells that were activated at the time of exposure to the drug.

**Lymphocytes from MTX-treated patients undergo apoptosis upon in vitro activation.** We verified in six rheumatoid arthritis patients, that a single injection of MTX during low-dose treatment was sufficient to prime lymphocytes to apoptosis upon subsequent mitogenic activation ex vivo (Fig. 8). In vitro apoptosis, determined after 24 h of activation by PHA, was completely inhibited in the presence of folic acid (10 μM) and folic acid (100 μM) (data not shown), indicating that it could be attributed to MTX or its polyglutamated derivatives.

**Discussion**

MTX was shown to exert a wide range of antiinflammatory activities (21, 25–27) that are primarily mediated by the release of adenosine from different cell types that express ecto-S-nucleotidase (28). Such antiinflammatory properties do not exclude a genuine immunosuppressive activity, in as much as ADA deficiency is associated with a severe combined immunodeficiency disease (29). The present study demonstrates that MTX induces apoptosis of PBL activated by mitogens or superantigens, and may induce clonal deletion of alloreactive T cells at concentrations that are achieved during low-dose clinical treatments (30–32). Remarkably, nonactivated T cells were resistant to apoptosis at up to 1 mM MTX, demonstrating a selectivity toward activated PBL. Furthermore, resting PBL incubated with MTX, and subsequently activated in drug-free medium, underwent apoptosis, suggesting that unactivated T cells may convert MTX to polyglutamate forms that are retained intracellularly. The ability to undergo apoptosis may reflect the capacity of PBL to convert MTX to MTX glu4 and MTX glu5, which were reported to be retained for up to 24 h in breast cancer cells (10, 11). In the present model, maximal apoptosis was achieved after 8 h of exposure to MTX and the half-life of the biological activity of polyglutamated MTX was around 3 d. Such information may be relevant for optimizing dosage and time interval of MTX administration during low-dose treatments. Indeed PBL from MTX-treated rheumatoid arthritis patients underwent apoptosis upon ex vivo activation, although the percentage of apoptotic cells remained lower than that observed in control PBL.
pressive activity of MTX may be primarily controlled by intracellular thymidine supports the prediction that this immunosuppressive activity of MTX was only reversed at high molar ratios of folate to MTX, but the results in the depletion of intracellular reduced folates, but the complete blockade of DHFR results in the depletion of intracellular reduced folates, but the apoptotic activity of MTX was only reversed at high molar ratios of folic acid to MTX, and much more efficiently by folic acid, which bypasses DHFR. Complete inhibition by exogenous thymidine supports the prediction that this immunosuppressive activity of MTX may be primarily controlled by intracellular levels of thymidine.

Adenosine accounted for only a minor part of MTX-induced apoptosis because adenosine-triggered apoptosis was restricted to a small subset (~15%) of activated PBL. As discussed below, programmed cell death of activated PBL after MTX treatment was initiated at the onset of the S phase of the cell cycle. It may be hypothesized that the apoptotic-triggering signal is generated by altered DNA strands produced by DNA polymerase in the absence of thymidine, thus inducing apoptotic pathways shared by various genotoxic agents, and involving p53, the product of the retinoblastoma susceptibility gene Rb, and multimeric kinase complexes that control the G1/S progression (33–35). However, the apoptotic-signaling pathways induced by altered DNA are still largely unknown. In this respect, it is noteworthy that the antiproliferative properties of MTX demonstrated at high (100 μM) and low (0.01 μM) concentrations are clearly independent from its apoptosis-inducing capacity, suggesting that G1/S progression and apoptosis may be controlled by distinct signaling pathways.

It has recently been reported that several cytotoxic drugs, including MTX, can trigger CD95 (Fas, APO-1) ligand expression in leukemia T cell lines (22) and hepatoma cell lines (36), and that CD95 ligand/CD95 interactions were critical in drug-induced apoptosis. However, in activated PBL, MTX triggered apoptosis by a CD95-independent pathway. This observation is of major relevance for therapeutic applications because CD95-dependent apoptosis is restricted to a subset of preactivated “memory” T cells that express the CD45 RO short isoform (37–39), and that have developed sensitivity to the CD95-mediated apoptosis pathway through an efficient IL-2 signal (23). Conversely, MTX induces apoptosis of naive as well as memory T cells, and may induce deletion of naive activated T cells, as shown by MLR experiments.

MTX is usually used as monotherapy in autoimmune diseases, but in association with other immunosuppressive drugs, such as CsA or FK 506, in graft-versus-host diseases, and organ allograft rejection (4–8). The present study indicates that blocking the IL-2 pathway by inhibition of gene transcription (CsA, FK 506), anti–IL-2 receptor antibodies (CD25) or drugs interfering with IL-2 signaling (rapamycin) that block activated T cells in the G1 phase of the cell cycle, prevents MTX-induced apoptosis in vitro. Therefore, one should not expect synergy between MTX and drugs that interfere with the IL-2 pathway, unless MTX deletes T cells that have escaped activation blockade by the associated drugs. This could be the case in autoimmune disorders where CsA is administered at much lower doses than in transplantation.

In conclusion, the present study demonstrates that low-dose MTX treatment can induce apoptosis and clonal deletion of activated T cells, and, thus, establishes the potent immunosuppressive properties of this drug. The model provides a rationale for intermittent administration and it could be applied to optimizing the pharmacokinetics of MTX treatment. However, the relative irreversibility of this effect may represent a risk of overimmunosuppression in long-term therapy.

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