Divergent effects of methotrexate on the clonal growth of T and B lymphocytes and synovial adherent cells from patients with rheumatoid arthritis

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Abstract

Objective—To define the mechanisms whereby methotrexate (MTX) manifests its effects in patients with rheumatoid arthritis.

Methods—T and B cells from peripheral blood and rheumatoid synovial tissues, synovial adherent cells, and the human fibrosarcoma cell line HT1080 and its mutant (defective in an enzyme in the nucleotide salvage pathway) were tested for clonal growth when cultured with MTX. Normal human fibroblasts and those with a deficiency in a salvage pathway were cultured with MTX in the presence or absence of purine and pyrimidine bases.

Results—Clonal growth of T and B cells, but not synovial cells, was inhibited by clinically relevant concentrations of MTX. Slowly proliferating fibroblast lines were resistant to MTX, whereas their rapidly proliferating counterparts were not. However, mutant fibroblast lines deficient in a salvage pathway were sensitive to MTX despite slow proliferation. Similarly, while skin fibroblasts were resistant to MTX, germ line mutant fibroblasts deficient in a salvage pathway were sensitive to small concentrations of MTX.

Conclusion—T and B lymphocytes, but not synovial cells, may be the target of MTX in vivo. Resistance to MTX may be associated with slow proliferation and the ability to synthesise nucleotides via salvage pathways. MTX can inhibit proliferation of even slowly growing cells by restricting the supply of nucleotides obtained via a salvage pathway, by removal of purine and pyrimidine bases, or by inducing a deficiency in a salvage pathway. It may be possible to manipulate the therapeutic effect of MTX by adjusting the amounts of purines and pyrimidines available to the cells in vivo.

Materials and methods

CELL PREPARATION
Peripheral blood mononuclear cells (PBMC) were obtained from the heparinised blood of healthy volunteers by a standard Ficoll-Hypaque density centrifugation method. RA
Synovial tissues were obtained during surgery from eight patients with active synovitis who fulfilled the 1987 American Rheumatism Association criteria for RA. Single cells were prepared by enzymatic digestion of the synovial tissues, as described previously.

T-CELL CLONING
Utilising techniques described earlier, we cloned T cells from PBMC obtained from healthy donors and from single cells prepared from RA synovial tissues obtained during surgical procedures. Briefly, PBMC and synovial single cells were inoculated into 96 well microtitre plates at 1–2 cells/well for PBMC and 2–20 cells/well for synovial single cells, with x irradiated (50 Gy) PBMC (2 x 10^4 cells/well) and x irradiated (100 Gy) Raji cells (1 x 10^4 cells/well). The cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 2 mmol/l L-glutamine, 0.5 μg/ml phytohaemagglutinin-P (Difco, Detroit, MI), and 0.5 ng/ml recombinant human interleukin-2 (Takeda Chemical Industries, Osaka, Japan). Various concentrations of MTX were added to the culture; 96 wells were prepared for each concentration. After two weeks of culture, each well was observed under an inverted microscope to determine the presence or absence of lymphocyte colonies. Cloning efficiencies were calculated for each concentration of MTX, assuming a Poisson distribution for the number of cells with the potential to form colonies, as follows:

\[
\text{cloning efficiency (%) = } \frac{1}{c} \ln (\text{number of negative wells / total number of wells}) \times 100\% 
\]

where \( c \) = number of plated cells/well.

Cloning efficiencies in cultures with MTX were compared with those in control cultures without MTX.

To examine the reverting effects of hypoxanthine and thymidine supplementation on growth inhibition by MTX in T cells, various concentrations of hypoxanthine and thymidine supplement (Gibco, Gaithersburg, MD) and 0.1 μmol/l MTX were added to the cloning cultures of T cells.

B CELL CLONING
B cells were cloned from PBMC and RA synovial single cells by procedures described previously, using Epstein-Barr virus (EBV) transformation with some modifications. CD19 positive cells were positively selected by using CD19 antibody coated magnetic beads (Dynal, Norway) from PBMC of healthy donors. CD19 PBMC and synovial single cells were incubated in the supernatant of the B95-8 cell line for one hour and then inoculated into 96 well plates at 100 cells/well for PBMC and 50–200 cells/well for synovial single cells, with x irradiated PBMC (5 x 10^4 cells/well). GIT medium (Nihonseiyaku Co Ltd, Tokyo, Japan) supplemented with 10% FCS was used for the cloning of B cells from single synovial cells; this medium, originally developed for serum free culture of hybridomas, was used because synovial B cell cloning was not successful in RPMI 1640 medium. After two weeks of culture, half of the medium was replaced with fresh medium; 96 wells were prepared for each concentration of MTX.

STATISTICAL ANALYSIS
Statistical significance of differences between plates with respect to numbers of wells showing cell growth was assessed by \( \chi^2 \) analysis. Differences were considered significant at \( p < 0.05 \).

Results

EFFECTS OF MTX ON THE CLONAL GROWTH OF T CELLS
The cloning efficiencies of the T cells from PBMC ranged from 12.5 to 36.8% (table 1). When MTX was added in concentrations greater than 0.05 μmol/l, none of the wells was positive for cell growth. Even with MTX 0.025 μmol/l, cloning efficiency was reduced to less than 10%. The cloning growth of T cells from RA synovium also was completely suppressed
Effect of MTX on clonal growth of lymphocytes and synovial cells in RA

Table 1  Effects of methotrexate on the clonal growth of T cells from normal blood and RA synovium

<table>
<thead>
<tr>
<th>Methotrexate (µmol/l)</th>
<th>0</th>
<th>0-001</th>
<th>0-01</th>
<th>0-025</th>
<th>0-05</th>
<th>0-075</th>
<th>0-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal blood</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expt 1</td>
<td>36-8</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0**</td>
</tr>
<tr>
<td>Expt 2</td>
<td>32-6</td>
<td>28-8</td>
<td>43-8</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0**</td>
</tr>
<tr>
<td>Expt 3</td>
<td>18-5</td>
<td>—</td>
<td>3-8</td>
<td>1-0**</td>
<td>0**</td>
<td>0**</td>
<td>0**</td>
</tr>
<tr>
<td>Expt 4</td>
<td>12-5</td>
<td>—</td>
<td>15-7</td>
<td>1-1**</td>
<td>0**</td>
<td>0**</td>
<td>0**</td>
</tr>
<tr>
<td>RA synovium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expt 1</td>
<td>5-8</td>
<td>—</td>
<td>5-8</td>
<td>3-7*</td>
<td>0**</td>
<td>—</td>
<td>0**</td>
</tr>
<tr>
<td>Expt 2</td>
<td>0-4</td>
<td>—</td>
<td>0**</td>
<td>0**</td>
<td>0**</td>
<td>—</td>
<td>0**</td>
</tr>
<tr>
<td>Expt 3</td>
<td>3-8</td>
<td>—</td>
<td>4-9</td>
<td>2-1**</td>
<td>0**</td>
<td>—</td>
<td>0**</td>
</tr>
</tbody>
</table>

Values represent cloning efficiency (%).
— = Not done.

Statistical significance of growth inhibition: *p < 0-05; **p < 0-01, compared with control (no methotrexate) (χ² analysis).

Table 2  Reversal of suppressive effects of methotrexate (MTX) on the T cell growth by hypoxanthine and thymidine supplementation

<table>
<thead>
<tr>
<th>MTX 0-1 µmol/l</th>
<th>No MTX</th>
<th>Hypoxanthine (µmol/l):</th>
<th>0</th>
<th>0-20</th>
<th>100</th>
<th>200</th>
<th>500</th>
<th>1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymidine (µmol/l):</td>
<td>0</td>
<td>0-32</td>
<td>16</td>
<td>32</td>
<td>80</td>
<td>160</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expt 1</td>
<td>4-5</td>
<td>0</td>
<td>0</td>
<td>5-5*</td>
<td>9-7*</td>
<td>9-1*</td>
<td>9-1*</td>
<td></td>
</tr>
<tr>
<td>Expt 2</td>
<td>2-0</td>
<td>0</td>
<td>0</td>
<td>6-5**</td>
<td>19-0*</td>
<td>17-9*</td>
<td>6-9**</td>
<td></td>
</tr>
</tbody>
</table>

Values represent cloning efficiency (%).
Statistical significance in the difference of T cell growth in the presence of 0-1 µmol/l MTX compared with plates with no hypoxanthine or thymidine: *p < 0-01 (χ² analysis).

by low concentrations of MTX (table 1). Thus MTX had potent inhibitory effects on the clonal growth of T cells from two different sources.

Partial rescue by hypoxanthine and thymidine of MTX inhibited T cell growth was obtained by adding the supplement solution at hypoxanthine and thymidine concentrations of 100 and 16 µmol/l, respectively (table 2). The greatest recovery was observed with hypoxanthine and thymidine concentrations of 200 and 32 µmol/l, respectively, at which the rescue effect reached a plateau (table 2).

**EFFECTS OF MTX ON THE CLONAL GROWTH OF B CELLS**

As observed in T cells, MTX had potent inhibitory effects on the clonal growth of B cells cloned directly from CD19 PBMC by EBV transformation; none of the wells was positive for cell growth when MTX was added in concentrations of 0-025 µmol/l or greater (table 3). The clonal growth of synovial B cells was relatively resistant to MTX compared with blood B cells: only partial inhibition was observed with a concentration of 0-025 µmol/l.

Table 3  Effects of methotrexate (MTX) on the clonal growth of B cells from normal blood and RA synovium

<table>
<thead>
<tr>
<th>Methotrexate (µmol/l)</th>
<th>0</th>
<th>0-001</th>
<th>0-01</th>
<th>0-025</th>
<th>0-05</th>
<th>0-075</th>
<th>0-1</th>
<th>1-0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal blood</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expt 1</td>
<td>26-0</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0**</td>
<td>0**</td>
</tr>
<tr>
<td>Expt 2</td>
<td>31-6</td>
<td>23-4</td>
<td>0**</td>
<td>0**</td>
<td>0**</td>
<td>0**</td>
<td>0**</td>
<td>0**</td>
</tr>
<tr>
<td>Expt 3</td>
<td>19-0</td>
<td>12-0</td>
<td>0**</td>
<td>0**</td>
<td>0**</td>
<td>0**</td>
<td>0**</td>
<td>0**</td>
</tr>
<tr>
<td>RA synovium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expt 1</td>
<td>15-1</td>
<td>21-1</td>
<td>11-0</td>
<td>1-1**</td>
<td>0**</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Expt 2</td>
<td>2-7</td>
<td>3-2</td>
<td>2-7</td>
<td>0**</td>
<td>0**</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Values represent cloning efficiency (%).
— = Not done.

Statistical significance of the growth inhibition in the presence of MTX: *p < 0-05; **p < 0-01, compared with control (no methotrexate) (χ² analysis).

MTX (table 3). However, as GIT medium contains 15-0 µmol/l hypoxanthine and 1-5 µmol/l thymidine, it is likely that these compounds reversed the inhibition of cell growth by MTX.

**EFFECTS OF MTX ON THE CLONAL GROWTH OF SYNOVIAL ADHENTER CELLS**

MTX had only marginal inhibitory effects on the clonal growth of synovial adherent cells in a concentration of 0-1 µmol/l (table 4)—the concentration that completely inhibited the growth of T and B cells. Even when the MTX concentration was increased to 1-0 µmol/l, 30–50% of the cells were found to be resistant (table 4), in sharp contrast to the data obtained from T and B cells. Surprisingly, the inhibitory effects of MTX 50 µmol/l on synovial adherent cells were similar to those of MTX 1-0 µmol/l (table 4). Thus the clonal growth of synovial adherent cells was found to be highly resistant to MTX compared with that of T and B cells.

**EFFECTS OF MTX ON THE CLONAL GROWTH OF A HUMAN FIBROSARCOMA CELL LINE, HT1080**

MTX had potent inhibitory effects on the clonal growth of large colony forming HT1080 cells; the results were similar to those observed in T and B cells (table 5). In contrast, and in common with synovial adherent cells, the small colony forming subpopulation was resistant to MTX, suggesting that sensitivity to MTX may relate to the rate of proliferation of the cells, and may not depend on cell type.

When dialysed FCS was substituted for regular FCS in the cloning medium, even the slowly proliferating subpopulations of HT1080 were quite sensitive to MTX (table 5). In addition, low concentrations of MTX completely inhibited the growth of both rapidly proliferating (large colony) and slowly proliferating (small colony) subpopulations of HT1080TG (table 5).

**EFFECTS OF MTX ON THE CLONAL GROWTH OF SKIN FIBROBLASTS**

In common with the synovial adherent cells and the slowing proliferating subpopulation of HT1080 cells, skin fibroblasts were resistant to MTX (table 6). In addition, clonal growth of a fibroblast line (MiTen) from a patient with genetic HPRT deficiency (Lesch-Nyhan syndrome) was completely inhibited by low concentrations of MTX, suggesting again that the salvage enzyme has an important role in MTX resistance (table 6).

**Discussion**

To investigate the cellular basis for positive effects of MTX in the treatment of RA, we studied the inhibitory effects of MTX on the clonal proliferation of T and B lymphocytes and synovial adherent cells. MTX completely inhibited the clonal growth of T and B cells obtained from both peripheral blood and
Table 4 Effects of methotrexate on the clonal growth of RA synovial adherent cells

<table>
<thead>
<tr>
<th>Methotrexate (μmol/L)</th>
<th>0</th>
<th>0-01</th>
<th>0-1</th>
<th>1-0</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt 1 (20 cells/well)</td>
<td>1.72</td>
<td>—</td>
<td>1.37</td>
<td>0.55**</td>
<td>0.91*</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Expt 2 (20 cells/well)</td>
<td>—</td>
<td>—</td>
<td>0.26</td>
<td>0.55</td>
<td>0.38</td>
<td>0.05</td>
<td>0.16</td>
<td>—</td>
</tr>
<tr>
<td>Expt 3 (50 cells/well)</td>
<td>0.78</td>
<td>0.75</td>
<td>0.66</td>
<td>0.37*</td>
<td>—</td>
<td>0.34**</td>
<td>—</td>
<td>0.29**</td>
</tr>
</tbody>
</table>

Values represent clonal efficiency (%).

Table 5 Effects of methotrexate on the clonal growth of human fibrosarcoma cell line HT1080

<table>
<thead>
<tr>
<th>Materials</th>
<th>Colony size</th>
<th>Methotrexate (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>0-001</td>
</tr>
<tr>
<td>HT1080</td>
<td>Large</td>
<td>19-5</td>
</tr>
<tr>
<td></td>
<td>Large</td>
<td>5-5</td>
</tr>
<tr>
<td>HT1080 (dialysed FCS)</td>
<td>Large</td>
<td>8-5</td>
</tr>
<tr>
<td></td>
<td>Large</td>
<td>4-4</td>
</tr>
<tr>
<td>HT1080TG (HPRT deficient)</td>
<td>Large</td>
<td>5-4</td>
</tr>
<tr>
<td></td>
<td>Large</td>
<td>2-9</td>
</tr>
<tr>
<td>HT1080TG (HPRT deficient)</td>
<td>Large</td>
<td>0-9</td>
</tr>
<tr>
<td></td>
<td>Large</td>
<td>1-2</td>
</tr>
</tbody>
</table>

Values represent clonal efficiency (%).

Table 6 Effects of methotrexate on the clonal growth of normal and hypoxanthine phosphoribosyltransferase deficient (HPRT-) skin fibroblasts

<table>
<thead>
<tr>
<th>Methotrexate (μmol/L)</th>
<th>0</th>
<th>0-1</th>
<th>1-0</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF-TY (normal)</td>
<td>3-71</td>
<td>3-34</td>
<td>2-08**</td>
</tr>
<tr>
<td>M/Ten (HPRT-)</td>
<td>0-63</td>
<td>0**</td>
<td>0**</td>
</tr>
</tbody>
</table>

Values represent clonal efficiency (%).
Effect of MTX on clonal growth of lymphocytes and synovial cells in RA

Inhibitory effect of MTX on the growth of HPRT deficient synovial adherent cells has not been examined (primarily because such biological specimens are not available).

Inhibition of the clonal growth of T cells by MTX was reversed at least partially by the addition of hypoxanthine and thymidine to the culture, confirming that the inhibition of de novo purine and pyrimidine synthesis is the mechanism by which growth is inhibited by MTX. The concentration of hypoxanthine required to achieve 80% recovery was 200 μmol/l in T cells. The concentration of hypoxanthine in the culture medium (10% FCS) was approximately 4 μmol/l (measured by HPLC, unpublished data); this concentration appears to have been insufficient to achieve complete reversal of inhibition of growth by MTX in T cells.

The clinical observation that the efficacy of MTX was decreased by supplementation with an active form of folic acid (folinic acid), while cessation of folinic acid led to the recovery of MTX effectiveness, suggests that inhibition of DHFR is also implicated in the mechanism for clinical efficacy of MTX. However, in other studies in which smaller doses of folinic acid or folate were administered in different regimens, no reduction in the efficacy of MTX was reported, even though a reduction in the side effects of MTX was observed in some.

Thus there are discrepancies concerning the effects of folinic acid on the effectiveness of MTX, which seem to be related to differences in the dosage and administration schedule of folinic acid.

Although our in vitro culture system may not be an accurate reflection of in vivo cell proliferation, the data from the present study may have in vivo relevance. The proliferation of T and B lymphocytes, but not synovial adherent cells, may be inhibited in vivo in patients with RA receiving low dose MTX treatment. The in vivo synovial cells may be resistant to MTX, as they utilise small concentrations of free bases in synovial fluid for the synthesis of nucleotides through the salvage pathway. This idea is supported by the observation that the concentration of hypoxanthine in RA synovial fluid is similar to that in our culture medium (approximately 4 μmol/l, unpublished data). Even when free bases are present in body fluids, they might not be available in quantities sufficient to maintain the proliferation of activated T and B cells. By adjusting the amounts of purines and pyrimidines present in vivo, it may be possible to manipulate the therapeutic effect of MTX.

Thus an ability to relate the resistance and sensitivity of various cells in vivo to the availability of purine and pyrimidine compounds may pave the way to a better understanding of, and better adjustment of, the effects of MTX in the treatment of RA.

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22. Allega C J, Drake J C, Jolivet J, Chabner B A. Inhibition of phosphoribosylaminomimidazolecarboxamide transformylase by methotrexate and dihydrolorotic acid and poly


