Inhibition of cytokine production by methotrexate. Studies in healthy volunteers and patients with rheumatoid arthritis

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Citation for published version (APA):

Chapter 6

Bioassay for detection of methotrexate in serum.

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Scand J Rheumatology 2004:33
Abstract

Objective: A bioassay for the measurement of methotrexate (MTX) in serum is developed. Methodes: The assay is based on the fact that MTX inhibits the proliferation of hypoxanthine-guanosine phosphoribosyl transferase (HGPRT) negative mouse B-cells (B9.H). HGPRT negative cells cannot use the salvage pathway of nucleotide synthesis to overcome inhibition by MTX.
Results: When B9.H cells are cultured with serial dilutions of serum, inhibition of proliferation is a measure for the amount of MTX in that serum. Circulating folates do not interfere with the assay.
Conclusion: This simple assay can detect low amounts of MTX in serum; it is therefore a useful assay to follow the pharmacodynamics of functional MTX after low dose MTX treatment.
Introduction

Methotrexate (MTX) inhibits the enzyme dihydrofolate reductase (DHFR) and several other enzymes, thereby inhibiting the folate metabolism and the de novo purine and pyrimidine synthesis (12;3). High-dose MTX therapy is well established for the treatment of several forms of cancer such as acute lymphoblastic leukemias (4). In addition, low-dose MTX therapy is widely used for the treatment of rheumatoid arthritis (RA) and other inflammatory diseases (5-8). The anti-inflammatory mechanism of low dose MTX treatment is still debated. Cronstein has suggested that MTX acts via the release of adenosine (9). We and others have proposed that MTX works via deletion of activated T-cells and the subsequent inhibition of cytokine production (10,11). MTX levels in serum of RA patients show a very strong increase directly after administration of the drug and after 1 or 2 hours the levels decrease (1213-15). MTX is transported into cells by folate receptors, but the majority is excreted by the kidneys. Intracellular MTX is partially metabolised to polyglutamate forms leading to accumulation of these forms in the cell. MTX levels in erythrocytes are stable over a period of several days (15). For a better understanding of the pharmacodynamics of the drug and its (side) effects it is useful to monitor circulating levels of functional MTX in the patients.

Various assays are available for the detection of MTX in biological fluids (serum, plasma or cerebrospinal fluid) or erythrocytes (16,17). The fluorescence polarisation immunoassay (TDx) and enzyme multiplied immunoassay technique (EMIT) are commercially available antibody based assays used to measure MTX in serum and plasma. Another frequently used assay for the analysis of MTX is high-pressure liquid chromatography (HPLC) which can be used for analysis in various body fluids or cells. With this technique it is possible to distinguish between different polyglutamate forms of MTX that accumulate in erythrocytes (18). Two other methods are based on the ability of MTX to inhibit the enzyme DHFR. When dihydrofolate is reduced to tetrahydrofolate by DHFR, NADPH is oxidised to NADP, which can be monitored by measuring the absorbency. This reaction is inhibited by the addition of serum containing MTX (17;19). The radio-ligand binding assay is also based upon the fact that MTX forms a stable complex with DHFR in the presence of NADPH. DHFR is sequentially incubated with samples containing MTX and with [3H]-MTX. Unbound [3H]-MTX is removed by dextran coated charcoal. The amount of radioactivity in the supernatant is inversely proportional to the concentration MTX in the sample (20,21).

Although there are many different assays for the measurement of MTX in serum and plasma, none of these assays measures biologically active and thus functional MTX. Here we present a simple bioassay suitable for the measurement of functional MTX in serum and plasma. Furthermore this assay is sensitive enough to measure serum of RA patients, treated with low dose MTX.
CHAPTER 6

Materials and methods

Cells and chemicals

B9 cells are IL-6 dependent mouse hybridoma cells, widely used to assay IL-6 activity (22). B9.H is subclone independent of IL-6 and negative for hypoxanthine-guanosine phosphoribosyl transferase (HGPRT, selected by Dr. S. Ebeling in our department). B9 and B9.H cells were grown in culture medium: Iscove’s Modified Dulbecco’s Medium (IMDM, Bio Whittaker, Verviers, Belgium) containing penicillin (100U/ml), streptomycin (100 μg/ml), 50 μM 2-mercaptoethanol and 5% FCS (Bodinco, Alkmaar, the Netherlands), supplemented with 8 pg/ml rIL-6 (CLB, Amsterdam, the Netherlands) for B9 cells. When indicated, heat inactivated (30°, 56°C) human pool serum (HPS) was used in addition to 5% FCS. Hypoxanthine and thymidine (cell culture tested) were obtained from Sigma (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). MTX was purchased from Lederle (Hoofddorp, the Netherlands). Polyglutamated MTX forms (Schircks, Jona, Switzerland) were a kind gift from Dr. G. Jansen (VU Medical Centre, Amsterdam, the Netherlands)

Proliferation assays

When proliferation was measured by cell counting 25,000 cells/well were cultured in 1ml for 3 days, at 37°C in the presence of 5% CO₂ in 24 well plates (Nunc, Roskilde, Denmark). The well was emptied and diluted in 9 ml isoton and counted on a Casy automated cell counter (Schärfe System GmbH, Reutlingen, Germany). For thymidine incorporation, cells (5000 cells/well) were cultured for 3 days in 200 μl at 37°C in the presence of 5% CO₂ in 96 well flat bottom plates (Nunc). [³H]-Thymidine (2 Ci/m mole, Amersham, Bucks, UK) was added at a final concentration of 1 μCi/ml (37 kBq/ml) the last 4 hours of culture. The cells were then harvested on glass fibre filters (Wallac, Turku, Finland) and radioactivity was measured by liquid scintillation counting.

MTX bioassay.

For the measurement of MTX in sera from RA patients, sera were heated for 30 minutes at 56°C and stored at −20°C until use. Serum was added to B9.H (5000 cells/well) in two-fold dilutions, starting with 25% of the total culture volume. All dilutions were tested in duplicate cultures. MTX was freshly diluted (2-fold) as a standard curve (highest concentration 100 ng/ml) and added to B9.H cells. After 3 days proliferation was measured using [³H]-thymidine incorporation as described above.

Patients

The study was approved by the local medical ethical committee and all patients gave written informed consent. Blood was taken from 23 RA patients, 2 hours after the first oral dose of MTX (10 mg). Serum was collected and heated for 30 minutes at 56°C to inactivate complement and stored at −20°C until use. MTX concentration in plasma stored at −20°C remains stable for at least 18 months (16).

Results

Effects of thymidine and hypoxanthine on the inhibition of proliferation by MTX.

MTX inhibits proliferation of a mouse B cell line, (figure 1a). Proliferation could be restored via the salvage pathway of nucleotide synthesis by addition of thymidine (T) and hypoxanthine (H) (figure 1a). Our aim was to measure MTX in serum and serum contains 1-2 mg/l H (23) When we analysed proliferation by [³H]-thymidine incorporation, we observed that the combination of serum H and the radioactive T led to decreased sensitivity for MTX.
Figure 1. Proliferation of cells in the presence of MTX. B9 (A) and B9.H (B) cells (25000 cells/ml) were incubated with 1μg/ml MTX, MTX and 100μM hypoxanthine (MTX + H), MTX and 25μM thymidine (MTX + T), or MTX, hypoxanthine and thymidine together (MTX + H/T), as indicated on the x-axis. Cells were counted at day 3. This is a representative of several experiments performed. Error bars indicate the SEM of duplicate cultures.

Figure 2. Sensitivity of B9 and B9.H to MTX. B9 (closed circles) and B9.H (open circles) cells were cultured in the presence of the indicated amounts of MTX. At day 3 [3H]-thymidine was added for 4 hours and cells were harvested. Error bars represent the SEM of 2 separate experiments. Data are represented as the % of the value (cpm) without MTX.

Effects of MTX on HGPRT negative cell line.
To circumvent this difficulty we decided to use B9.H, a subclone of B9 that is negative for the enzyme HGPRT, an enzyme of the salvage pathway of nucleotide synthesis. As expected, the proliferation of this cell line could not be rescued by the addition of hypoxanthine and thymidine (figure 1b). Indeed we found that B9.H is more sensitive to MTX than the parental B9 (figure 2).

Recovery of MTX after heating.
Before testing patient sera, the recovery of MTX in different individual sera was investigated. Sera of 5 normal donors were spiked with 200 nM, 20 nM and 5 nM MTX
and heated for 30 minutes at 56°C to avoid complement-induced toxicity to the murine B9.H cells. The concentration of MTX was determined with the bioassay as described in the materials and methods section. The recovery of MTX in these sera is close to 100% (table 1). Two sera spiked with 5 nM were excluded because at a serum concentration of 25%, these sera proved to be toxic for the cells without addition of MTX. As this serum toxicity at high serum concentrations is a frequent finding this could limit the sensitivity of the assay. When full sensitivity is required we can eliminate toxicity of serum by boiling samples for 10 minutes. All (toxic) proteins are precipitated with this procedure, and, if needed, serum can be tested at 1:2 dilution. To assess the effects of this boiling procedure, various concentrations of MTX were added to 1ml HPS. We boiled 0.5 ml for 10 minutes and heated 0.5 ml at 56°C for 30 minutes. Precipitated proteins were spun down and in the supernatant, MTX was measured with the bioassay. As expected boiling did not affect MTX activity (figure 3).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Sera spiked with MTX (nM)</th>
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<tr>
<td>Input</td>
<td>measured (mean)</td>
</tr>
<tr>
<td>200</td>
<td>208</td>
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<tr>
<td>20</td>
<td>24.1</td>
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<td>5</td>
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Table 1. Sera spiked with MTX. Human sera were spiked with the indicated concentration of MTX and heated at 56°C for 30 minutes. MTX concentration was determined using the bioassay.

Figure 3. Recovery of MTX after heating. B9.H cells were cultured in the presence of heated sera containing different amounts of MTX. MTX was added to the sera before heating. The input is plotted against the values measured.

**MTX measurement in sera from RA patients.**

MTX was measured in 23 sera of RA patients. Sera were collected from these patients 2 hours after their first oral dose of MTX. The concentration MTX measured in the individual sera ranged from 32 to 269 ng/ml. The results of two independent measurements are plotted against each other in figure 4. The mean of the first measurement was 315 nM (SEM 25) and the second 270 nM (SEM 25).
Figure 4. **MTX measurements in serum of RA patients.** Two hours after an oral dose of 10 mg MTX, serum MTX concentration of 23 RA patients was determined in two separate experiments using the bioassay.

Figure 5. **Comparison of bioassay and TDx assay.** Two hours after an oral dose of 10 mg MTX, serum MTX concentration of 23 RA patients was determined using the bioassay and the commercially available TDx assay (Abbott). The results are also depicted in an Altman and Bland plot. Dotted lines indicate 2xSD from the mean difference (striped line).

**Comparison of bioassay with TDx assay.**
To confirm the results obtained with the bioassay, we have compared our results with the results of the frequently used and commercially available TDx assay. All 23 RA sera that were tested in the bioassay were send to a facility were MTX concentration in serum is routinely measured with the TDx assay. The outcome of both measurements is depicted in figure 5a. With the TDx assay the mean MTX concentration was 343 nM (SEM 26) and with the bioassay the mean concentration was 290 nM (SEM 25). The Altman and Bland plot (fig 5b) shows that there is reasonable agreement between the methods and that the difference between the two is not dependent of the actual MTX concentration.

**Effect of MTX polyglutamation on proliferation**
In the cell the majority of the MTX is polyglutamated. We have tested the effects of polyglutamated forms of MTX on the proliferation of B9.H cells. Of the polyglutamated forms tested, only MTX-glu2 is capable of inhibiting B9.H proliferation but at approximately 5-fold higher concentrations than MTX (figure 6). The activity of the MTX-glu4 and -glu5 forms is 400-fold lower.
Figure 6. Effect of MTX polyglutamation on proliferation. Concentration curves of MTX and MTX-glu2, -glu4 and -glu5 were added to B9.H cells. At day 3 [\textsuperscript{3}H] thymidine was added for 4 hours and cells were harvested. Error bars represent the SEM of duplicate cultures.

Discussion

For the measurement of MTX in human serum we have developed a bioassay. This assay makes use of mouse B9 cells that are negative for the enzyme HGPRT (B9.H); proliferation of these cells is measured by the incorporation of radiolabelled thymidine. MTX present in serum samples inhibits proliferation of B9.H cells. HGPRT-negative B9.H cells cannot use the salvage pathway of nucleotide synthesis. Consequently, the presence of nucleotides, either present in FCS, released by dying cells in the culture, or radiolabelled thymidine, can not disturb the assay. In addition, this clone proved to be more sensitive to MTX. Very likely the absence of HGPRT accounts for the increased sensitivity of B9.H cells compared to B9 cells. It has been reported that certain HGPRT-negative cells, derived from the cell line HL-60, are 3 times more sensitive to the effects of MTX than wild type cells (24).

Several groups have studied the pharmacodynamics of MTX for different reasons (12-15;26-31). Many have used the commercially available TDx method for measurement of MTX in plasma (1213;14;28-31). Although MTX dose and administration vary between the studies the reported mean plasma levels measured in the first two hours are similar. However, the half life of MTX that is reported is more variable, individuals that have a half life of more than 30 hours are reported (13,29). Several hours after intake of MTX an inactive metabolite, 7-OH-MTX, rises in plasma. This metabolite can cross react with the TDx assay. Although cross reactivity is reported to be lower than 1.5% (13) this can present a problem when MTX is measured at levels are around the sensitivity limit of the assay, for example at 24 hours after oral intake. A late time point, such as 24 hours, is especially important for the calculation of half-lives. It is possible that interference of 7-OH-MTX in the assay can be the reason for the discrepancy in half-lives that are reported in the studies mentioned before. The advantage of measurement of MTX with the bioassay is that only functional MTX can be measured. These measurements are therefore independent of differences in MTX metabolism of patients.

MTX serum levels were measured with the bioassay in 23 patients, two hours after they received their first oral dose of 10 mg MTX. These levels correlate well with the levels measured with the TDx assay. The mean MTX concentration we have measured lies within the reported range of MTX levels described by others (30,31).
With this bioassay it is possible to measure low amounts of MTX in serum especially after boiling of the serum samples. Here we showed that a spiked 5 nM could be recovered by testing the sample at a 1:4 dilution. The EMIT has a sensitivity of 200 nM, the HPLC method of 30 nM and the TDX assay of 10 nM (16). The radio-ligand binding assay reaches a sensitivity of 1 nM (15,20) and is slightly more sensitive than the bioassay. The enzymatic assay has a comparable sensitivity (3 nM) (17). To achieve maximal sensitivity, without possible serum effects on the viability of B9.H cells, it is best to boil all serum samples before use. This results in precipitation of all serum proteins and allows testing in a 1:2 serum dilution shifting the theoretical detection limit to 2.5 nM. A concentration method as described by Sinnet et al. is also suitable for use in the bioassay (12). Concentration of the sample will lead to a further lowering of the detection limit.

In table 1 we reported about toxicity of serum even in the absence of MTX. These effects of were only observed at serum concentrations of more than 12.5% of the total culture volume. In this case it was known that the samples did not contain MTX, hence the observed toxicity was due to other factors. Occasionally it can be questionable whether toxicity is induced by contaminated serum or MTX. In that case it is possible to discriminate between the two because inhibition by MTX can be reversed by addition of folic acid (32).

Folate supplementation is given to RA patients treated with MTX to reduce MTX related toxicity. In most cases folic acid is given (30,33;34). After 48 weeks of MTX therapy with folic acid supplementation mean total folate levels in serum rise to 59.7 nM (34). Folic acid, up to 90 µM, does not have an effect on proliferation of B9.H cells, nor on MTX induced inhibition of proliferation (not shown). Folic acid supplementation is exceptional. However, folic acid can prevent MTX induced inhibition of proliferation of B9.H cells at 0.32 µM, these concentrations are probably not reached in plasma after a weekly oral dose of 2.5 mg (34). Therefore, it is unlikely that folate supplementation of patients interferes with the bioassay. The bioassay described here is a simple and sensitive assay to measure MTX in serum and can be performed using techniques which are available at many rheumatological or immunological laboratories. Although thorough validation is necessary, this assay might be useful to study the pharmacodynamics of MTX in RA patients. Only future clinical studies will show if it has a place in routine monitoring.

Acknowledgements.

This study was supported by a grant of the Dutch League Against Rheumatism (NR 98-1-402).

References


