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Methotrexate ameliorate T cell dependent autoimmune arthritis and encephalomyelitis but not antibody- or fibroblast-induced arthritis

Franziska Lange1,2, Estelle Bajtner1, Carola Rintisch1, Kutty Selva Nandakumar1, Ulrich Sack2, Rikard Holmdahl1

1 Medical Inflammation Research, Lund University
2 Dept of Clinical Immunology and Transfusion Medicine, Leipzig University

Corresponding author: Rikard Holmdahl, Section for Medical Inflammation Research, BMC I11, Lund University, S-22184 Lund, Sweden, Tel: +(46)462224607, Fax: +(46)462223110, e-mail: rikard.holmdahl@inflam.lu.se

Objective. To investigate the mode of action of methotrexate (MTX) in different types of models for RA and MS

Methods. We selected models for RA and MS known to have different pathogenesis i.e. fibroblast induced arthritis in SCID mice, collagen induced arthritis (CIA), anti-collagen II antibody induced arthritis (CAIA) and experimental autoimmune encephalomyelitis (EAE) in (Balb/c x B10.Q)F1 and B10.Q mice and pristane induced arthritis in DA rats (PIA). The MTX treatment was started one day after the onset of disease and continued for 14 days to properly compare the effects on the different models.

Results. All models known to be critically dependent on T cell activation (CIA, PIA and EAE) were effectively downregulated by titrated doses of MTX. In contrast no effects were seen on fibroblast induced arthritis or CAIA. No effects were seen on the levels of anti-CII antibodies in the CIA experiment.

Conclusion. The data show that MTX has strong ameliorative effect on both classical models of RA, like CIA and PIA, but also on a model for MS, EAE. It also suggests that MTX operates only in diseases which are preceded by, and dependent on, T cell activation. A comparison between CAIA and CIA suggest that MTX operates independently of arthritogenic antibodies. These results demonstrate that different animal models reflect the complexity of the corresponding human diseases and suggest that several models should be used for effective screening of new therapeutics.

Key Words. Collagen induced arthritis, fibroblast induced arthritis, experimental allergic encephalomyelitis, methotrexate, pristane
Introduction

Several disease modifying antirheumatic drugs (DMARD) are used in the treatment of RA. The two most important DMARD’s today are inhibitors of TNFa and methotrexate (MTX) (1). MTX is an analogue of folic acid and as such it is an anti-metabolite and a potent inhibitor of dihydrofolate reductase, which subsequently inhibits the de novo purine and pyrimidine synthesis. Based on these properties it was developed as a cytostatic agent. Several pharmacological mechanisms for MTX have been reported. MTX has been shown to act through various mechanisms such as direct promotion of synovial cell apoptosis and thereby reducing the pannus formation (2), blocking proliferation of lymphocytes (3), inhibition of IL-1 production (4) and vascular endothelial cell proliferation (5) and increase in adenosine release (6). The arthritis suppressive effect of the today used low dose MTX treatment is not clarified although cytokine patterns are disturbed but it is unclear whether the disease promoting mechanism also involves immune priming or only effector functions of the inflammatory attack. Differences in MTX response would not only depend on the state of the disease progress in the RA patient but also the type of molecular pathway involved, as RA most likely is a heterogeneous syndrome rather than a specific disease entity.

To investigate the effects of MTX on experimental models of RA we have used four different models that represent various facets of RA: the well known collagen induced arthritis model (CIA) in mice (7), the anti-collagen II (CII) antibody induced arthritis (CAIA) in mice (8), the pristane induced arthritis model (PIA) in rats (9) and the new fibroblast induced arthritis model (LS48-SCID) in mice (10). To compare the effects of these disease models we initiated treatment one day after the onset of disease and continued the treatment for 14 days.

Methods

Animals
The animals for the CIA, CAIA, PIA and EAE experiments were bred and kept at the animal department of Medical Inflammation Research, which is a conventional department with a climate-controlled environment with 12h light/dark cycles and water and food ad libitum. For the CIA and CAIA experiments we used (C57/BL10.Q x Balb/c)F1 mice (termed QB) . For the EAE experiments we used C57Bl/10.Q mice (termed BQ) and for the PIA experiments we used DA rats. The LS48 induced arthritis was performed in the Medizinisch-Experimentelles-Zentrum of the university of Leipzig (Germany) under specific pathogen free conditions (27°C, 80% air humidity, 12h light/dark cycles) and with water and food ad libitum. The mice were female C.B-17-scid/IcrCrl mice purchased from Charles River (Wiga, Sulzfeld, Germany) and acclimatized for at least 1 week prior to any experimental manipulation and kept in laminar-flow filter cabinets. All animals were used sex- and age-matched and the animals were marked, randomly mixed in the cages and scored by investigators in a blinded fashion. Animal experiments followed national guidelines for animal experiments and was approved by local animal protection committees in Sweden and Germany, respectively.

Methotrexate (MTX) treatment
MTX was purchased from Medac (Hamburg, Germany) and diluted in PBS in accordance with the weight of the animals and the needed conc of the experiment. It was kept in darkness at 4°C. Animals were treated by daily intraperitoneal injections of indicated concentrations of MTX in PBS, in mice using a volume of 200 μL and in rats 1 mL per injection. Identical
CIA
CIA was induced using native rat CII prepared from a rat chondrosarcoma after pepsin digestion as previously described (11). The CII was emulsified in complete Freund’s adjuvant (CFA, Difco, Detroit, MI) to a final concentration of 1mg/ml and 100 µl was injected intradermally at the base of the tail of QB mice on day 0. The mice were boosted on day 21 with a 50µl injection intradermally at the base of the tail with a homogenate containing 50µg CII suspended in incomplete Freund’s adjuvant (IFA, Difco), prepared as described above. Clinical scoring was performed as described earlier (11). Briefly, each inflamed toe or knuckle gives one point, whereas an inflamed wrist or ankle gives five points, resulting in a maximal score of 15 (5 toes + 5 knuckles + 1 wrist/ankle) for each paw and 60 points for each mouse. All mice were scored daily. One day after onset of the disease (score ≥ 1 point) the treatment was started. Arthritic mice received PBS or MTX daily by i.p. injection (200µl) for 14 days. The animals were sacrificed on the last day of the treatment. The mice were bled on day 0, day 21 and then individually on day 7, 10 and 14 after disease onset. The levels of anti-CII IgG were determined using quantitative ELISA as described earlier (11). For the induction of CAIA we used purified CII-specific mAbs M2139 (IgG2b) and CIIC1 (IgG2a). At least 16 weeks old males were injected i.v. with a combination of the two mAbs (M2139 and CIIC1) at a total concentration of 9 mg per mouse. Control mice received an equivalent volume of PBS. On day 5, all mice were injected i.p. with LPS (50µg/mouse). The scoring and treatment was performed as described above.

PIA
For the PIA experiment we used male and female rats between 8 and 10 weeks of age were immunized intradermally at the base of the tail with 150 µl pristane (Sigma). Scoring and treatment was performed as described above, however rats were injected with PBS or MTX in a volume of 1mL for each injection.

LS48 induced arthritis
The LS48 cell line (German collection of microorganisms and cell cultures, Braunschweig [DSMZ] accession number DSM ACC 2455; Biotectid, Leipzig, Germany) is a permanently growing murine cell line with characteristics of fibroblasts and without the tendency to express subclones. Cells were cultured using 75cm² culture flasks (Greiner, Solingen, Germany) with 1x10⁵ cells/ml in a cell culture medium containing DMEM and RPMI 1640 (1:2), 10 % FCS, 25 mM HEPES, penicillin (100 U/ml) and streptomycin (100 µg/ml; all Gibco, Eggenstein, Germany; hereafter called culture medium). At confluency, the cells were trypsinized with trypsin-EDTA (0.25 % trypsin/0.2 % EDTA; Gibco) for 5 min and diluted 1:2 in 150 cm³ culture flasks. To induce arthritis the mice were anesthesized by an intraperitoneal application of a combination of metomidate (6 mg/100 g BW) and fentanyl (0.006 mg/100 g BW); the injection site was shaved and disinfected. 5x10⁵ cells suspended in 20 µl PBS were instilled into the right knee joints through the patellar tendon. Joint swelling (both the injected and non-injected side) was monitored daily by measuring the diameter with caliper. Treatment was performed as described above starting one day after injection of the cells and continuing for 14 days. The mice were sacrificed at the last day of the treatment. To prepare serial paraffin sections, the knee joints were fixed in 4 % paraformaldehyde for two days, then placed in a decalcifying solution (70 ml of 85 % formic acid, 10% glutaraldehyde and 1 % sodium metaperiodate).
acid, 85 ml of 30% hydrochloric acid, 70 g AlCl₃, 1 liter of distilled water) for 24 h. The joints were transferred into a graded series of ethanol (70%, 80%, 96%, 100%) for several hours and placed subsequently in methylbenzoate (10 h), xylene (3 h) and paraffin (20 h) before they were embedded in paraffin blocks. The extent of joint destruction was assessed histologically evaluating hematoxylin/eosin-stained paraffin sections (5-7 μm). All histology reagents were obtained from Merck.

EAE
EAE was induced using 150 μg synthetic MOG 79-90 peptide, emulsified in *Mycobacterium tuberculosis* H37Ra in complete Freund’s adjuvant (CFA, Difco, Detroit, MI) (equal volume of peptide/PBS and CFA). This solution was homogenised, and 100μl of the homogenate was injected intradermally at the base of the tail. 500 ng pertussis toxin was given i.p. immediately after the immunization and 48 h post immunization. All animals were weighed and scored daily. Clinical score was designated as follows: 0, no detectable signs of EAE; 1, affected tail tonus; 2, tail paralysis; 3, mild hind leg paresis; 4, severe hind leg paresis; 5, one hind leg paralysis; 6, complete hind leg paralysis; 7, complete hind leg paralysis and fore legs paresis; 8, death as described earlier (12).

At score 5 or more, the mice got 0.5-1.0 ml 0.1% NaCl s.c. in the neck. Affected mice received PBS or well-defined amounts of MTX daily by i.p. injection (200μl) for 14 days. The animals were sacrificed individually on the last day of the treatment.

Statistics
All comparisons of disease scores include all animals in the experiment and the statistical analysis is determined using the software Statview. A non-parametric test, Mann-Whitney, was used when two groups or Kruskal Wallis when more than two groups were compared. A p value of less than 0.05 is regarded as significant.

Results

Dose titration and treatment of collagen induced arthritis (CIA)
To determine the most effective dose of MTX treatment we first performed a dose titration study on CIA. The mice showed the first signs of arthritis around day 21 after immunization and thereafter each mouse with arthritis were randomly selected to be treated with different doses of MTX (0.1mg/kg; 2.5mg/kg; 5mg/kg) or PBS. The MTX treatment had a significant and dose dependent effect on the score of the disease (fig.1) with the maximal effect by the intermediate dose of 2.5 mg/kg. No toxic effects were seen at any of the used doses. All the mice developed a high anti-CII antibody titre independent of the arthritis severity and the MTX dose (fig. 1b).

CAIA
For the treatment of the CAIA model we selected the most efficient MTX dose (2.5 mg/kg) and the treatment started after onset of arthritis, which was around 5 days after the monoclonal anti-CII antibody injection. All mice continued the expected development of arthritis as seen in this model but with no effect of MTX treatment (fig. 2).

PIA
All animals developed a severe PIA with a sudden onset around day 12 after immunization (Fig 3). The doses we used for the mice were found to be toxic for the rats in a previous pilot experiment (data not shown). We therefore predetermined a series of lower doses that showed
no toxicity. Thus the doses used in the experiment shown in Fig 3 were titrated it from 0.1mg/kg to 0.075mg/kg and 0.05mg/kg. All doses of MTX used had a significant ameliorative effect on the arthritis but with no obvious dose-titration effect. It is possible that the effect seen was maximal as the joint in this model rapidly gets destroyed and the healing process is difficult to differ from the inflammatory process.

**LS48 induced arthritis**

To bypass both antibody and T cell mediated pathways in the development of arthritis we used a previously undescribed model which is induced by injection of a fibroblast cell line (LS48) into SCID mouse. Arthritis developed as expected, starting at day 1, and was recorded by measuring the thickness of the knee joints every day (Fig 4). The mice were treated with the same doses as used for the CIA experiment (0,1mg/kg; 2,5mg/kg and 5mg/kg MTX or PBS) starting after the onset of arthritis. To confirm the development of arthritis histology sections were made at the end of the experiment (day 14) (Fig 5). Surprisingly we could not see any effect by the MTX treatment.

**EAE**

To determine the effect of MTX on another inflammatory disease with a clearly documented T cell dependent pathway we selected an EAE model. The mice developed the first signs of the disease 10 days after MOG peptide injection and as with the arthritis model we started the treatment the first day after onset in each mouse (Fig 6). The mice were treated with the same doses of MTX as used in the CIA experiment (0,1mg/kg; 2,5mg/kg; 5mg/kg) or PBS. The treatment had a significant and dose dependent effect on the severity of the disease with the highest dose (5 mg/kg) as the most efficient.

**Discussion**

The finding that MTX treatment efficiently modulates some models for RA but not others indicates that MTX is not a general anti-inflammatory or joint-protective agent but is more specifically directed to pathways operating more efficiently in only some forms of arthritis. Clearly, MTX efficiently suppress the classical CIA model even when administered after the priming period and after the onset of arthritis. Since the treatment started after the onset of arthritis it did not affect the immune priming following immunization and not the earliest inflammatory events with synovial hyperplasia and infiltration of inflammatory cells. Thus, as expected, no effect was seen on the levels of antibodies to CII, which is determined by activation of T cell dependent B cells during the immune priming process. The joint targeted effector mechanism of the classical CIA model is probably quite complex involving T cell stimulation of synovial cells, T cell independent mesenchymal activation and an arthritogenic effect by antibodies binding to cartilage. It is generally believed that, at least in the acute phase, the antibody mediated component is dominating (13-17). Consequently, serum from CII immunized mice and also CII-specific monoclonal antibodies transfer arthritis (8, 18-20). It was therefore surprising to find that MTX treatment had no effect on the CAIA model indicating that MTX operates through another pathway not associated with arthritogenic antibodies in the CIA model. To test the possibility that MTX treatment is efficient in T cell dependent inflammatory models we used a model for multiple sclerosis induced with a peptide from MOG(12). This is a T cell dependent model, which in contrast to CIA develops without the involvement of pathogenic antibodies (21, 22). In addition, TNFa has been suggested to play no or actually a protective role in MOG induced EAE (23). Thus, TNFa has different roles in the CIA and EAE, however, the effect of MTX was very similar in the CIA and EAE models underlining that MTX operates on mechanisms shared between these two models.
To further address the possibility that MTX influence T cell dependent pathways leading to arthritis we used the PIA model in DA rats. PIA is known to be both dependent and mediated by T cells (9, 24). The disease can be transferred with CD4+ αβ TCR+ T cells (24). MTX turned out to be more potent but also more toxic in the rat but at doses 10 times lower than in the mouse dosage it was possible to very efficiently suppress the development of established PIA. The mechanisms whereby T cells mediate arthritis are poorly known but they are likely to involve many types of synovial cells like macrophages and fibroblasts. These can be activated to secrete cytokines like TNFa or proteases like MMP13 or differentiate into destructive cells like osteoclasts (25-27). Both macrophages and fibroblasts can also be activated without the help of T cells and the role of T cells in their activation is not clear. However, to address such downstream pathways without dissecting the precise mechanisms we used an arthritis model induced with injection of activated fibroblasts into SCID mouse. In this fibroblast-mediated and T cell independent model MTX treatment had clearly no effect again indicates that the therapeutic effect is dependent on mechanisms in the joints that are T cell dependent. There is one earlier experiment describing low dose MTX treatment of the CIA model, the effects seen were similar to the results described here although the treatment was started before onset (28). A difference was however that in their experiments both antibodies and TNFa production by T cells were lowered. This effect is likely to be an effect by MTX on the immune priming in lymph nodes, a process not likely to be important for the results in our experimental set up in which immune priming had already occurred.

Our goal with these series of treatment experiments was not to identify the precise mechanisms of MTX, which are likely to be very complex, but rather to document the usefulness of various arthritis models. Clearly, the CIA and PIA models respond to MTX treatment whereas the CAIA and fibroblast models do not. Thus, in the hypothetical case in which MTX was developed for human treatment today then MTX would have to be tested on animal models if it would comply with the FDA guidelines. Obviously in this case scenario the MTX only would be looked upon favourably if the CIA or PIA models but not if the CAIA and SCID were used. However, this is only a short-term treatment and it is possible that longer treatment as is the standard treatment in humans, would have been more predictive. Obviously, treatment with MTX 1 day after onset of RA has not been performed and in addition it is difficult to predict the doses for treatment to be used as even mouse and rats differed dramatically in dose response. Treatment mimicking the actual treatment in humans would have also required the use of chronic arthritis models but this is not normally achievable easily for testing new drugs, i.e. anti-TNFα was not tested in such models although it has later been shown to be efficient also in chronicity(29). In addition, RA is a complex and heterogeneous disease, and develops in discrete stages, and it is important to develop treatments for different variants and the effect on different models are therefore of value. As we have a limited knowledge of the variants of RA and the fact that the in vivo mechanisms of newly developed drugs is unknown, it is difficult to predict which animal model should be used to verify the proof of principle of a drug. Therefore, it would be more beneficial to use a selected number of animal models that are well characterized in pathways and molecular mechanism are valuable and will increase our knowledge about RA and shorten the time to reach new beneficial treatments.

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The authors declare no competing commercial interest
**Figure legends**

**Figure 1a**
MTX treatment of CIA. Day 0 indicates onset of arthritis of each mouse and MTX is given at different doses starting 1 day after the onset. The mice are treated with 0.1 mg MTX/kg (n=5), 2.5 mg MTX/kg (n=3), 5 mg MTX/kg (n=3) and a control group treated with PBS (n=5). The mean score of all mice each day is given ± SEM. * indicate significant differences in severity scores, p<0.05, for the groups treated with 2.5 mg/kg and 5 mg/kg in comparison with the control group.

**Figure 1b**
Anti-CII antibody levels in the CIA experiment. The mice were bled at day 14 after onset of arthritis. Mean values ±SEM are indicated. No significant differences by MTX treatment were observed.

**Figure 2**
MTX treatment of CAIA. One group of mice are treated with MTX (2.5 mg/kg)(n=13) and a control group treated with PBS (n=13) starting 1 day after the onset of arthritis. Control mice are treated with PBS only. The mean score of all mice each day is given ± SEM. No significant effect of MTX was seen.

**Figure 3**
MTX treatment of PIA. Day 0 indicates onset of arthritis of each rat and MTX is given at different doses starting 1 day after the onset. The rats are treated with 0.005 mg MTX/kg (n=6), 0.075 mg MTX/kg (n=6), 0.1 mg MTX/kg (n=13) and a control group treated with PBS (n=6). The mean score of all rats each day is given ± SEM. * indicate significant differences in severity scores, p<0.05, for all MTX treated groups in comparison with the control group. In the group with highest MTX dose (0.1 mg MTX/kg) 8 rats died at different time points during the experiment.

**Figure 4**
MTX treatment of fibroblast induced arthritis in SCID mice. MTX is given at different doses (5 mg/kg, 2.5 mg/kg, 0.1 mg/kg and PBS in the control group, n=9 in each group) starting 1 day after the injection of the fibroblasts, when all mice already had developed arthritis. Scoring starts at day 1 when the arthritis commence and the treatment is given from this day and continuous daily until day 14. The mean diameter of the injected left knee of all mice in each group each day is given ± SEM. No significant effect of MTX was seen.

**Figure 5**
Histological aspect of left SCID mouse knee joint 14 days after injection of 5 x 10^5 LS48 cells into the articular space. Original magnification 16x (a) and 40 X (b). LS48 cells form a dense tissue attaching to and invading into articular structures. Stained with hematoxylin-eosin.

**Figure 6**
MTX treatment of EAE. Day 0 indicates onset of encephalomyelitis of each mouse and MTX is given at different doses starting 1 day after the onset. The mice are treated with 0.1 mg MTX/kg (n=11), 2.5 mg MTX/kg (n=10), 5 mg MTX/kg (n=10) and a control group treated with PBS (n=12). The mean score of all mice each day is given ± SEM. Stars indicate significant differences in encephalomyelitis severity scores, p<0.05 in comparison with the control group.
References

Fig1b anti-CII-antibody levels in CIA
Fig2a  MTX-Treatment in CAIA

Mean Clinical Score vs Day After Onset

- 0 mg/kg
- 2.5 mg/kg
Fig3  MTX-Treatment in PIA

Mean Clinical Score vs Day After Onset for different doses of MTX:
- 0 mg/kg
- 0.05 mg/kg
- 0.075 mg/kg
- 0.1 mg/kg

* denotes statistically significant difference.
Fig 4. MTX-Treatment in fibroblast induced arthritis.
Fig 6  MTX-Treatment in EAE

![Graph showing mean clinical score over days for different doses of MTX in EAE, with error bars and a trend line. The x-axis represents days after onset, ranging from d0 to d14, and the y-axis represents mean clinical score from 0 to 4.5. Different doses are indicated by different markers: 0 mg/kg (square), 0.1 mg/kg (triangle), 2.5 mg/kg (cross), and 5.0 mg/kg (pentagon). There is a starred area indicating a significant difference.]
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