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Effect of methotrexate on Th1 and Th2 immune responses in mice

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Abstract

We investigated the effect of the anti-rheumatic drug methotrexate (MTX) on Th1 and Th2 immune responses in mice. For this investigation, mice were immunized subcutaneously at the base of the tail with ovalbumin (OVA) emulsified with complete Freund's adjuvant (day 0). Varying doses of MTX were orally administered daily from days 0 to 20. On day 21, anti-OVA IgG2a and interferon-γ (IFN-γ) as indicators of Th1 responses and anti-OVA IgG1 and interleukin-10 (IL-10) as those of Th2 responses were measured. The results showed that treatment with MTX was followed by decreases in OVA-specific IgG and proliferation of spleen cells to the antigen. The anti-rheumatic drug inhibited both anti-OVA IgG2a and IgG1production, although the inhibitory effect of MTX on the antigen-specific IgG2a production appeared to be greater than that on IgG1 production. IFN-γ, but not IL-10, secretion was markedly downregulated by MTX. Administration of MTX resulted in suppression of antigen (OVA)-induced arthritis in mice. The suppression of the joint inflammation by MTX was associated with inhibition of OVA-specific proliferative responses of spleen cells, anti-OVA IgG, IgG2a and IgG1 production, and IFN-γ and IL-10 secretion, although more pronounced decreases in IgG2a and IFN-γ were observed compared with those in IgG1 and IL-10 in MTX-treated mice. These results indicate that MTX appears to suppress Th1 and, to a lesser extent, Th2 immune responses and its anti-arthritic effect on human rheumatoid arthritis might be at least in part explained by down-regulation of Th1 responses involved in the disease.

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Introduction

Two subsets of helper T cells, types I (Th1) and II (Th2) (Mossmann et al 1986; Mossmann & Coffman 1989), have distinct roles in the immune system. Th1 cells modulate cellular immunity by producing interleukin-2 (IL-2) and interferon-γ (IFN-γ) whereas Th2 cells are implicated in humoral responses by secreting IL-4, IL-5, and IL-6. In addition, IFN-γ suppresses Th2 immune responses (Dickensheets et al 1999), while IL-4 (van Roon et al 1995) and IL-10 downregulate Th1 responses (Yin et al 1997). These Th1 and Th2 cytokines have been shown to play an important role in a number of diseases in man. For example, IFN-γ is produced in joints of patients with rheumatoid arthritis (Liblau et al 1995) and neutralization of IFN-γ attenuates the severity of the disease (Sigidin et al 2001). An increased expression of IL-4 mRNA is observed in the sputum of patients with asthma (Oliverstein et al 1999) and neutralization of IL-4 blocks the development of acute airway hypersensitivity in a murine asthma model (Corry et al 1996).

Methotrexate has been used to treat patients with cancer. Treatment with methotrexate results in marked inhibition of dihydrofolate reductase followed by decreases in purine and thymidine associated with anti-tumour effects. Methotrexate is also used to treat patients with rheumatoid arthritis, although doses used for the treatment are lower than those given to patients with cancer (First & Kremer 1988). The mechanisms of the anti-rheumatic effects of methotrexate include suppression of neutrophil leukotriene synthesis (Speling et al 1990), downregulation of protein and lipid methylation (Nesher & Moore

1990) and upregulation of secretion of anti-inflammatory autocoid adenosine (Cronstein et al 1991). However, few studies demonstrated the effect of methotrexate on Th1 and Th2 immune responses.

In this study, we show that treatment with methotrexate results in marked suppression of Th1 responses, including antigen-specific IgG2a antibody production as well as secretion of IFN-γ, while the anti-rheumatic drug showed relatively mild suppression of Th2 responses, including IgG1 production. Methotrexate failed to affect secretion of the Th2 cytokine IL-10.

Materials and Methods

Materials

Female DBA/1J mice, 7–9 weeks of age, were used in all experiments. The mice were maintained in a temperature-controlled environment with free access to standard rodent chow and water. Experiments were performed in accordance with the ethical guideline of Kobe Pharmaceutical University.

Immunization with ovalbumin

One hundred micrograms of ovalbumin (Sigma-Aldrich Fine Chemicals, St Louis, MO) was dissolved in 50 μL of phosphate-buffered saline (PBS) and emulsified with an equal volume of complete Freund's adjuvant (CFA; Difco Laboratories, Detroit, MI). One hundred microlitres of the emulsion was injected subcutaneously into the base of the tail (day 0).

Administration of methotrexate

Varying doses, including 0.1, 0.5 and 2.5 mg kg^{-1}, of methotrexate were dissolved in 500 μL of saline containing 1% bicarbonate buffer (pH 9.6) and orally administered daily on days 0–20. Five hundred microlitres of saline containing 1% bicarbonate buffer (pH 9.6) alone was given as a control. There was no significant difference in body-weight gain between control- and methotrexate-treated mice (Table 1).

Induction of antigen-induced arthritis (AIA)

Mice were immunized with ovalbumin as described above and 21 days later the mice were intra-articularly challenged by injection of 25 μL of PBS containing 50 μg of ovalbumin into left ankle joints (Yoshino 1995). The right ankle joints were injected with 25 μL of PBS alone as a control. To evaluate the severity of arthritis, the thickness of the both ankles was measured using a dial gauge caliper calibrated with 0.01-mm graduations. The net increase in joint thickness attributable to the antigenic challenge was calculated by subtracting the increase in thickness of the right ankle from that of the left ankle. There was no net joint swelling after injection of ovalbumin in non-immunized mice.

Table 1 Effect of methotrexate on mice body-weight gain.

Methotrexate dose (mg/kg)	Body weight (g)	
	Day 0	Day 21
0 (Saline)	23.1 ± 0.7	23.8 ± 0.9
0.1	22.7 ± 0.5	23.6 ± 0.5
0.5	22.8 ± 0.9	23.8 ± 1.2
2.5	22.7 ± 0.6	23.7 ± 0.8

Mice were orally given methotrexate at the time of immunization with ovalbumin (day 0) and then daily up to day 20. Values are shown as mean ± s.e.m. of 5 or 6 mice. Data are representative of three experiments. There were no significant differences in body weights among the above groups when data were analysed using a one-way analysis of variance followed by Bonferroni's test.

Measurement of antibodies to ovalbumin

Sera were obtained on day 21 and heat inactivated at 56 °C for 30 min. Anti-ovalbumin IgG, IgG1, and IgG2a were measured using an ELISA as described previously (Yoshino et al 1999). In brief, 96-well flat-bottom microtitre plates were incubated with 100 μL/well of ovalbumin (100 μg mL^{-1}) at 37 °C for 1 h and washed three times with PBS containing 0.05% Tween-20. The wells were then blocked by incubation with 100 μL of PBS containing 1% casein (Sigma) at 37 °C for 30 min. After washing, the plates were incubated with 100 μL of a 1:5000 (for IgG and IgG1 measurement) or 1:500 (for IgG2a measurement) dilution of each serum sample at 37 °C for 30 min. The plates were washed, and 100 μL/well of a 1:2000 dilution of alkaline phosphatase-labelled rat anti-mouse IgG (Sigma) or 1:1000 dilution of alkaline phosphatase-labelled anti-mouse IgG1 and IgG2a (PharMingen, San Diego, CA) was added and incubated at 37 °C for 1 h. After washing, 100 μL of 3 mM *p*-nitrophenylphosphate (Bio-Rad, Richmond, CA) was added per well and the plates were incubated in the dark at room temperature for 15 min. The absorbance was then measured at 405 nm by IMMUNO-MINI NJ-2300 (Nalge Nunc International K.K., Tokyo, Japan). The results were expressed as absorbance units at OD_{405} ± s.e.m.

Measurement of cytokines

Spleens were removed from the mice on day 21 and cell suspensions prepared (Yoshino et al 1998). Erythrocytes in the cells were lysed with Tris-NH_4Cl. A total of 5×10^6 cells in 1 mL of RPMI1640 (Sigma) containing 1 mM L-glutamine, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 5×10^{-5} M 2-mercaptoethanol and 2% heat-inactivated autologous mouse serum was cultured in 24-well tissue culture plates either in medium alone or with 50 μg mL^{-1} ovalbumin. Forty-eight hours later supernatants were harvested and stored at −20 °C until assayed. Cytokine production was quantified by commercially

available ELISA kits (Endogen Inc., Woburn, MA) for IL-10 and IFN-γ. No IL-4 was measured as a Th2 cytokine by an ELISA since the cytokine secretion was under the limit of detection.

Proliferation assay

A total of 5×10^5 spleen cells in 0.1 mL of RPMI1640 (Sigma) containing 1 mM L-glutamine, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 5×10^{-5} M 2-mercaptoethanol and 2% heat-inactivated autologous mouse serum was cultured with 50 μg mL^{-1} of ovalbumin in each well of 96-well tissue culture plates. Seventy-two hours later, each well was pulsed with 0.5 μCi of tritiated thymidine and the cells were cultured for another 17 h. The cultures were harvested onto fibreglass filters using a multiharvester and counted using standard liquid scintillation techniques. Results, expressed in counts min^{-1}, were the average of triplicate cultures of cells pooled from 5 or 6 mice.

Statistics

The effects of methotrexate concentration on the various experimental outcomes were statistically evaluated using a one-way analysis of variance followed by Bonferroni's test for comparison of individual treatments or, alternatively, using a Mann–Whitney U-test. In this study, $P < 0.05$ was accepted to denote significance.

Results

Effect of methotrexate on ovalbumin-specific immune responses

Treatment with methotrexate was followed by a decrease in anti-ovalbumin IgG antibodies in sera in a dose-related fashion (Table 2). The serum level of anti-ovalbumin IgG in mice treated with 2.5 mg kg^{-1} of methotrexate was 49% lower than that in control mice. Proliferative responses of spleen cells to ovalbumin were also dose-dependently suppressed in mice given methotrexate; approximately 53% inhibition (vs control) of cell proliferation was observed in mice treated with 2.5 mg kg^{-1} of methotrexate.

Effect of methotrexate on Th1 and Th2 immune responses

Administration of methotrexate resulted in decreases in both ovalbumin-specific IgG2a and IgG1, with the decrease in anti-ovalbumin IgG2a appearing to be greater than that in the antigen-specific IgG1 (Figure 1). The highest dose (2.5 mg kg^{-1}) of methotrexate showed 76% inhibition of IgG2a production, whereas 43% inhibition of IgG1 production was seen in mice given the same dose.

To investigate the effect of methotrexate on secretion of Th1 and Th2 cytokines, IFN-γ and IL-10 were measured, respectively. Treatment with methotrexate resulted in marked suppression of IFN-γ production (up to 56% inhibition), whereas no inhibitory effect of the drug on IL-10 secretion was observed (Figure 2).

Effect of methotrexate on AIA

To study the effect of methotrexate on AIA induced by immunization with ovalbumin (day 0) followed by intra-articular injection of the antigen on day 21, mice were orally given methotrexate daily over a period of 21 days commencing on day 0. Treatment with methotrexate was followed by significant suppression of AIA (Figure 3). The suppressive effect of methotrexate on joint inflammation was associated with a significant decrease in ovalbumin-specific proliferative responses of splenic cells as well as antigen-specific IgG. Both Th1 responses, including anti-ovalbumin IgG2a and IFN-γ production, and Th2 responses, such as the antigen-specific IgG1 and IL-10 secretion, were downregulated by methotrexate, with the downregulation of the Th1 response by the anti-rheumatic agent appearing to be greater than that of the Th2 response (Table 3).

Discussion

This study demonstrates that methotrexate appears to have marked inhibitory effects on Th1 responses since treatment of mice with methotrexate was followed by a pronounced decrease in Th1 responses including anti-ovalbumin IgG2a and IFN-γ production. Methotrexate seems to have less effect on Th2 responses since the

Table 2 Effects of methotrexate on production of anti-ovalbumin IgG and proliferative responses of spleen cells to the antigen.

Methotrexate dose (mg/kg)	Anti-ovalbumin IgG (A405)[a]	Proliferation (counts min^{-1})[b]
0 (Saline)	0.79 ± 0.04	60 167 ± 5595
0.1	0.65 ± 0.06	47 094 ± 1889
0.5	0.52 ± 0.08	31 185 ± 887
2.5	0.40 ± 0.03*	28 081 ± 720

Mice were orally given methotrexate at the time of immunization with ovalbumin (day 0) and then daily up to day 20. On day 21, anti-ovalbumin IgG in sera and proliferative responses of spleen cells to the antigen were measured as described in Materials and Methods. [a]Mean ± s.e.m. of 5 or 6 mice; [b]mean ± s.e.m. of triplicated cultures of cells pooled from 5 or 6 mice. Data are representative of three experiments. *$P < 0.05$ vs control, a one-way analysis of variance followed by Bonferroni's test.

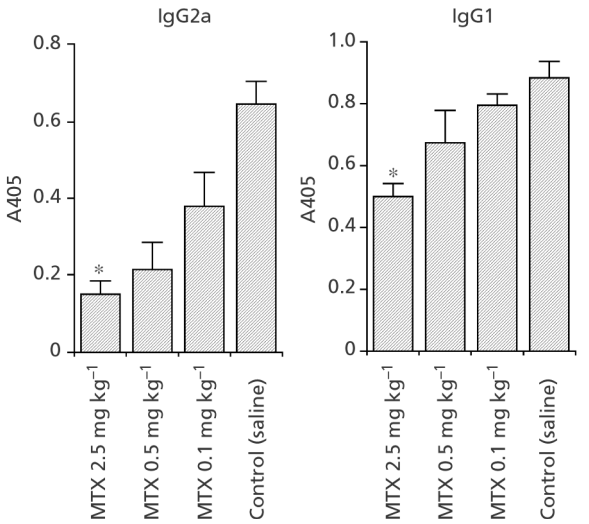

Figure 1 Effect of methotrexate on anti-ovalbumin IgG1 and IgG2a production. Mice were immunized with ovalbumin on day 0. Five hundred microlitres of saline containing the indicated doses of methotrexate were orally administered daily on days 0–20. Five hundred microlitres of saline alone was given as a control. On day 21, the mice were sacrificed and anti-ovalbumin IgG2a and IgG1 antibodies were measured as described in Materials and Methods. Bars show the mean ± s.e.m. of 5 or 6 mice. Data are representative of three experiments. $*P < 0.05$ vs control, a one-way analysis of variance followed by Bonferroni's test.

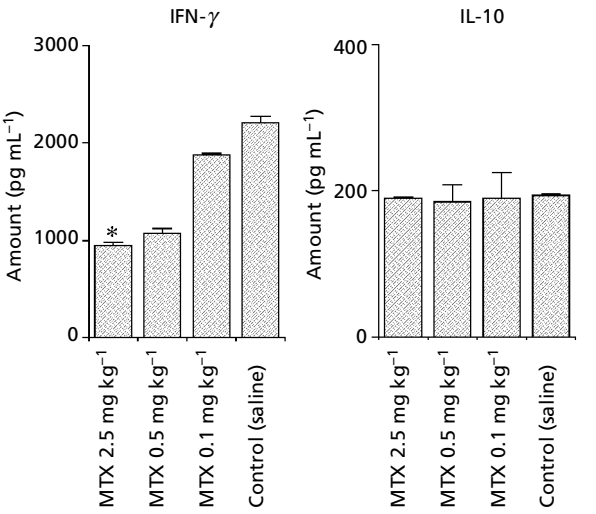

Figure 2 Effect of methotrexate (MTX) on IFN-γ and IL-10 production by spleen cells. Mice were immunized with ovalbumin on day 0. Five hundred microlitres of saline containing containing the indicated doses of methotrexate were orally administered daily on days 0–20. Five hundred microlitres of saline alone was given as a control. On day 21, the mice were sacrificed and spleen cells were cultured with ovalbumin for 48 h. IFN-γ and IL-10 were measured as described in Materials and Methods. Spleen cells from naive non-immunized mice produced an undetectable level (less than 5 pg mL^{-1}) of IFN-γ and IL-10. Bars show the mean ± s.e.m. of 5 or 6 mice. Data are representative of three experiments. $*P < 0.05$ vs control, a one-way analysis of variance followed by Bonferroni's test.

suppressive effect of the anti-rheumatic drug on the Th2 antibody anti-ovalbumin IgG1 production was relatively mild. Moreover, methotrexate had no significant effect on secretion of IL-10.

Methotrexate has been shown to inhibit production of antigen-specific IgG (Shikata et al 1996), total IgG (Rackham et al 2002) and rheumatoid factors (Spadaro et al 1993). In our studies, methotrexate also suppressed anti-ovalbumin IgG production, supporting previous results. The suppression of anti-ovalbumin production by methotrexate seems to be explained by inhibition of the antigen-specific proliferative responses of spleen cells, since anti-ovalbumin IgG production is T-cell dependent. Furthermore, the decrease in lymphoid cell proliferation in methotrexate-treated mice may be due to induction of apoptosis of activated T cells (Cronstein et al 1993) or production of adenosine (Huang et al 1997) by the drug, which should inhibit T-cell activation and proliferation via the cAMP-dependent signaling pathway (Genestier et al 1998).

There are only a few studies demonstrating the effect of methotrexate on Th1 and Th2 responses. For instance, Constantin et al (1998) previously reported that methotrexate enhanced IL-10 gene expression but suppressed IL-2 and IFN-γ gene expression in peripheral blood mononuclear cells from rheumatoid arthritis patients, which had been stimulated with the mitogen phytohemagglutinin (PHA) in-vitro. They therefore concluded that the in-vivo beneficial effect of methotrexate in rheumatoid

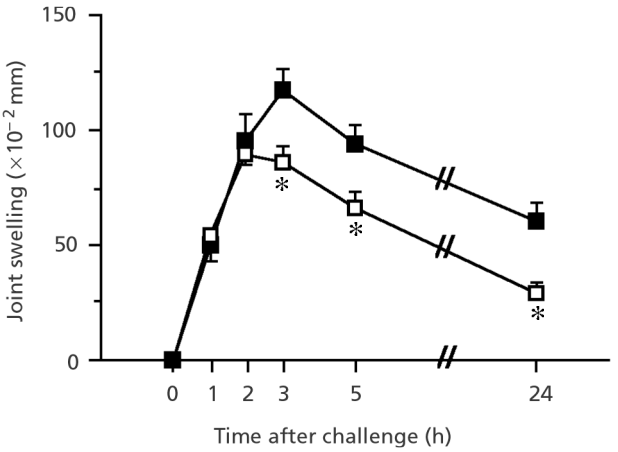

Figure 3 Effect of methotrexate on AIA. Mice were immunized with ovalbumin on day 0. Five hundred microlitres of saline containing the indicated doses of methotrexate were orally administered daily on days 0–20. Five hundred microlitres of saline alone was given as a control. On day 21, AIA was induced by intra-articular injection of ovalbumin into ankle joints as described in Materials and Methods. Closed square, saline alone (control); open square, saline containing 2.5 mg kg^{-1} of methotrexate. Vertical bars show s.e.m. of 5 or 6 mice. Data are representative of three experiments. $*P < 0.05$ vs control, Mann–Whitney analysis.

Table 3 Effect of methotrexate on Th1 and Th2 responses in mice with AIA.

Methotrexate dose (mg/kg)	Anti-ovalbumin IgG (A405)[a]	Proliferation (counts min^{-1})[b]	Th1 responses		Th2 responses	
			Anti-ovalbumin IgG2a (A405)[a]	IFN-γ (pg mL^{-1})[b]	Anti-ovalbumin IgG1 (A405)[a]	IL-10 (pg mL^{-1})[b]
0 (Saline)	1.1 ± 0.04	$68\,203 \pm 3832$	0.93 ± 0.06	1804 ± 155	0.98 ± 0.03	901.3 ± 12
2.5	0.65 ± 0.07**	$21\,214 \pm 3931$	0.42 ± 0.04**	476.9 ± 37	0.75 ± 0.07**	514.6 ± 31

Mice were orally given methotrexate at the time of immunization with ovalbumin (day 0) and then daily up to day 20. On day 21, anti-ovalbumin IgG in sera and proliferative responses of spleen cells to the antigen were measured as described in Materials and Methods. [a]Mean ± s.e.m. of 5 or 6 mice; [b]mean ± s.e.m. of triplicated cultures of cells pooled from 5 or 6 mice. Data are representative of three experiments. **$P < 0.01$ vs control, Mann–Whitney analysis.

arthritis might be explained by upregulation of Th2 cytokine secretion and downregulation of Th1 cytokine production. Our ex-vivo studies demonstrated that treatment with methotrexate was followed by a decrease in IFN-γ secreted from spleen cells stimulated with the antigen ovalbumin, supporting the in-vitro finding by Constantin et al (1998). However, our ex-vivo studies showed that administration of methotrexate failed to modulate secretion of IL-10. In contrast, the Th2 cytokine production was suppressed by methotrexate in mice with AIA. Methotrexate suppressed anti-ovalbumin IgG2a and IgG1 production, with the suppression of the antigen-specific IgG2a production appearing to be greater than that of IgG1 production. Therefore, we conclude that the anti-rheumatic actions during the treatment of rheumatoid arthritis might be due to downregulation of Th1 responses but not upregulation of Th2 responses. Differences in the methods of treatment with methotrexate may explain the differences between the above effects of the drug and results of previous studies.

The precise mechanism by which methotrexate suppressed Th1 responses more effectively than Th2 responses remains to be investigated. However, possible mechanisms may include production of adenosine by methotrexate (Cronstein et al 1993), which increases intracellular cAMP levels in T cells (Huang et al 1997) since an increase of intracellular cAMP level results in inhibition of Th1 but not Th2 cells (Munoz et al 1990; Heo et al 1998). Another possible mechanism is selective death of Th1 but not Th2 cells caused by the cytotoxicity of methotrexate. The cytotoxic effect of methotrexate can be reduced by p-glycoprotein, one of the multidrug-resistance proteins (de Graaf et al 1996; Norris et al 1996). Activated, but not resting, Th1 and resting and activated Th2 cells express such protein (Lohoff et al 1998), indicating that resting Th1 cells have higher risk of death when treated with methotrexate compared with activated Th1 cells and resting and activated Th2 cells. It is of note that in our experiments mice were treated with methotrexate from the time of immunization with ovalbumin (i.e. before T cells, including Th1 cells, were activated by the antigen).

As shown in Figure 2, the secretion of IL-10 in mice used in our studies was much weaker than that of IFN-γ, suggesting that Th2 induction was minimal. Therefore, there is a possibility that treatment with methotrexate might significantly affect Th2 responses in mice in which IL-10 is preferentially produced.

Treatment with methotrexate resulted in significant suppression of AIA associated with marked decreases in anti-ovalbumin IgG2a and IFN-γ and with relatively mild decreases in anti-ovalbumin IgG1 and IL-10. This suggests that the downregulation of Th1 responses by the anti-rheumatic agent may be critically involved in the suppression of the joint inflammation more than that of Th2 responses. Novaes et al (1996) also demonstrated that methotrexate suppressed AIA in rabbits and that the suppression of the disease was related to decreases in inflammatory mediators including prostaglandin E_2, thromboxane B_2 and IL-1β. However, no investigation was carried out in terms of the role of Th1 and Th2 responses in the suppression of AIA by methotrexate. To our knowledge, this is the first demonstration that methotrexate modulates Th1 and Th2 immune responses in AIA.

The relatively distinct correlation of the suppression of AIA by methotrexate with the downregulation of IFN-γ and IgG2a production by the drug suggests that Th1 responses may play a central role in arthritis. The role of Th1 responses in arthritis has also been demonstrated in collagen-induced arthritis in mice, which is another experimental model of rheumatoid arthritis (McIntyre et al 1996). Furthermore, a higher ratio of IFN-γ to IL-4 mRNA expression in synovial tissues of patients with rheumatoid arthritis has been observed (Scola et al 2002), indicating that rheumatoid arthritis may be a Th1-dominant disease. Therefore, the beneficial effect of methotrexate on rheumatoid arthritis may be at least in part explained by downregulation of Th1 responses.

Conclusions

Methotrexate inhibited Th1 and, to a lesser extent, Th2 immune responses in mice. Treatment with methotrexate was followed by suppression of AIA which was associated with a marked decrease in Th1 responses, suggesting that the mechanisms of the beneficial efficacy of the anti-

rheumatic agent in rheumatoid arthritis may include downregulation of Th1 immune responses involved in the disease.

References

Constantin, A., Loubet-Lescoulie, P., Lambert, N., Yassine-Diab, B., Abbal, M., Mazieres, B., de Preval, C., Cantagrel, A. (1998) Antiinflammatory and immunoregulatory action of methotrexate in the treatment of rheumatoid arthritis: evidence of increased interleukin-4 and interleukin-10 gene expression demonstrated in vitro by competitive reverse transcriptase-polymerase chain reaction. *Arthritis Rheum.* **41**: 48–57

Corry, D. B., Folkesson, H. G., Warnock, M. L., Erle, D. J., Matthay, M. A., Wiener-Kronish, J. P., Locksley, R. M. (1996) Interleukin 4, but not interleukin 5 or eosinophils, is required in a murine model of acute airway hypersensitivity. *J. Exp. Med.* **183**: 109–117

Cronstein, B. N., Eberle, M. A., Gruber, H. E., Levin, R. I. (1991) Methotrexate inhibits neutrophil function by stimulating adenosine release from connective tissue cells. *Proc. Natl Acad. Sci. USA* **88**: 2441–2445

Cronstein, B. N., Naime, D., Osted, E. (1993) The antiinflammatory mechanism of methotrexate. *J. Clin. Invest.* **92**: 2675–2682

de Graaf, D., Sharma, R. C., Mechetner, E. B., Schimke, R. T., Roninson, I. B. (1996) P-glycoprotein confers methotrexate resistance in 3T6 cells with deficient carrier-mediated methotrexate uptake. *Proc. Natl Acad. Sci. USA* **93**: 1238–1242

Dickensheets, H. L., Venkataraman, C., Schindler, U., Donnelly, R. P. (1999) Interferons inhibit activation of STAT6 by interleukin 4 in human monocytes by inducing SOCS-1 gene expression. *Proc. Natl Acad. Sci. USA* **96**: 10800–10805

First, D. E., Kremer, J. M. (1988) Methotrexate in rheumatoid arthritis. *Arthritis Rheum.* **31**: 305–314

Genestier, L., Paillot, R., Fournel, S., Ferraro, C., Miossec, P., Revillard, J.-P. (1998) Immunosuppressive properties of methotrexate: apoptosis and clonal deletion of activated peripheral T cells. *J. Clin. Invest.* **102**: 322–328

Heo, Y., Lee, W. T., Lawrence, D. A. (1998) Differential effects of lead and cAMP on development and activities of Th1- and Th2-lymphocytes. *Toxicol. Sci.* **43**: 172–185

Huang, S., Apasov, S., Koshiba, M., Sitkovsky, M. (1997) Role of A2a extracellular adenosine receptor-mediated inhibition of T-cell activation and expansion. *Blood* **90**: 1600–1610

Liblau, R. S., Singer, S. M., McDevitt, H. O. (1995) Th1 and Th2 CD4+ T cells in the pathogenesis of organ-specific autoimmune diseases. *Immunol. Today* **16**: 34–38

Lohoff, M., Prechtl, S., Sommer, F., Roellinghoff, M., Schmitt, E., Gradehandt, G., Rohwer, P., Stride, B. D., Cole, S. P., Deeley, R. G. (1998) A multidrug-resistance protein (MRP)-like transmembrane pump is highly expressed by resting murine T helper (Th2), but not Th1 cells, and is induced to equal expression levels in Th1 and Th2 after antigenic stimulation in vivo. *J. Clin. Invest.* **101**: 703–710

McIntyre, K. W., Shuster, D. J., Gillooly, K. M., Warrier, R. R., Connaughton, S. E., Hall, L. B., Arp, L. H., Gately, M. K., Magram, J. (1996) Reduced incidence and severity of collagen-induced arthritis in interleukin-12-deficient mice. *Eur. J. Immunol.* **26**: 2933–2938

Mossmann, T. R., Coffman, R. L. (1989) TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol.* **7**: 145–173

Mossmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A., Coffman, R. L. (1986) Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* **136**: 2348–2357

Munoz, E., Zubiaga, A. M., Merrow, M., Sauter, N. P., Huber, B. T. (1990) Cholera toxin discriminates between T helper 1 and 2 cells in T cell receptor-mediated activation: role of cAMP in T cell proliferation. *J. Exp. Med.* **172**: 95–103

Nesher, G., Moore, T. M. (1990) The in vitro effects of methotrexate on peripheral blood mononuclear cells: modulation by methyl donors and spermidine. *Arthritis Rheum.* **33**: 954–959

Norris, M. D., de Graaf, D., Haber, M., Kavallaris, M., Madafiglio, J., Gilbert, J., Kwan, E., Stewart, B. W., Mechetner, B. B., Gudkov, A. V., Roninson, I. B. (1996) Involvement of MDR1 P-glycoprotein in multifactorial resistance to methotrexate. *Int. J. Cancer* **65**: 613–619

Novaes, G. S., Mello, S. B., Laurindo, M., Cossermelli, M. (1996) Low dose methotrexate decreases intraarticular prostaglandin and interleukin 1 levels in antigen induced arthritis in rabbits. *J. Rheumatol.* **23**: 2092–2097

Oliverstein, R., Taha, R., Minshall, E. M., Hamid, Q. A. (1999) IL-4 and IL-5 mRNA expression in induced sputum of asthmatic subjects: comparison with bronchial wash. *J. Allergy Clin. Immunol.* **103**: 238–245

Rackham, O. J., Sills, J. A., Davidson, J. E. (2002) Immunoglobulin levels in methotrexate treated paediatric rheumatology patients. *Arch. Dis. Child.* **87**: 147–148

Scola, M. P., Thompson, S. D., Brunner, H. I., Tsoras, M. K., Witte, D., van Dijk, M. A., Grom, A. A., Passo, M. H., Glass, D. N. (2002) Interferon-γ:interleukin 4 ratios and associated type 1 cytokine expression in juvenile rheumatoid arthritis synovial tissue. *J. Rheumatol.* **29**: 369–378

Shikata, Y., Murai, E., Ito, M., Kitta, T., Sakyo, K., Hidaka, M., Shiba, T., Kishi, I. (1996) Evaluation of antirheumatic action of methotrexate (MTX) (1) Effects of MTX and 7-hydroxymethotrexate on collagen-induced arthritis and studies on the analgesic, anti-inflammatory and antipyretic actions in rats. *Ouyou Yakuri* **52**: 425–436

Sigidin, Y. A., Loukina, G. V., Skurkovich, B., Skurkovich, S. (2001) Randomized, double-blind trial of anti-interferon-γ antibodies in rheumatoid arthritis. *Scand. J. Rheumatol.* **30**: 203–207

Spadaro, A., Riccieri, V., Sili Scavalli, A., Taccari, E., Zoppini, A. (1993) One year treatment with low dose methotrexate in rheumatoid arthritis: effect on class specific rheumatoid factors. *Clin. Rheumatol.* **12**: 357–360

Speling, R. I., Coblyn, J. S., Larkin, J. K., Benincaso, A. I., Austen, K. F., Weinblatt, M. E. (1990) Inhibition of leukotriene B4 synthesis in neutrophils from patients with rheumatoid arthritis by a single oral dose of methotrexate. *Arthritis Rheum.* **33**: 1149–1155

van Roon, J. A., van Roy, J. L., Duits, A., Lafeber, F. P., Bijilsma, J. W. (1995) Proinflammatory cytokine production and cartilage damage due to rheumatoid synovial T helper-1 activation is inhibited by interleukin-4. *Ann. Rheum. Dis.* **54**: 836–840

Yin, Z., Braun, J., Neure, L., Wu, P., Liu, L., Eggens, U., Sieper, J. (1997) Crucial role of interleukin-10/interleukin-12 balance in the regulation of the type 2 T helper cytokine response in reactive arthritis. *Arthritis Rheum.* **40**: 1788–1797

Yoshino, S. (1995) Antigen-induced arthritis in rats is suppressed by the inducing antigen administered orally before, but not after immunization. *Cell. Immunol.* **163**: 55–58

Yoshino, S., Murata, Y., Ohsawa, M. (1998) Successful induction of adjuvant arthritis in mice by treatment with a monoclonal antibody against IL-4. *J. Immunol.* **161**: 6904–6908

Yoshino, S., Sasatomi, E., Mori, Y., Sagai, M. (1999) Oral administration of lipopolysaccharide exacerbates collagen-induced arthritis in mice. *J. Immunol.* **163**: 3417–3422