Enhanced *in Vitro* Induced Production of Interleukin 10 by Peripheral Blood Mononuclear Cells in Rheumatoid Arthritis Is Associated with Clinical Response to Methotrexate Treatment

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ABSTRACT. ***Objective.*** To investigate the effect of *in vivo* treatment with methotrexate (MTX) on the regulation of *ex vivo* interleukin 10 (IL-10) production by peripheral blood mononuclear cells (PBMC) derived from patients with rheumatoid arthritis (RA).

Methods. Spontaneous as well as lipopolysaccharide (LPS) and phytohemagglutinin (PHA) induced IL-10 release was assessed by a specific immunoassay in culture supernatants of PBMC derived from 32 patients with active RA before and 6, 12, and 24 weeks after MTX treatment. IL-10 production was correlated to the clinical response. As a control, IL-10 release from PBMC of 7 healthy blood donors was determined.

Results. PBMC of patients with RA showing > 50% improvement of the Paulus index after 3 and 6 months of MTX treatment (responders; n = 18) exhibited significantly enhanced IL-10 production after *in vitro* stimulation with LPS, whereas constitutively released IL-10 was below the detection limit of the immunoassay in all patients and controls. In contrast, IL-10 release from LPS stimulated PBMC of RA patients who showed < 20% improvement by Paulus index (nonresponders; n = 14) or who even deteriorated compared to baseline disease activity was markedly downregulated during MTX treatment *in vivo*. PHA-induced IL-10 release from PBMC *in vitro* was not significantly affected by MTX *in vivo* whether RA patients responded or not to MTX.

Conclusion. Enhanced *ex vivo* LPS induced IL-10 production by PBMC of patients with RA is associated with a favorable therapeutic response to MTX treatment, whereas reduced production coincides more closely with disease deterioration or insufficient response. This may reflect both disease outcome upon treatment and/or the mode of the antiinflammatory action of MTX in RA. Because the LPS — but not the PHA — induced *ex vivo* IL-10 production by PBMC was stimulated by MTX *in vivo*, monocytes seem to be the prominent target cells for this drug mediated antiinflammatory cytokine regulation. (J Rheumatol 2001;28:496–501)

Key Indexing Terms:
RHEUMATOID ARTHRITIS INTERLEUKIN 10 METHOTREXATE
PERIPHERAL BLOOD MONONUCLEAR CELLS

Low dose methotrexate (MTX) has proven efficacy in controlling the inflammatory manifestations of rheumatoid arthritis (RA) in short term and longterm prospective studies[1-3]. In RA, the immunosuppressive properties of MTX seem to play a secondary role[4], whereas its antiinflammatory actions are much more prominent: inhibition of neutrophil chemotaxis and adherence[5-7], inhibition of neovascularization[8], coordinated suppression of inflammatory cytokines like interleukin 1ß (IL-1ß) and IL-8 and stimulation of cytokine inhibitors such as IL-1 receptor antagonist (IL-1ra) and soluble tumor necrosis factor receptors (sTNFR)[9-11], stimulation of tissue inhibitor of metalloproteinases 1 (TIMP-1)[12] in monocytes, and a shift from Th1 to Th2 responses[13] in peripheral blood mononuclear cells (PBMC).

Among Th2 lymphokines, IL-10 has attracted particular attention because of its possible therapeutic potential in RA[14]. IL-10 inhibits proinflammatory cytokine and chemokine production and stimulates natural cytokine inhibitor production in blood mononuclear cells and synovial fibroblasts[15] in addition to blocking T cell responses to specific antigens through inhibition of costimulatory properties of macrophages[16]. Preclinical studies in a

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variety of animal models including collagen induced arthritis[17-19] have shown that IL-10 is effective in preventing[18] or inhibiting[19] inflammation and autoreactivity[20-22]. Moreover, the exogenous addition of IL-10 to RA cell cultures *in vitro* has been shown to affect several immunopathological processes in RA[23-29].

Although circulating and synovial levels of IL-10 are increased in RA[30,31], evidence suggests that there may be a relative deficit of available IL-10[32]. We investigated whether IL-10, which has immunoregulatory and antiinflammatory properties, is one of the therapeutic targets of MTX in RA. The recent observation that MTX stimulated IL-10 gene expression by PHA stimulated PBMC from RA patients *in vitro*[13] prompted us to carry out an *ex vivo* study by monitoring spontaneous and LPS, as well as PHA, induced production of IL-10 by PBMC of RA patients before and after MTX treatment *in vivo* and by correlating the results to the clinical response.

MATERIALS AND METHODS

Patients. Thirty-two patients with active RA were treated for up to 24 weeks with weekly intramuscular or subcutaneous injections of MTX (15 mg). Active RA was defined by fulfillment of at least 3 of the following 4 criteria: 6 or more joints tender or painful on motion, 3 or more swollen joints, erythrocyte sedimentation rate (ESR) > 28 mm/h, and morning stiffness > 45 min duration. MTX and dosage of concomitant nonsteroidal antiinflammatory drugs (NSAID) and steroids (≤ 7.5 mg prednisone/day) were kept constant during the whole study. Clinical assessment was performed before and 6, 12, and 24 weeks after MTX therapy. Laboratory assessment before and during treatment included ESR, routine hematology, erythrocyte folinic acid, serum transaminases, alkaline phosphatase, and creatinine.

After 12 weeks of MTX treatment at a constant weekly dosage of 15 mg the patients were divided into 2 groups, responders and nonresponders, according to a composite activity index[33]. Patients showing improvement of > 50% compared to baseline were defined as responders, and patients with improvement < 20% or those who deteriorated upon treatment were classified as nonresponders.

Cells. Venous blood was drawn from RA patients 24 h after the last intramuscular or subcutaneous injection of MTX, and PBMC were isolated by Ficoll-Hypaque fractionation[34]. The cells were washed 3 times in phosphate buffered saline (PBS) and resuspended in culture medium (10^6 cells/ml). The number of monocytes was determined by differential counting after staining for nonspecific esterase[35]. Monocyte counts in patients' PBMC preparations ranged between 19 and 36% before treatment, and no statistically significant intra or intergroup or intraindividual differences were observed throughout the study. Cells (2×10^5) in 0.2 ml RPMI 1640 supplemented with 100 IU/ml penicillin/streptomycin (Gibco, Basel, Switzerland) and 1% pasteurized plasma protein solution (5% PPL SRK, Swiss Red Cross) were incubated with or without lipopolysaccharide from *E. coli* (100 ng/ml; Gibco), phytohemagglutinin (1 µg/ml; Sigma Chemicals, Buchs, Switzerland), IL-1ß (10 ng/ml; R&D Systems, London, UK), and with or without MTX (10 ng/ml; AHP AG, Zug, Switzerland) in flat-bottom microtiter plates (Nunc, Roskilde, Denmark) in a humidified atmosphere of 5% CO_2 at 37°C for 48 h. These culture conditions proved to be optimal for assessing IL-10 after LPS and PHA stimulation in PBMC supernatants in prior experiments. Cell-free culture supernatants were collected and stored at –70°C until use.

Cytokine assay. IL-10 was determined by a specific human IL-10 ELISA (R&D Systems). The lower detection limit of this assay was 3.9 pg/ml.

Statistics. Intragroup comparisons were performed using Student's t test (responders and nonresponders). Intergroup comparisons were assessed by Wilcoxon ranked sum test. Results were considered statistically significant at $p < 0.05$.

RESULTS

Characteristics of patients at study entry. Patients were retrospectively classified as responders and nonresponders based on a composite activity index for evaluation of the efficacy of second-line drugs[33]: 18 patients (56.3%) fulfilled the criteria of responders, whereas 14 patients (43.7%) had to be classified as nonresponders after a 12 week treatment with low dose MTX. Table 1 shows that the clinical entry variables did not differ significantly between the 2 groups of patients with RA.

Clinical variables of disease activity and ESR before and during MTX treatment. Table 2 shows the changes of clinical disease activity variables like tender and swollen joint score, duration of morning stiffness, and ESR before and during 12 weeks of MTX treatment. Responding patients exhibited a significant reduction of the number of tender and swollen joints, duration of morning stiffness, and ESR ($p < 0.05$–0.005). In contrast, nonresponding patients exhibited a similar number of tender and swollen joints and ESR values as before MTX treatment.

IL-10 production by PBMC before MTX treatment. As shown in Table 3, spontaneous IL-10 production by PBMC of responders as well as nonresponders and healthy controls was below the detection limit of 3.9 pg/ml. However, incubation of PBMC with LPS and PHA markedly induced *in vitro* IL-10 production. The stimulated IL-10 release from PBMC of nonresponders was slightly higher than that from cells of responders ($p < 0.05$) and that from cells of healthy controls was lower than that from PBMC of nonresponders ($p < 0.025$).

Spontaneous and in vitro LPS and PHA stimulated IL-10 production of PBMC during MTX treatment. Figure 1 shows the differences between patients with and without clinical

Table 1. Characteristics of patients at study entry.

	Responders, n = 18	Nonresponders, n = 14
Age, yrs	52.4 ± 12.8*	48.5 ± 8.7
Male/female	4/14	2/12
Duration of RA, mo	16.5 ± 21.3	23.6 ± 22.8
ESR, mm/h	46.4 ± 28.5	42.5 ± 22.7
Positive RF, n (%)	12 (67)	8 (57)
Swollen joint count (0–58)	27.8 ± 18.5	23.6 ± 9.9
Tender joint count (0–60)	32.3 ± 19.6	29.7 ± 16.8
Morning stiffness, min	142 ± 102	114 ± 88
Erosive disease, no. of patients (%)	9 (50)	5 (38)
Current therapy		
NSAID, no. of patients	18	14
NSAID + prednisone, ≤ 7.5 mg/day	8	5

*Values are mean ± SD, unless indicated otherwise.

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Table 2. RA disease activity variables before and after 12 and 24 weeks of MTX treatment in responders and nonresponders.

Variable	Responders, (n = 18)	Nonresponders, (n = 14)
Tender joint score (0–60)		
Before	32.3 ± 19.6	29.7 ± 16.9
12 weeks MTX	19.4 ± 7.9*	25.8 ± 14.2
24 weeks MTX	14.6 ± 5.4*	22.6 ± 12.7
Swollen joint score, 0–58		
Before	27.8 ± 18.5	23.6 ± 9.9
12 weeks MTX	15.4 ± 7.6*	24.5 ± 10.2
24 weeks MTX	10.6 ± 4.8†	21.8 ± 8.6
Morning stiffness, min		
Before	142 ± 102	114 ± 88
12 weeks MTX	62 ± 61**	92 ± 66
24 weeks MTX	25 ± 32†	78 ± 58
ESR, mm/h		
Before	46.4 ± 28.5	42.5 ± 22.7
12 weeks MTX	24.8 ± 16.7***	37.8 ± 21.4
24 weeks MTX	20.4 ± 14.6**	34.8 ± 20.6
Patient's global assessment, 1–5		
Before	3.2 ± 1.2	3.6 ± 1.4
12 weeks MTX	1.6 ± 1.0†	3.2 ± 1.2
24 weeks MTX	1.4 ± 0.9†	3.0 ± 1.2
Physician's global assessment, 1–5		
Before	3.6 ± 1.6	3.6 ± 1.4
12 weeks MTX	1.9 ± 1.2†	3.0 ± 1.3
24 weeks MTX	1.6 ± 1.0†	2.8 ± 1.5

*p < 0.01; **p < 0.0001; ***p < 0.005, †p < 0.001; Student's t test for intragroup comparisons.

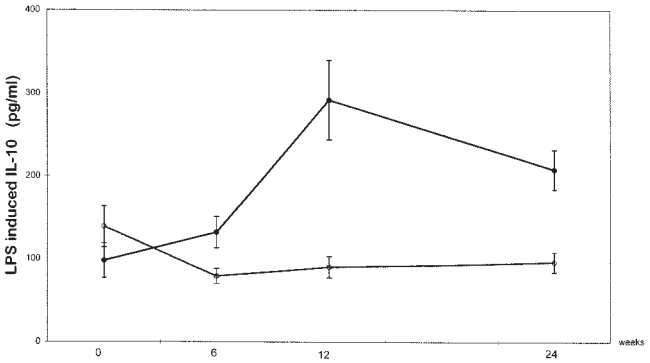

Figure 1. LPS induced IL-10 release from PBMC of patients responding and not responding to methotrexate (MTX) after a treatment period of 6, 12, and 24 weeks; 2 × 10^5 PBMC of RA patients were incubated 2 days with or without LPS (100 ng/ml), and IL-10 was measured in the cell culture supernatants. Figure illustrates mean values ± SEM of responding (●; n = 18) and nonresponding (○; n = 14) patients, with significant differences from baseline values for responders after 6 ($p < 0.05$), 12 ($p < 0.001$), and 24 weeks ($p < 0.01$) and for nonresponders after 6, 12, and 24 weeks ($p < 0.01$) of treatment with MTX. The intergroup comparisons showed a statistically significant difference of $p < 0.001$ after 12 weeks and $p < 0.01$ after 24 weeks between responders and nonresponders.

improvement after 6, 12, and 24 weeks of MTX treatment. There was no significant change of spontaneous IL-10 release from PBMC, which was below the detection limit of the immunoassay in cell cultures from all patients.

However, we found a differentially regulated IL-10 production after stimulation of PBMC with LPS *in vitro*. In the responding patients MTX treatment induced markedly enhanced LPS stimulated IL-10 production after 6 ($p < 0.05$), 12 ($p < 0.001$), and 24 weeks ($p < 0.01$), whereas in nonresponding patients the LPS stimulated IL-10 production by PBMC was already significantly downregulated after 6 weeks of treatment ($p < 0.01$) and remained constant at a low level thereafter. In a few experiments we also examined IL-10 production by PBMC after *in vitro* stimulation with IL-1ß, which showed the same pattern of IL-10 regulation as observed after LPS stimulation both in responders and nonresponders to MTX treatment *in vivo* (data not shown). The PHA induced IL-10 production by cells from responding and nonresponding patients was very close to that observed after LPS stimulation; however, the differences between the 2 patient groups were statistically not significant. These data are shown in Figure 2. In some selected experiments we examined the effect of MTX on spontaneous and LPS and PHA induced IL-10 production of

Table 3. Pretreatment concentrations of spontaneous and LPS and PHA induced IL-10 production by PBMC of responding and nonresponding patients with RA and healthy controls.

Group	Spontaneous IL-10, ng/ml	LPS Induced IL-10, ng/ml	PHA Induced IL-10, ng/ml
Responders, n = 18			
Mean ± SD	< 0.005	0.098 ± 0.095	0.183 ± 0.164
Range	< 0.005	< 0.005–0.423	0.067–0.570
Nonresponders, n = 14			
Mean ± SD	< 0.005	0.139 ± 0.093*	0.229 ± 0.149
Range	< 0.005	0.042–0.364	0.091–0.980
Healthy controls, n = 7			
Mean ± SD	< 0.005	0.087 ± 0.02**	0.169 ± 0.043
Range	< 0.005	0.064–0.110	0.045–0.340

*p < 0.05, Wilcoxon ranked sum test (nonresponders vs responders). ** < 0.025, Wilcoxon ranked sum test (controls vs nonresponders).

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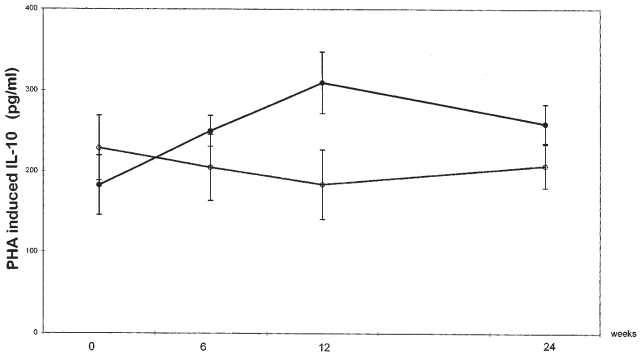

Figure 2. PHA induced IL-10 release from PBMC of patients responding and not responding to MTX after a treatment period of 6, 12, and 24 weeks; 2×10^5 PBMC of RA patients were incubated 2 days with or without PHA (1 µg/ml) and IL-10 was measured in the cell culture supernatants. Illustration shows mean values ± SEM of responding (●; n = 18) and nonresponding patients (○; n = 14). Intraindividual and intergroup differences were not statistically significant.

PBMC of patients *in vitro* before and after MTX treatment *in vivo*. We constantly found a slight but not significant decrease of constitutive and stimulated IL-10 release from PBMC of these patients that was equal whether they belonged to the group of responders or nonresponders (data not shown).

Pretreatment cellular IL-10 production in responding and nonresponding patients. As shown in Table 3 there was a slightly significant difference of LPS induced IL-10 production by PBMC of responding and nonresponding RA patients before MTX treatment, in that nonresponding patients started with a higher IL-10 production ($p < 0.05$) than their responding counterparts, although there were no differences in clinical and humoral disease activity variables between the 2 groups at study entry. No differences between the 2 patient groups or between patients and controls were observed with regard to spontaneous and PHA induced IL-10 release.

DISCUSSION

We show for the first time that *in vivo* treatment with MTX increased the *ex vivo* LPS stimulated IL-10 release from PBMC of patients with RA along with the most pronounced therapeutic response after 3 and 6 months, whereas IL-10 production significantly decreased in nonresponding patients. The marked increase of IL-10 production in responding patients after 3 and 6 months is in agreement with the clinical course of disease upon MTX treatment reaching a plateau effect after 6 months, with a consecutive period of stabilization of disease activity along with a simultaneous stabilization in IL-10 production. In nonresponding patients a different picture of the kinetics of IL-10 production emerged. Although starting at a higher level before treatment compared to nonresponders, the cellularly produced IL-10 already declined after 6 weeks of treatment in nonresponding patients, remaining at a constant low level thereafter.

In contrast to the previous result that MTX stimulated IL-10 production by PHA activated PBMC of MTX treated or nontreated RA patients on the messenger RNA level *in vitro*[13], this result was not confirmed by the *ex vivo* data on the protein level in our study. However, our observations are in accord with a study describing enhanced circulating IL-10 levels in RA patients treated with MTX[36], and also fit quite well to the stimulation of IL-10 release from monocytes, as seen with adenosine[37], provided that one considers adenosine an important effector molecule of the antiinflammatory action of MTX[38]. MTX treatment significantly stimulated the *in vitro* LPS induced — but only marginally stimulated the PHA induced — IL-10 production of PBMC of responding patients, which adds to the known antiinflammatory cellular effects of MTX, as recently reviewed[39]. Again, considering the data of Constantin, *et al*[13], who showed enhanced MTX mediated IL-10 production by PHA stimulated PBMC of RA patients *in vitro* by reverse transcription polymerase chain reaction, it cannot be absolutely ruled out that activated T cells might also contribute to enhanced IL-10 production. However, IL-10 upregulation has so far only been observed with *in vitro* application of MTX to cell cultures, but not in the *ex vivo* situation as in our study. An explanation for this discrepancy could be that kinetics of IL-10 production under *ex vivo* conditions, as in this study, are differently regulated by MTX compared to the *in vitro* situation, considering in particular the different time periods of cellular exposure to pharmacologically relevant amounts of the drug in either clinical or experimental settings. It is possible that after a 2 day culture of PHA stimulated PBMC we were too late to find any significant increase of IL-10 protein, in contrast to enhanced IL-10-specific mRNA levels, which were already detectable after 8 h of PHA stimulation and MTX co-cultivation[13].

In patients with RA as well as in healthy individuals application of IL-10 leads to a decrease of TNF-α and of IL-1ß production[14,40]. In addition, IL-10 dose dependently stimulates cytokine induced IL-1ra release and sTNFR p75 and/or sTNFR p55 shedding from human PBMC and/or synovial fibroblasts[15]. These divergent cellular effects on cytokine agonist and antagonist regulation reflect the coordinated antiinflammatory effects of IL-10 on different cell types contributing to rheumatoid inflammation. In particular, IL-10 might either generate a less inflammatory type of monocyte and/or suppress aberrant T cell function, both leading to downregulation of the immunologically mediated inflammatory process. Based on our present and previous findings[9,11], such a favorable coordinated antiinflammatory effect can obviously be attributed to MTX and may explain the therapeutic efficacy of this drug in the treatment of RA.

The differences of pretreatment levels of LPS induced IL-10 production in responding and nonresponding patients

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may implicate this cytokine as a possible prognostic marker of response to MTX treatment. This has yet to be confirmed with a much larger cohort of RA patients. In conclusion, our findings did not clearly confirm that enhanced IL-10 production is actually the result of specifically MTX mediated effects *in vivo* or merely the reflection of disease remission. However, our observation of a nearly 3-fold increase of *ex vivo* stimulated IL-10 production in patients responding to MTX compared to the marked decline of this antiinflammatory lymphokine in nonresponding patients suggests that IL-10 might be at least a surrogate marker of RA disease activity, which is differentially influenced by the patient's responsiveness to MTX treatment *in vivo*. This is particularly relevant when monocytes are activated by exposure to inflammatory stimuli like LPS or IL-1ß *in vitro*, which may fairly reflect the *in vivo* situation in different tissue compartments in RA[41].

Our observations that an increase of IL-10 production resulted only from stimulation with LPS (and to a much lesser degree with PHA) suggests that monocytes were more important than lymphocytes among PBMC as target cells for IL-10 upregulation under the influence of MTX. However, in a small group of patients with early RA it has recently been shown that during treatment with MTX the number of IL-10 producing CD4+ T cells increases[42], indicating that regulation of IL-10 production by MTX may also occur on the T cell level. One plausible explanation why PBMC did not show increased spontaneous IL-10 production from either patient group treated with MTX is that circulating monocytes among these PBMC were not exposed sufficiently *in vivo* to stimulating factors like circulating primary inflammatory cytokines to allow this upregulation. In rheumatoid synovial fluids or synovial membranes, where relatively high local concentrations of IL-1ß and TNF-α and a much higher degree of monocyte activation are found[41] compared to peripheral blood, IL-10 upregulation upon MTX treatment might be much more prominent than in the circulation and might essentially contribute to the antiinflammatory mode of action of MTX in RA.

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