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Enhanced production of tissue inhibitor of metalloproteinases by peripheral blood mononuclear cells of rheumatoid arthritis patients responding to methotrexate treatment

M. Seitz and J.-M. Dayer[1]

Department of Rheumatology and Clinical Immunology/Allergology, University Hospital, Inselspital, Berne and [1]Division of Immunology and Allergy, University Hospital, Geneva, Switzerland

Abstract

Objective. To determine the effects of methotrexate (MTX) treatment of rheumatoid arthritis (RA) patients (a) on the circulating levels and (b) on the *ex vivo* production of matrix metalloproteinase-1 (MMP-1) and tissue inhibitor of metalloproteinases-1 (TIMP-1) by peripheral blood mononuclear cells (PBMNC).

Methods. Circulating levels, spontaneous *ex vivo* and *in vitro* production of MMP-1, TIMP-1 and interleukin-6 (IL-6) were assessed by immunoassays in sera and culture supernatants of PBMNC derived from 27 patients with active RA before and 3 months after beginning MTX treatment and from seven healthy subjects. The production and serum levels of MMP-1, TIMP-1 and IL-6 were correlated to the clinical response.

Results. PBMNC of RA patients showing $\geqslant 20\%$ improvement of the Paulus index after 3 months of MTX treatment (responders; $n = 16$) exhibited a significantly enhanced production of spontaneous TIMP-1 *ex vivo* which was associated with the enhanced synthesis of IL-6. In contrast, PBMNC of 11 patients with $<20\%$ improvement and/or progression of disease showed a marked reduction of TIMP-1 and IL-6 secretion. Circulating levels of TIMP-1 remained unchanged in both groups whereas serum IL-6 levels declined in the responder group. MMP-1 was detectable only in very few culture supernatants and RA sera. Moreover, PBMNC of healthy donors revealed that MTX also stimulated TIMP-1 and IL-6 release *in vitro*, IL-6 being partially responsible for the induction of TIMP-1 production.

Conclusions. Both *ex vivo* and *in vitro*, the enhanced TIMP-1 production by PBMNC of RA patients and healthy individuals upon MTX treatment is associated with simultaneously enhanced IL-6 release, and enhanced *ex vivo* production of both is clearly associated with short-term clinical efficacy. This may reflect disease remission and favourable effects on host defence mechanisms against aberrant inflammation and extracellular matrix turnover in RA patients undergoing MTX treatment.

KEY WORDS: TIMP-1, Methotrexate, Rheumatoid arthritis, Peripheral blood mononuclear cells.

Low-dose methotrexate (MTX) has proven anti-inflammatory properties, according to both short-term and long-term prospective studies in rheumatoid arthritis (RA) [1–4]. This beneficial clinical effect is attributed to its inhibitory effects on neutrophil function [5–7] and neovascularization both *in vivo* and *in vitro* [8]. More recent studies have shown that monocytes/macrophages are very important cellular targets of MTX-mediated effects [9] and that many anti-inflammatory properties of the latter are presumably due to its interference with pro-inflammatory cytokines such as interleukin-1 (IL-1) [10–14]. Monocytes/macrophages are of particular importance in the pathogenesis of RA [15] owing to their immunoregulatory function, inflammatory potential and their capacity to modulate the turnover of extracellular matrix both directly by producing metalloproteinases and proteinase inhibitors [16, 17] and indirectly by secreting catabolic cytokines like IL-1 [18] and tumour necrosis factor-α (TNF-α) [19] which potently induce metalloproteinase gene expression by resident synovial fibroblasts. It was shown that upon MTX treatment of RA patients, both IL-1 [13] and collagenase [20] gene expression and protein production were markedly down-regulated in inflammatory joints. However, there is no

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Correspondence to: M. Seitz, Department of Rheumatology and Clinical Immunology/Allergology, University Hospital, Inselspital, CH-3010 Berne, Switzerland.

evidence as to whether MTX might also be able to modulate the production of proteinase inhibitors by monocytes/macrophages and lymphocytes, both of which contribute mostly to leucocyte infiltration of the inflamed synovial membrane in RA. The aim of this study, therefore, was to examine the influence of MTX treatment on the *ex vivo* and *in vitro* production of tissue inhibitor of metalloproteinases (TIMP-1) and matrix metalloproteinase-1 (MMP-1) by peripheral blood mononuclear cells (PBMNC) of RA patients and healthy subjects and to correlate the results with the clinical response to treatment.

Methods

Patients

Twenty-seven patients with active RA were treated for up to 12 weeks with weekly intramuscular injections of 15 mg MTX. Active RA was defined by fulfilment of at least three of the following four criteria: six or more joints tender or painful on motion, three or more swollen joints, erythrocyte sedimentation rate (ESR) $\geqslant$28 mm/h and morning stiffness $\geqslant$45 min in duration. The dosage of MTX and of associated non-steroidal anti-inflammatory drugs (NSAIDs) and steroids ($\leqslant$7.5 mg prednisone/day) was kept constant during the whole study. Clinical assessment was performed before and after 12 weeks of MTX therapy. Laboratory tests before and during treatment included ESR, routine haematology, erythrocyte folinic acid, serum transaminases, alkaline phosphatase and creatinine.

After 12 weeks of treatment the patients were retrospectively divided into two groups, 'responders' and 'non-responders', according to a composite activity index which has been previously described [21]. 'Study responders' had to fulfil the criteria of $\geqslant$20% improvement from baseline for morning stiffness, Westergren ESR, tender joint and swollen joint scores (60 and 58 joints, respectively, hips not assessed for swelling graded 0–3), and the patient's and physician's assessment of disease severity [on a five-point scale, ranging from 1 (symptom-free) to 5 (very severe)]. Patients whose condition improved $\leqslant$20% or deteriorated upon treatment were defined as 'non-responders'.

Cells

Venous blood was drawn from RA patients 24 h after the last intramuscular injection of MTX, and blood mononuclear cells were isolated by Ficoll-Hypaque fractionation [22]. The cells were washed three 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 non-specific esterase [23]. Monocyte counts in PBMNC of patients ranged between 17 and 38% before MTX treatment, and no statistically significant intra- and intergroup or intra-individual differences were observed throughout the study. Cells (2×10^5) in 0.2 ml of 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 *Escherichia coli* (LPS, 100 ng/ml; Gibco) in flat-bottom micro-titre plates (Nunc, Merelbeke, Denmark) in a humidified atmosphere of 5% CO_2 at 37°C for 48 h. Cell culture supernatants were collected and stored at -70°C until use. In selected experiments, PBMNC of seven healthy donors were co-cultured for up to 8 days with MTX (1, 10 or 100 ng/ml; Methotrexat Lederle, Zug, Switzerland), a polyclonal goat anti-human IL-6 antibody (300 ng/ml; R&D Systems, Abingdon, UK) or a combination of both. In prior experiments it was found that this antibody was able to capture 100% of human recombinant IL-6 up to a concentration of 20 pg/ml and about 50% up to a concentration of 300 ng/ml of this cytokine in cell-free culture medium. In addition, we used a polyclonal goat anti-human IL-10 antibody (R&D Systems); 100 ng/ml of this antibody was able to capture 100% of recombinant human IL-10 up to a concentration of 100 pg/ml in cell-free culture medium.

Immunoassays

Samples of cell culture supernatants and patient sera were subjected to specific enzyme-linked immunosorbent assays (ELISAs) for the determination of MMP-1 and TIMP-1 as described previously [24–26]. The lower detection limit for MMP-1 was 1 ng/ml and 2 ng/ml for TIMP-1. IL-6 and IL-10 were measured by specific ELISAs purchased from R&D Systems. Laboratory personnel were blind to the clinical data.

Statistics

Intragroup comparisons were performed using Student's *t*-test (responders and non-responders). Intergroup comparisons were assessed by the Wilcoxon ranked sum test. The results were considered statistically significant at $P < 0.05$.

Results

Characteristics of the patients at study entry

Patients were classified retrospectively as responders and non-responders based on a composite activity index for the evaluation of the efficacy of second-line drugs [21]. Sixteen patients (59.3%) fulfilled the criteria of 'responders', whereas 11 patients (40.7%) were classified 'non-responders' after a 12-week treatment with low-dose MTX. The clinical entry variables did not differ significantly between the two groups of RA patients (Table 1).

Characteristics of the patients before and after MTX treatment

Disease activity parameters of patients responding and those not responding to MTX treatment after 3 months were compared with baseline values before treatment. 'Responders' showed a highly significant ($P < 0.0005$ to < 0.001) reduction of all disease activity parameters including swollen joint count, tender joint count, morning stiffness, ESR, patient's and physician's assessment.

TABLE 1. Characteristics of the patients at study entry

	Responders ($n = 16$)	Non-responders ($n = 11$)
Age (yr)	51.8 ± 11.7[a]	51.2 ± 8.8
Males/females	2/14	1/10
Duration of RA (months)	34.6 ± 26.5	38.8 ± 34.6
ESR (mm/h)	52.7 ± 30.8	48.4 ± 28.7
Positive rheumatoid factor	13 (82)[b]	9 (82)
Swollen joint count (0–58)	28.7 ± 16.2	26.5 ± 10.4
Tender joint count (0–60)	32.8 ± 20.4	29.7 ± 13.1
Morning stiffness (min)	142 ± 116	112 ± 82
Erosive disease (% of patients)	12 (75)	8 (73)
Current therapy		
NSAID (number of patients)	16	11
NSAID + prednisone ($\leqslant$ 7.5 mg/day)	5	3

[a]Values are given as the mean ± S.D.
[b]Percentage in parentheses.

In contrast, all these parameters did not vary significantly in 'non-responders' (Table 2).

Spontaneous release of TIMP-1 and MMP-1 from PBMNC

At the cellular level, PBMNC of patients responding well to a 3-month MTX treatment secreted significantly more TIMP-1 during *in vitro* culture than at baseline before therapy ($P < 0.001$) (Fig. 1). In contrast, PBMNC of RA patients whose condition did not improve or even deteriorated upon MTX treatment, showed a marked reduction of TIMP-1 production ($P < 0.01$). There was no difference in proteinase inhibitor production by PBMNC between the two patient groups at baseline before starting MTX. We detected very low levels of spontaneous MMP-1 protein production in PBMNC culture supernatants before MTX treatment in only three patients. MMP-1 secretion was stimulated by LPS but not significantly modulated by MTX treatment in either patient group, whereas LPS-stimulated TIMP-1 production was enhanced in PBMNC cultures of clinical responders. These data are not shown as the number of patient samples investigated was too low.

Serum levels of MMP-1 and TIMP-1

In order to examine if circulating TIMP-1 behaves like an acute phase protein, we measured TIMP-1 serum concentrations before and after MTX treatment in RA patients. As shown in Fig. 2, there were no significant differences in TIMP-1 serum levels in responding and non-responding RA patients before and after 3 months of treatment with MTX. Circulating levels of MMP-1 were below the detection limit of the immunoassay used except in two patients.

IL-6 release by PBMNC

To ascertain whether enhanced TIMP-1 synthesis of PBMNC from patients responding to MTX treatment would be mediated by the concomitantly enhanced secretion of TIMP-1-inducing factors we tested the same cell culture supernatants for IL-6. As shown in Fig. 3, the production of IL-6 was markedly induced ($P < 0.025$) in PBMNC of clinical responders after 3 months of MTX treatment. In contrast, in non-responding patients the low baseline IL-6 released from PBMNC was further reduced ($P < 0.025$) upon MTX treatment.

Serum levels of IL-6

In contrast to the cellular production of IL-6, circulating IL-6 levels significantly decreased in 'responders' to MTX treatment ($P < 0.0005$), whereas in 'non-responders' a slight, statistically non-significant increase was observed (Fig. 4).

In vitro production of TIMP-1 and IL-6 by PBMNC

In order to examine the hypothesis of a possible causal interrelationship between IL-6 and TIMP-1 induction we performed the appropriate *in vitro* experiments with

TABLE 2. Parameters of disease activity before and after 3 months of treatment with MTX in responding and non-responding RA patients

	Responders ($n = 16$)		Non-responders ($n = 11$)	
	Baseline	3 months	Baseline	3 months
Swollen joint count (0–58)	28.7 ± 16.2	11.8 ± 7.5*	26.5 ± 10.4	29.4 ± 12.7
Tender joint count (0–60)	32.8 ± 20.4	13.5 ± 8.6**	29.7 ± 13.1	24.6 ± 14.4
Morning stiffness (min)	142 ± 116	20 ± 16*	112 ± 82	94 ± 56
ESR (mm/h)	52.7 ± 30.8	27.6 ± 15.4	48.4 ± 28.7	42.5 ± 23.7
Patient's assessment (0–5)	3.2 ± 0.8	1.4 ± 0.8*	2.9 ± 0.7	2.8 ± 0.8
Physician's assessment (0–5)	3.1 ± 0.9	1.6 ± 0.7*	2.9 ± 0.8	3.1 ± 1.1

*$P < 0.0005$, **$P < 0.001$.
Results represent means ± S.D.

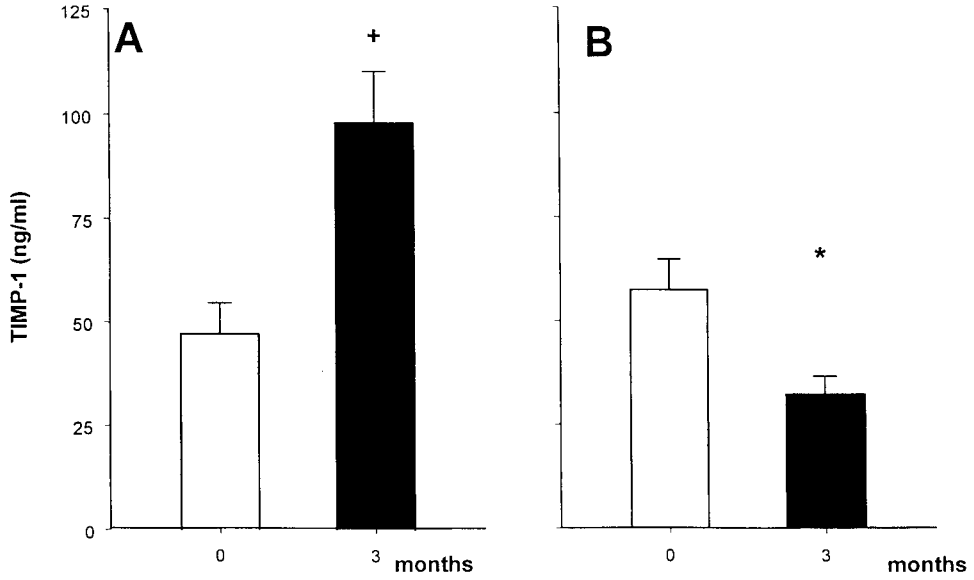

FIG. 1. Spontaneous TIMP-1 release by PBMNC of patients with RA. TIMP-1 release before MTX treatment (open columns) and after 3 months of treatment (filled columns) in responding (A; $n = 16$) and non-responding patients (B; $n = 11$). The results represent mean $\pm$ standard error of the mean (S.E.M.). $^{*}P < 0.01$; $^{+}P < 0.001$.

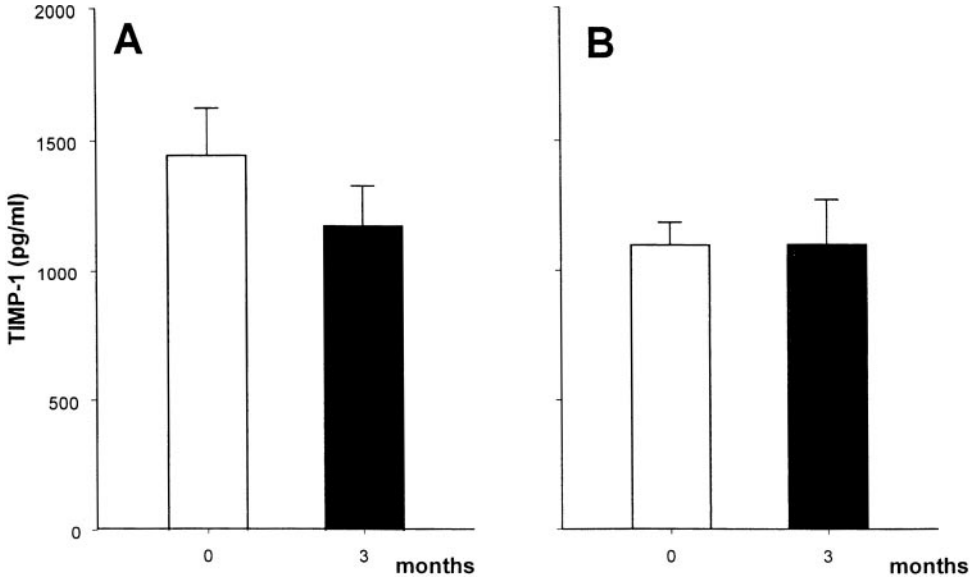

FIG. 2. Concentrations of TIMP-1 in the serum of patients with RA. Circulating TIMP-1 levels before MTX treatment (open columns) and after 3 months of treatment (filled columns) in responding (A; $n = 16$) and non-responding patients (B; $n = 11$). Mean values $\pm$ standard error of the mean (S.E.M.) are shown. The differences were not significant.

low-dose MTX (1, 10 and 100 ng/ml). Table 3 shows that pharmacologically relevant amounts of MTX induced a marked increase in TIMP-1 and IL-6 release by PBMNC from three normal healthy donors after 2 days of cell culture. To determine if IL-6 was responsible for the simultaneous induction of TIMP-1 in PBMNC exposed to MTX, we performed experiments in the presence of a polyclonal antibody to IL-6. Figure 5 shows that PBMNC cultured over a period of 8 days continuously released substantial amounts of TIMP-1 and that this production was markedly stimulated by incubation with MTX. Both spontaneous and MTX-induced production of TIMP-1 by PBMNC were partially inhibited by the addition of anti-IL-6 antibodies to cell cultures but not by anti-IL-10 antibodies. The most pronounced effects of anti-IL-6 on TIMP-1 release were observed after 5 and 8 days of co-incubation with MTX.

Discussion

In the course of this study involving a cohort of RA patients we observed that the spontaneous TIMP-1 release by PBMNC was increased in those patients that

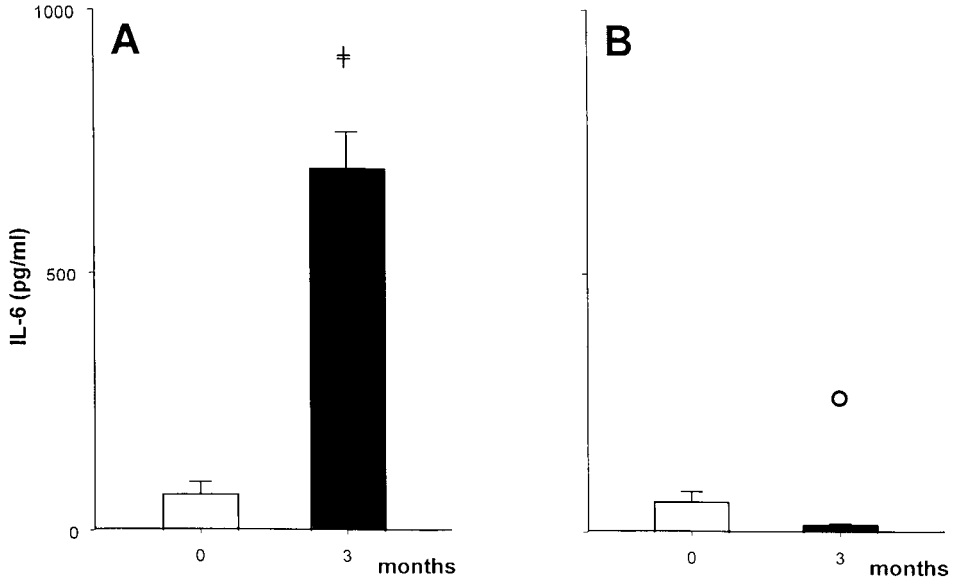

FIG. 3. Spontaneous IL-6 release from PBMNC of patients with RA. IL-6 release before MTX treatment (open columns) and after 3 months of treatment (filled columns) in responding (A; $n = 16$) and non-responding patients (B; $n = 11$). The results represent mean values ± standard error of the mean (S.E.M.). ‡ $P < 0.0005$, ○ $P < 0.025$.

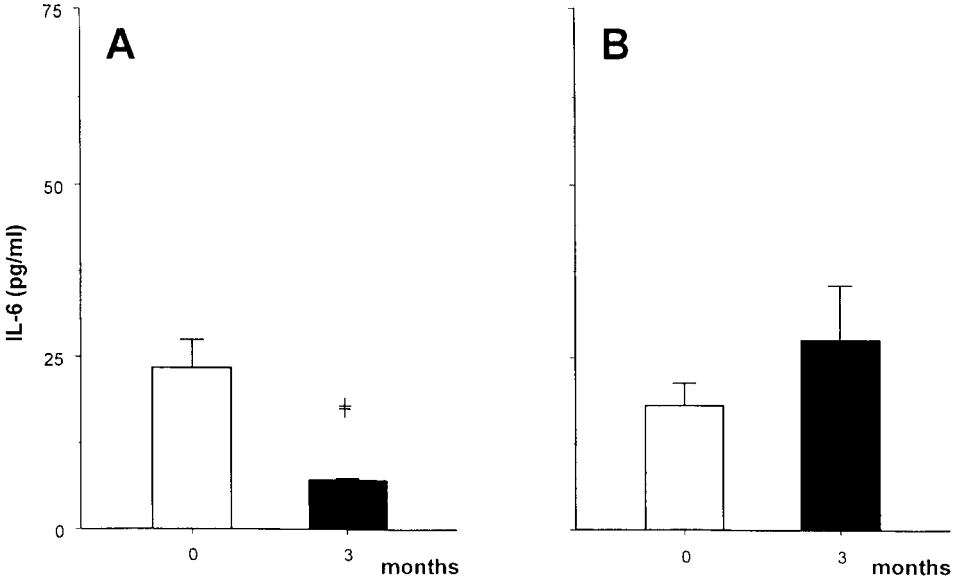

FIG. 4. Concentrations of IL-6 in the serum of patients with RA. Circulating IL-6 levels before MTX treatment (open columns) and after 3 months of treatment (dark columns) in responding (A; $n = 16$) and non-responding patients (B; $n = 11$). Mean values ± standard error of the mean (S.E.M.) are shown. ‡ $P < 0.0005$.

TABLE 3. Stimulation of the production of TIMP-1 and IL-6 on PBMNC by MTX *in vitro*

	TIMP-1 (ng/ml)			IL-6 (pg/ml)		
	1	2	3	1	2	3
Control	28.95[a]	20.68	41.00	9.28	7.83	11.26
+ MTX (1 ng/ml)	31.20	21.04	40.85	12.52	9.52	13.07
+ MTX (10 ng/ml)	46.81	48.46	138.74	33.94	14.27	156.79
+ MTX (100 ng/ml)	48.15	45.48	122.52	31.07	14.05	142.84

[a]The results represent the means of a 2-fold determination of TIMP-1 or IL-6 release by PBMNC of three individual healthy subjects.

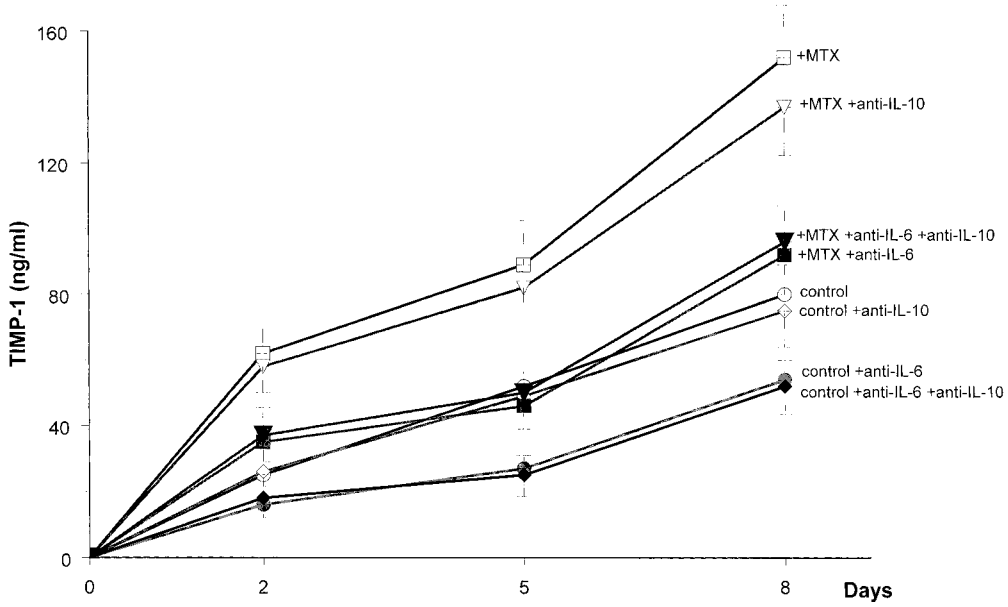

FIG. 5. Kinetics of TIMP-1 production by PBMNC of normal individuals with or without *in vitro* treatment with anti-IL-6 antibodies, anti-IL-10 or both. Normal PBMNC were incubated for up to 8 days with (●) or without (○) anti-human IL-6 polyclonal antibodies (300 ng/ml), with anti-IL-10 (open diamond) or with a combination of anti-IL-6 and anti-IL-10 (filled diamond), with MTX (□), with MTX and anti-IL-6 (■), with MTX and anti-IL-10 (△) or with MTX and a combination of anti-IL-6 and anti-IL-10 (▲). After 2, 5 and 8 days of culture, supernatants were removed for the determination of TIMP-1. Mean values ± standard error of the mean (S.E.M.) of experiments using PBMNC of three different healthy blood donors are shown. Differences were statistically significant when control cells were compared with those incubated with anti-IL-6 or when MTX-treated cells were compared with those additionally treated with anti-IL-6 *in vitro* after 5 ($P < 0.05$) and 8 ($P < 0.05$) days.

responded to MTX treatment after 3 months, whereas in non-responding patients TIMP-1 was down-regulated.

This is the first report of drug-mediated enhancement of natural metalloproteinase inhibitors in RA patients. In contrast, down-regulation of collagenase gene expression has been observed in RA synovium during treatment with MTX in previous studies [20]. It might be argued that this was probably due to the simultaneous down-regulation of IL-1 which strongly induces MMP-1 in synovial fibroblasts [18] and which is a well-known target of MTX effect *in vivo* and *in vitro* [9].

As shown in previous studies, the administration of MTX treatment to RA patients stimulates the production of cytokine inhibitors by circulating blood monocytes, accompanied by the inhibition of IL-1 and IL-8 release [14, 27]. This is consistent with our present finding of enhanced TIMP-1 synthesis, indicating that down-regulation of the inflammatory potential of the monocyte may also coincide with enhanced TIMP-1 release by these cells.

Because the data in this study were obtained on PBMNC it is not possible to distinguish whether TIMP-1 was produced by monocytes due to a direct effect of MTX on monocytes or due to cell–cell interaction between lymphocytes and monocytes or other cells affected by MTX *in vivo*. Cytokines known to induce TIMP-1 in monocytes/macrophages are IL-6 and IL-10 [28, 29] as are IL-6 in fibroblasts [30] and CC-chemokines in $CD4^+$ lymphocytes [31]. In our experiments, however, monocyte chemoattractant protein-1 (MCP-1) cannot have been a significant inducer of TIMP-1 since in previous studies this chemokine was not produced spontaneously in detectable amounts by PBMNC of responding RA patients after treatment with MTX for 3 months [14, 27]. Nevertheless, it cannot be ruled out that other CC-chemokines such as macrophage inflammatory protein MIP-1α, MIP-1β or RAUTES (regulated on activation, normal T cell expressed and secreted) have induced TIMP-1 synthesis in T-helper lymphocytes among PBMNC. Moreover, another potential inducer like IL-10 was not detected under basal conditions in cell culture supernatants of RA patients below a threshold of 50 pg/ml. It is therefore quite unlikely that IL-10 should play an important part in TIMP-1 stimulation. With regard to the possible induction of TIMP-1 in monocytes, we also tested cell culture supernatants for IL-6 secretion. It was only in PBMNC cultures of responding patients that IL-6 release was induced spontaneously after 3 months of treatment, at which time a significant stimulation of TIMP-1 release by the same cells occurred. However, we cannot account for the reduced spontaneous TIMP-1 release from PBMNC of clinically non-responding patients. An explanation might be that in clinical terms the majority of non-responding patients actually deteriorated upon MTX treatment. Reduced TIMP-1 secretion may reflect disease progression rather than merely a lack of response to treatment. This phenomenon could be independent of the specific effects of MTX, while the relative deficit of potentially protective molecules like TIMP-1 and

IL-6 could create an environment for uncontrolled inflammation and tissue degradation.

Of particular interest, MTX when applied in pharmacologically relevant concentrations to normal PBMNC stimulated both IL-6 and TIMP-1 production *in vitro.* TIMP-1 release was inhibited at least partially by antibodies to IL-6, suggesting that IL-6 might have been an important factor in the induction of TIMP-1 upon MTX treatment *in vivo* and *in vitro*. Presumably, TIMP-1 release was only partially inhibited by anti-IL-6 antibodies because these were only partially effective in capturing IL-6. It could be argued that IL-6 induction *in vivo* could be countering the anti-inflammatory effect of MTX seen in clinical terms. In contrast to IL-1 and TNF-α, IL-6 represents a mechanism of host defence and on stimulation by IL-1 and TNF-α it increases several acute phase proteins, such as protease inhibitors. Indeed, many studies have demonstrated that upon MTX treatment circulating serum levels of IL-6 decline along with the remission of RA [32]. One plausible explanation would be that circulating levels of cytokines like IL-6 and cytokine inhibitors which correlate quite well with clinical RA disease activity upon MTX treatment [14, 32], reflect inflammatory activity of the synovial compartment where they are abundantly produced in active stages of the disease [33–36]. However, regulation at the primary cellular level and/or in other compartments like peripheral blood might be totally different, as shown for IL-1Ra and the soluble TNF receptors in recent studies [14, 27].

Apart from IL-6 induction, there may be other reasons for TIMP-1 stimulation. Recent studies have shown that MTX administered to RA patients up-regulated IL-1Ra while down-regulating IL-1 production by PBMNC [14, 27]. This may reflect not only the anti-inflammatory action of this drug but might also support the notion that it enhances monocyte differentiation *in vivo*. In fact, recent experimental results from our laboratory indicate that this assumption might be true [37]. Thus, it is also possible that enhanced *ex vivo* TIMP-1 production upon MTX treatment could be due to stimulated monocyte differentiation *in vivo*. This is supported by the observation that TIMP-1 synthesis increases in differentiated mononuclear phagocytes [38].

Because the slight possibility remained that apart from IL-6, IL-10 in very low amounts could also have stimulated TIMP-1 production by PBMNC in our system, we performed *in vitro* experiments with capturing antibodies to human IL-10 to elucidate this issue. In contrast to anti-IL-6 antibodies, anti-IL-10 antibodies did not even partially block the MTX-induced TIMP-1 production by PBMNC *in vitro*.

Mononuclear phagocytes are capable of mediating matrix degradation by two distinct biological pathways. First, the production of cytokines such as IL-1 and TNF-α, which stimulate metalloproteinase release by fibroblasts and synoviocytes, and second, by secreting their own proteolytic enzymes into the tissue microenvironment. Our data suggest that treatment with MTX might counteract these deleterious pathophysiological events by stimulating the synthesis of a natural metalloproteinase inhibitor which is mediated at least in part by enhanced IL-6 synthesis.

In conclusion, the data presented in this and previous reports [14, 27] suggest that MTX exerts an anti-inflammatory effect and may inhibit extracellular matrix degradation by blood mononuclear cells in RA patients. MTX has obviously divergent effects on circulating and cellularly produced protective molecules. These comprise cytokine inhibitors such as IL-1Ra, soluble TNF receptors, Th2 lymphokines like IL-4 and IL-10 [39] and, as mentioned in this study, IL-6 and TIMP-1. In the circulation, these molecules, except for TIMP-1, seem to behave like acute phase proteins which are down-regulated by MTX, reflecting a secondary net result of anti-inflammatory drug action. At the primary cellular level, however, MTX stimulates these acute phase protein-like molecules, which may antagonize locally the deleterious catabolic effects of IL-1 and TNF-α as well as the activity of tissue-degrading metalloproteinases at the site of inflammation. Thus, MTX may enhance the local natural host defence against uncontrolled inflammation and tissue breakdown.

In particular, the potential role of MTX on extracellular matrix repair has to be further elucidated in long-term studies by correlating the modulation of MMP-1 and TIMP-1 production by circulating and synovial cells with radiological progression of erosion and degree of joint destruction having been reported to be slowed more upon MTX treatment than upon other disease-modifying anti-rheumatic drugs (DMARDs) [40–44].

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