Relationship between serum RANTES levels and radiological progression in rheumatoid arthritis patients treated with methotrexate

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Abstract

Objective

The aim of this study was to evaluate the relationship between serum chemokines and the clinical and radiological response to a one-year course of methotrexate (MTX) in patients suffering from rheumatoid arthritis (RA).

Methods

Twenty out-patients suffering from active RA entered a one-year open prospective study on the effects of low dose MTX therapy. Plain radiographs of the hands and feet were taken at study entry and at the end of the follow-up, and were compared for the number of eroded joints. Serum levels of both C-X-C and C-C chemokines were obtained before the initation of MTX and after 6 and 12 months of treatment.

Results

The levels of serum RANTES before treatment were significantly higher in RA patients than in the controls and returned to normal levels after one year of treatment. Serum levels of the other chemokines were either in the normal range or undetectable. Twelve patients (60%) did not show any new eroded joints at the end of the follow-up period and were considered as radiological responders (RR). Serum levels of GRO-α and RANTES after 6 months of treatment were significantly higher among the patients with radiological progression than in RR patients.

Conclusions

We observed high levels of serum RANTES in a series of RA patients during the active stage of the disease. MTX treatment significantly lowered the serum levels of RANTES, GRO-α and MCP-1. High levels of serum RANTES or GRO-α after 6 months of MTX treatment seem to be predictive of radiological erosions after one year.

Key words

Rheumatoid arthritis, methotrexate, serum chemokines.

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Introduction

A central role in the cellular recruitment and activation of leukocytes at the sites of inflammation is played by a family of structurally related cytokines known as the chemokines. These proteins can be divided into four groups with different chemotactic specificities and molecular organization. The C-X-C chemokines, with the first two cystine residues separated by an amino acid, include interleukin(IL)-8 and growth-related-gene-product-alpha (GRO-α), and exert their function mainly on neutrophils. The C-C chemokines, with the first two cysteine located in adjacent position, such as RANTES (regulated upon activation, normal T cell expressed and secreted), monocyte chemotactic protein-1 (MCP-1) and macrophage inflammatory protein-1α (MIP-1α) mediate the recruitment and activation of monocytes and lymphocytes (1-4). Two other groups are now recognized, namely the C and the C-XXX-C chemokines, which exert their activity mainly on monocytes, resting T cells and NK cells.

The pivotal role of these molecules in RA is underlined by the findings of high levels of IL-8, GRO-α, MIP-1α and MCP-1 in both the serum and synovial fluid of rheumatoid arthritis (RA) patients (5-8). RANTES molecules have been observed in T cells of the peripheral blood, synovial fluid and synovial tissue of RA patients. In the same group of patients MIP-1α has been observed both in T and non-T cells of the peripheral blood, synovial fluid and synovial tissue, while MCP-1 has been detected only in the non-T cellular component of the same specimens (9). The stimulation of RA synovial fibroblasts with IL-1 has induced the production of RANTES, GRO-α, MIP-1α and MCP-1 (10).

Five C-X-C and 8 CC receptors have been described and are expressed in different combinations on human leukocytes. In particular, resting T cells express only the CXCR4 receptor while after IL-2 stimulation they produce CCR 1,2,3 and 5 and CXCR3 and 4 on their surfaces. The expression of these receptors allows the T cell to become responsive to the chemotaxis and cell activation mediated by the interaction with different chemokines such as RANTES, MIP-1α, GRO-α and MCP-1 (1).

Several controlled studies have clearly demonstrated the clinical efficacy and tolerability of methotrexate (MTX) in the treatment of RA and the drug is presently considered as the first choice therapy for RA (11). However, it appears that MTX only slows the progression of radiological damage but does not halt the appearance of new joint erosions.

The mechanism of the anti-inflammatory action of MTX seems to be related to its ability to increase the cellular release of adenosine in the extra-cellular space. Adenosine, through its interaction with specific leukocyte receptors, has an immunosuppressive and anti-inflammatory activity as demonstrated *in vitro* on lymphocytes, monocytes and neutrophils (12). Adenosine reduces the monocyte synthesis of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, IL-6 and IL-8 and increases the production of the anti-inflammatory IL-10 (13-16). In RA patients treated with MTX the reduction of serum levels of IL-1 (17), IL-2 (17), IL-6 (17-20), IL-8 (17), soluble IL-2 receptor (sIL-2R) (21), and IL-1 receptor antagonist (IL-1RA) (22) has been reported. Only two studies have evaluated the effect of MTX on the production of some chemokines (MCP-1 and IL-8) by peripheral blood monocytes in RA patients treated with the drug. It was found that the spontaneous production of IL-8 by peripheral blood mononuclear cells (PBMC) *in vitro* after MTX treatment was increased among non-responder patients and was reduced in patients clinically responsive to the drug. No significant change was observed in MCP-1 production (23).

To further evaluate the immunoregulatory effect of methotrexate we measured the serum concentration of different chemokines during the acute stage of RA before the begining of MTX treatment and during a one-year follow-up period. We also looked for correlations between serum chemokine concentrations and clinical, laboratory and radiological evolution of the disease.

Patients and methods

Twenty patients fullfilling the 1987 ARA criteria for RA (24) were enrolled in the study. Their clinical, demographic and

Table I. Patients' characteristics at entry.

Number of patients	20
Mean age (yrs.)	64 ± 13
Mean age at onset (yrs.)	50 ± 14
Mean duration of RA (mos.)	69 ± 90
Sex (M/F)	2/18
Previous use of 2 or more DMARDs	70%
Use of systemic steroids	45%
Rheumatoid factor positivity	50%
ANA positivity	30%

Values are expressed as the mean ± SD.
DMARDs: disease modifying anti-rheumatic drugs.

laboratory characteristics at entry are shown in Table I. No patient had a family history of spondylitis, psoriasis, psoriatic arthritis, uveitis, inflammatory bowel diasease (IBD), Behçet's disease, or reactive arthritis, nor a personal history of cutaneous psoriasis, IBD, aphthous stomatitis, or previous infection of the genital, urinary or alimentary tracts.
All of the patients had active disease, defined by the presence of at least 3 of the following 4 criteria: morning stiffness > 45 minutes; more than 6 swollen joints; more than 9 tender joints; and erythrocyte sedimentation rate (ESR) > 30 mm/hour. The patients had taken no second line drugs for at least six weeks prior to the study.
MTX was administered at an initial single weekly dosage of 10 mg. Nine patients received systemic steroids at a dosage of < 5 mg prednisone equivalent per day and all patients were taking a concomitant full dosage of non-steroidal anti-inflammatory drugs (NSAIDs). No patient had received intra-articular steroids for at least one month before entering the study.

Clinical examination
Clinical assessment of the RA patients was performed at entry, every 2 weeks for the first month, and monthly thereafter by the same rheumatologist (CS or PM) throughout the study period. The rheumatological assessment included: the number of tender joints, number of swollen joints, articular index (number of tender and/or swollen joints), grip strength, duration of morning stiffness, global assessments of disease activity by the patient and the physician using a 5 grade (0 - 4) numerical scale, and joint pain using a 10 cm horizontal visual analogue scale (VAS).
At every visit the patient's response to treatment was classified according to the the ACR 50% criteria (25).

Laboratory studies
The following tests were performed at entry and at each visit: complete blood count, transaminase, total bilirubin, alkaline phosphatase, electrolytes, serum creatinine, urine analysis, and ESR. C-reactive protein (CRP) was determined by the nephelometric method (Behringwerke, Marburg, Germany).
The serum IL-8, GRO-α, MCP-1, and RANTES MIP-1α were measured using a sandwich ELISA, following the manufacturer's instructions (Quantikine, R&D Systems, Minneapolis, MN). Eighteen healthy subjects matched for age and sex were used as controls.
The peripheral blood lymphocyte subpopulations were determined by the FACS method as described elsewhere (26).

Radiological examination
Plain radiographs of the hands and feet were taken both at the beginning of the MTX treatment and at the end of the follow-up and were compared for the presence of erosions and joint damage (scored according to Larsen, ref. 27). For the comparison we considered the following joints: wrists, metacarpophalangeals, proximal and distal interphalangeals of the hands, metatarsophalangeals and the interphalangeal of the first toe. We obtained two scores for each patient: the number of joints with at least one erosion (erosion score: ES) and the sum of the Larsen's severity index for each of the joints considered (Larsen Score: LS).
The radiographs were scored independently by two of the authors (PM, CS) without knowledge of the patient's name or the order of the radiographs. When the data were discordant, the final score for each joint was obtained by general consensus.
Patients were considered as radiological responders (RR) if the number of eroded joints did not increase at the end of the follow-up period.

Statistical analysis
Paired and unpaired T-tests and chi-square tests were used for the analysis. Mann-Whitney and Wilcoxon tests were used when necessary. Correlations were determined by the Pearson method. Multivariate analysis of variance was used to compare variables between patients with and without radiological deterioration at each follow-up time.

Results

Before treatment, serum RANTES levels were significantly elevated in the RA patients compared to the controls; in only 45% were levels within the normal range (mean ± 2 SD). The serum levels of GRO-α and MCP-1 in the RA patients did not significantly differ from the controls while IL-8 and MIP-1α were undetectable in the majority of patients (Table II).
The basal serum RANTES levels showed a significant negative correlation with the basal serum MCP-1 concentration (-0.48, p = 0.03) and a positive correlation with the average grip strength (+0.53, p = 0.02) and NK peripheral blood cells (CD57+CD8-).
Basal serum MCP-1 levels showed a significant positive correlation with the serum CRP concentration at entry (+0.49, p = 0.03).

Table II. Basal serum chemokines in rheumatoid arthritis patients and controls before treatment.

Serum chemokines	Patients (n = 20)	Controls (n = 18)	P
RANTES (ng/ml)	60 ± 58	30 ± 7	0.03
GRO-α (pg/ml)	155 ± 115	149 ± 44	ns
MCP-1 (pg/ml)	411 ± 202	359 ± 135	ns

Values are expressed as the mean ± SD.

Table III. Clinical and laboratory evolution of the disease in RA patients treated with MTX.

Outcome measure	First visit	12 months	p
Articular index	13.7 ± 5.6	4.0 ± 4.1	0.0001
Swollen joints	8.9 ± 3.8	2.6 ± 3.3	0.0001
Duration of morning stiffness (min)	119 ± 84	29 ± 52	0.001
Mean grip strength (mm/Hg)	68 ± 67	134 ± 62	0.0001
Pain-VAS (mm)	72 ± 15	24 ± 27	0.0001
Patient's global assessment (0-4)	3.0 ± 0.6	1.4 ± 1.0	0.0001
Physician's global assessment (0-4)	2.8 ± 0.6	1.3 ± 0.9	0.0001
ESR (mm/1st hr)	39 ± 22	23 ± 19	0.003
CRP (mg/dl)	2.5 ± 2.1	0.85 ± 0.96	0.003

Values are expressed as mean ± SD or percentage.

At the end of one year of MTX treatment we observed significant improvement in all of the clinical and laboratory parameters considered (Table III) and only 3 patients were found to be clinically non-responsive.

One year of treatment with MTX induced a significant reduction in the serum levels of RANTES, GRO-α and MCP-1 with normalization of the RANTES level at the end of the follow-up period (Table IV).

The mean number of eroded joints per patient and the LS did not increase significantly during the follow-up period (Table V). Despite good control of the clinical parameters, 8 patients (40%) had at least one new eroded joint at the end of the study year.

The delta value of the number of eroded joints per patient (time 0 vs end of follow-up) showed a significantly negative correlation with the delta value of the number of tender joints (time 0 vs 6 months) ($r = -0.44$, $p = 0.05$) and the number of swollen joints (time 0 vs 6 months) ($r = -0.55$, $p = 0.012$).

The delta value of the number of eroded joints was positively correlated with the number of tender and swollen joints after 6 ($r = 0.73$,$p = 0.0001$ and $r = 0.60$,$p = 0.005$ respectively) and 12 months ($r = 0.49$,$p = 0.03$ and $r = 0.51$,$p = 0.02$ respectively) of treatment. Interestingly the delta value of the number of eroded joints had a significantly positive correlation with the serum concentration of GRO-α and RANTES at 6 month of follow-up ($r = 0.61$, $p = 0.006$ and $r = 0.49$, $p = 0.03$, respectively).

Table IV. Serum chemokine concentration in RA patients during methotrexate treatment.

Serum chemokines	Before treatment	6 months	12 months	p*
RANTES (ng/ml)	60.3 ± 58.0	71.2 ± 71.9	36.3 ± 38.2	0.005
GRO-α (pg/ml)	155 ± 115	172 ± 174	137 ± 131	0.058
MCP-1 (pg/ml)	411 ± 202	356 ± 143	316 ± 137	0.048

Values are expressed as the mean ± SD. *p = base values vs 12 months.

Table V. Radiological evolution of the disease in RA patients treated with MTX.

Outcome measure	First radiographs	Last radiographs (12 months)	p
Percentage of patients with articular erosions	65%	75%	
Mean Larsen's Score	39.4 ± 34.6	41.2 ± 33.5	Ns
Mean number of eroded joints	5.8 ± 7.9	6.2 ± 7.3	Ns
Patients without new eroded joints (%)		60	

Values are expressed as the mean ± SD.

We compared the radiological, clinical and laboratory parameters of the patients with radiological progression (radiological non-responders: RNR) and patients with stable radiological disease (RR) (Table VI). We found that RNR patients had significantly higher serum levels of GRO-α and RANTES at six months than RR patients (Table VI). After 6 months of MTX therapy 83% of the RR patients had serum RANTES levels in the normal range compared to 37% of the RNR patients ($p = 0.03$). Patients with high serum RANTES after 6 months had a 2.7 (CI 0.8 - 9.0) relative risk of developing new erosions compared to patients with normal values.

Among the 12 RR patients we observed a statistically significant reduction in the serum levels of MCP-1, GRO-α, RANTES, CRP and ESR at the end of the follow-up period (Table VI b). At the same time serum RANTES levels were in the normal range in 92% of these patients. In the RNR patients, at the end of follow-up period the mean serum levels of MCP-1α, GRO-α, RANTES, CRP and ESR were unchanged compared to the initial levels.

Discussion

An inflammatory insult produces a cascade of events that, by the production of inflammatory cytokines (mainly IL-1, IL-6 and TNF-α), leads to the synthesis and release of various chemokines which promote the recruitment of leukocytes at the site of inflammation. Chemokines induce the firm adhesion of leukocytes on the endothelial cells, their subsequent migration into the interstitial tissue, and also cellular activation. Leukocyte activation promotes the stimulation of cytokines and chemokine production, with the induction of a loop of amplification of the inflammatory response (1-4).

High expression of different chemokines has been observed in the serum, synovial fluid and synovial tissue of RA patients during phases of active disease (5-10). Unlike other studies (5-8), we were unable to find high levels of serum IL-8, GRO-α or MIP-1α, which in our patients were either undetectable or in the normal range. Probably differences in the selection of patients (duration of previous treatments, duration of the disease,

Table VI. (a) Comparison of radiological and demographic data between patients with radiological evolution and patients without radiological deterioration.

Demographic and radiological features	Patients without radiological progression (n = 12)	Patients with radiological progression (n = 8)	P
Mean age	55 ± 13	57 ± 12	ns
Mean disease duration	92 ± 105	32 ± 47	ns
Sex (F/M)	12/ 0	6/2	ns
Percentage of patients without erosions	42%	25%	ns
Rheumatoid factor positivity	50%	50%	ns
Larsen score 1 (basal)	38.2 ± 37.4	41.1 ± 32.5	ns
Larsen score 2** (at the end of follow-up)	36.8 ± 36.5	47.8 ± 29.4	ns
Delta Larsen score	-1.4 ± 2.9	6.6 ± 7.3	0.003
Erosion score 1 (basal)	6.8 ± 8.9	4.5 ± 6.3	ns
Erosion score 2 (at the end of follow-up)	6.1 ± 8.3	6.3 ± 6.1	ns
Delta erosion score	-0.6 ± 0.9	1.7 ± 1.0	0.0001

Values are expressed as the mean ± SD.

(b) Comparison of clinical and laboratory data between patients with radiological progression and those without radiological progression.

Clinical features	Patients without radiological progression (n = 12)		Patients with radiological progression (n = 8)		P °
Articular index (basal values)	13.3 ± 4.8		14.1 ± 6.8		Ns
Articular index at 6 months	1.8 ± 2.5	(0.0001)§	5.0 ± 4.4	(0.017)§	Ns
Articular index at 12 months	3.3 ± 3.5	(0.0001)^	5.3 ± 4.8	(0.023)^	Ns
Swollen joints (basal values)	8.4 ± 3.1		9.8 ± 4.8		Ns
Swollen joints at 6 months	1.6 ± 1.5	(0.0001)§	1.0 ± 1.7	(0.003)§	Ns
Swollen joints at 12 months	1.9 ± 2.6	(0.0001)^	3.6 ± 4.2	(0.005)^	Ns
Basal ESR (mm/1st hr)	45.1 ± 23.2		28.5 ± 15.7		Ns
ESR at 6 months (mm/1st h)	25.1 ± 22.9	(0.007)§	19.1 ± 9.0	(ns)§	Ns
ESR at 12 months (mm/1st h)	25.7 ± 24.0	(0.015)^	19.0 ± 9.7	(ns)^	Ns
Basal CRP (ng/ml)	2.7 ± 2.3		2.2 ± 1.8		Ns
CRP at 6 months (ng/ml)	0.8 ± 0.7	(0.009)§	1.4 ± 1.6	(ns)§	Ns
CRP at 12 months (ng/ml)	0.8 ± 1.2	(0.028)^	0.9 ± 0.5	(ns)^	Ns
Basal MCP-1 (pg/ml)	439 ± 238		370 ± 138		Ns
MCP-1 at 6 months (pg/ml)	373 ± 131	(ns)§	333 ± 164	(ns)§	Ns
MCP-1 at 12 months (pg/ml)	288 ± 120	(0.016)^	354 ± 157	(ns)^	Ns
Basal GRO-α (pg/ml)	136 ± 112		183 ± 120		Ns
GRO-α at 6 months (pg/ml)	109 ± 97	(ns)§ß	259 ± 202	(ns)§	0.046
GRO-α at 12 months (pg/ml)	98 ± 78	(0.033)^	184 ± 171	(ns)^	Ns
Basal RANTES (ng/ml)	63 ± 61		56 ± 57		Ns
RANTES at 6 months (ng/ml)	44 ± 47	(ns)§	112 ± 86	(ns)§	0.05
RANTES at 12 months (ng/ml)	27 ± 10	(0.02)^	49 ± 59	(ns)^	Ns

Values are expressed as mean ± SD.
° P values of patients with radiological progression vs those without radiological progression; § p values baseline vs 6 months; ^ p values baseline vs 12 months.

etc.) or in the drug treatment at the time of the chemokine determination can explain these discrepancies. Only serum RANTES levels were higher than in the control population.

We observed similar findings during the active phase of polymyalgia rheumatica (28) and psoriatic arthritis (29). This, however, represents the first study of serum RANTES levels in RA patients. The source of this chemokine probably is the synovial RA fibroblasts which, under the influence of different cytokines [mainly IL-1 and the combination of TNF-α and interferon (IFN)-γ], are able to produce and release high levels of RANTES (30). Furthermore, in RA synovial and peripheral T cells have been reported to produce and release RANTES (10). We also found a positive correlation between peripheral NK cells and

serum RANTES, suggesting, as reported in a recent study, a possible contribution of these cells to the serum level of the chemokine (31). Barnes *et al.* described high levels of RANTES in the whole blood and joints of the Lewis rat during the course of adjuvant-induced arthritis, and polyclonal antibodies directed against human RANTES greatly ameliorated the symptoms in the same arthritis model (32).

The role of RANTES in T-cell and monocyte recruitment at the site of inflammation is underlined by the high levels of expression of the chemokine receptor CCR5 (ligand of RANTES, MIP-1α and MIP-1α) on activated monocytes and T lymphocytes observed in the synovial fluid and tissue of RA patients. This activation can induce a T-helper (TH)1 shift in the immune-mediated response (33).

The immunosuppressive activity of MTX is in part related to its ability to reduce the concentration of different cytokines and their receptor in serum (IL-6, sIL-2R and TNF-α receptor 55), synovial fluid (IL-1β) and synovial membrane (IL-1β, TNF-γ) in RA patients (17-23, 34).

Furthermore, a significantly decreased percentage of synovial membrane macrophages (CD68+ cells) has been reported, along with a reduction in the T cell percentage (CD3+ and CD8+) and in the expression of adhesion molecules (E-selectin and VCAM-1) (35) in RA patients after MTX treatment. The mechanism of the immunosuppressive effect of the drug is still unknown, but is probably linked to the increased concentration of adenosine in the extra-cellular space induced by MTX (12).

We observed a significant reduction in serum concentration of GRO-α, MCP-1 and RANTES only after 12 months of MTX treatment, while a positive clinical response was seen after just 3 months. In an experimental model of rat arthritis only the synovial concentration of RANTES was correlated with the clinical symptom of joint inflammation, and high blood RANTES concentrations persisted several weeks after recovery (31).

We also evaluated the relationship between serum chemokines levels and the development of joint erosions after one year of MTX therapy. A recent study has demonstrated a time lag between high serum levels of CRP and ESR and the subsequent development of joint erosions. The number of swollen joints and the serum levels of these acute phase reactants were correlated with the number of new erosions observed at radiological examination 6 months later (36). In our study GRO-α and RANTES levels in the serum after 6 months of MTX treatment showed a good correlation with the delta value of the erosive score. In the RNR group a trend toward increased levels of serum RANTES was observed after 6 months of treatment. The lack of effect of MTX treatment on RANTES production during the first 6 months of therapy seems to have a detrimental effect on the radiological evolution of RA seen 6 months later.

A significant reduction in the serum levels of RANTES, GRO-α and MCP-1 was observed in the RR group after one year of treatment, while in the RNR patients no significant changes were observed. An analogous significant reduction in the acute phase reactants (CRP and ESR) was observed after 6 and 12 months of MTX treatment only in the RR patients, suggesting that in the RNR group MTX treatment induced a partial inhibition of the synovial inflammation which was sufficient to control only the clinical signs of the disease.

In conclusion, we observed high serum levels of the chemokine RANTES during the active stage of RA and a significant reduction in serum RANTES, GRO-α and MCP-1 in a group of RA patients treated with MTX. The serum levels of RANTES and GRO-α after 6 months of treatment showed a good correlation with the appearance of new joint erosions after 12 months of MTX therapy. Determination of the serum levels of these chemokines after 6 months of MTX treatment could be useful in monitoring the efficacy of the therapy. Our study was not designed to yield information about the clinical usefulness of the chemokine determination in the assessment of erosive progression in RA patients during MTX treatment. Only a randomized, long-term study on larger group of patients will allow us to better define the practical use of this laboratory determination.

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