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Long-term study of the impact of methotrexate on serum cytokines and lymphocyte subsets in patients with active rheumatoid arthritis: correlation with pharmacokinetic measures

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ABSTRACT

Objective: To describe changes in immune parameters observed during long-term methotrexate (MTX) therapy in patients with active rheumatoid arthritis (RA) and explore correlations with simultaneously measured MTX pharmacokinetic (PKC) parameters.

Design: Prospective, open-label, long-term mechanism of action study.

Setting: University clinic.

Methods: MTX was initiated at a single weekly oral dose of 7.5 mg and dose adjusted for efficacy and toxicity for the duration of the study. Standard measures of disease activity were performed at baseline and every 6–36 months. Serum cytokine measurements in blood together with lymphocyte surface immunophenotypes and stimulated peripheral blood mononuclear cell (PBMC) cytokine production were assessed at each clinical evaluation.

Results: Cytokine concentrations exhibited multiple significant correlations with disease activity measures over time. The strongest correlations observed were for interleukin (IL)-6 ($r=0.45$, $p<0.0001$ for swollen joints and $r=0.32$, $p=0.002$ for tender joints) and IL-8 ($r=0.25$, $p=0.01$ for swollen joints). Significant decreases from baseline were observed in serum IL-1B, IL-6 and IL-8 concentrations. The most significant changes were observed for IL-6 ($p<0.001$). Significant increases from baseline were observed in IL-2 release from PBMCs ex vivo ($p<0.01$). In parallel, multiple statistically significant correlations were observed between MTX PKC measures and immune parameters. The change in swollen joint count correlated inversely with the change in area under the curve (AUC) for MTX ($r=-0.63$, $p=0.007$).

Conclusions: MTX therapy of patients with RA is accompanied by a variety of changes in serum cytokine expression, which in turn correlate strongly with clinical disease activity and MTX pharmacokinetics (PKCs). These data strongly support the notion that MTX mediates profound and functionally relevant effects on the immunological hierarchy in the RA lesion.

Key messages

What is already known about this subject?

There are no studies of the mechanism of action of methotrexate (MTX) which include serial prospective measures of serum cytokines and simultaneous measures of pharmacokinetics and clinical variables. This study was carried out over a 3-year period.

What does this study add?

The study shows for the first time that MTX treatment is associated with significant decreases in serum interleukin (IL)-6 and IL-8 and that these decreases correlate well with both long-term, sequential measures of MTX PK and with clinical outcomes.

How might this impact on clinical practice?

The recognition that MTX has a significant effect on serum IL-6 will provide insight into its MOA. Perhaps more importantly, it explains the further changes in transaminase levels and possible additive effects on IL-6 when used with these biological response modifiers and Janus kinase inhibitors.

INTRODUCTION

Rheumatoid arthritis (RA) affects ~0.5–1% of the world's population with an estimated prevalence of up to 2 million cases in the USA alone.[1] In numerous clinical guidelines, methotrexate (MTX) is the anchor treatment for RA management.[2–8] Patients exhibit dose-dependent clinical improvements while on MTX.[9] Toxicity is a more common reason to discontinue therapy than is lack of efficacy.[6 10] Recent guidelines of the American College of Rheumatology have reinforced the central role of MTX in the treatment of RA.[11]

In spite of a proliferation of reports of the effect of MTX on various in vitro, ex vivo or animal models of inflammation, its mechanism of action in patients with RA remains incompletely understood.[12] Few investigations have examined directly the potential effects of MTX on biochemical, immune or inflammatory parameters in vivo or ex vivo in high-intensity, near-patient immune functional assays. Nor has the relationship between MTX pharmacokinetics (PKCs) and immune functional parameters been examined formally or in detail. In particular, although the PKCs of MTX in RA are well described,[13–17] we are unaware of prior attempts to seek correlations between PKC measures in patients with RA on MTX and simultaneously assessed immune markers of disease activity.

We report here prospective measurements of immune and PKC parameters in a cohort of patients with active RA beginning treatment with MTX and remaining on therapy over a period of 3 years. We describe MTX-associated changes in serum cytokines and lymphocyte phenotypic markers which correlate with the measurements of disease activity as well as with drug PKCs. We provide for the first time convincing PKC evidence supporting the immune modulatory role of MTX in RA over long treatment intervals.

METHODS

Patients: Seventeen patients with definite RA[18] were recruited from the outpatient population of the Division of Rheumatology at Albany Medical College and consecutively enrolled. Patients had active disease as previously defined[2] and signed informed consent. Patients were approached consecutively and no patient who was asked to participate refused. Patients had never before received MTX and discontinued their prior slow-acting antirheumatic drug at least 1 month prior to beginning MTX therapy. Patients continued non-steroidal anti-inflammatory drugs or prednisone <10 mg daily throughout the study according to prior prescription. The prednisone dose was held constant for 1 month prior to study entry and no intra-articular steroid dose was allowed 1 month prior to initiation of the study. MTX was initiated at a dose of 7.5 mg weekly and increased incrementally to achieve maximal clinical efficacy and decreased for toxicity as previously described in a different cohort.[2] All patients were followed by the same clinical investigator (JMK) throughout the study. Patient demographic and clinical characteristics are shown in table 1.

Clinical evaluations: Clinical evaluations were performed at baseline and every 6 months thereafter through 36 months. Clinical evaluations recorded at each visit consisted of the number of tender and swollen joints (66 diarthrodial joints examined), the duration of morning stiffness in minutes, the interval from time awakening to the first onset of fatigue in minutes, grip strength, and patient and physician evaluation of pain and global arthritis activity using a five-point scale were 0=absent, 1=mild, 2=moderate, 3=severe and 4=very severe. Adverse events were recorded at the time of each clinical evaluation after questioning the patient regarding possible toxic reactions to MTX.

Table 1 Clinical and demographic features of patients with rheumatoid arthritis prior to MTX therapy (N=17)

Age (years) (mean, SD)	60.9 (12.1)
Sex (F:M)	10:7
RA disease duration (months)	133 (130)
NSAIDs (%)	17 (100)
Prior DMARDs (n)	0=3
	1=8
	2=5
	3=1
Haemoglobin (g/dL) (mean, SD)	12.6 (2.0)
Western ESR (mm/hour) (mean, SD)	67.9 (34.1)
RF (%)	14 (82)
Prednisone (N) (mean dose (SD))	10 (5.4 (2.4))

DMARDs, disease-modifying anti-rheumatic drugs; ESR, erythrocyte sedimentation rate; F, female; M, male; MTX, methotrexate; RA, rheumatoid arthritis; RF, rheumatoid factor; NSAIDs, non-steroidal anti-inflammatory drugs.

Laboratory: Laboratory values determined at each visit consisted of a complete blood count, platelet count, Westergren erythrocyte sedimentation rate, rheumatoid factor (nephelometry) and serum chemistry including serum aspartate aminotransferase γ-glutamyl transferase, alkaline phosphatase, albumin, total bilirubin, total protein and creatinine. The absolute number of neutrophils and lymphocytes were calculated at each visit by multiplying the total white cell count (WCC) by the percentage of these cells reported on the differential count.

Immune studies: In order to establish whether sampling for immune parameters would be acutely affected by weekly MTX dosing, blood samples for cytokines and lymphocyte phenotypic analyses were obtained on two occasions at the time of each study evaluation. The first sample was obtained at 8:00 of the day of the clinical evaluation and initiation of PKC blood sampling but prior to the weekly MTX dose given to the patient that day (pre). The second sample was obtained 24 hours after the first, following the weekly MTX dose (post). Sera were aliquoted and frozen at −70°C and all longitudinal samples of each individual later were thawed and quantified by ELISA for the following cytokines: interleukin (IL)-1B (T-cell Diagnostics, Cambridge, Massachusetts, USA); IL-2 and IL-6 (Biosource, Camarillo, California, USA); IL-4 and IL-8 (R&D Systems, Minneapolis, Minnesota, USA). All ELISAs were performed in accordance with the appropriate manufacturer's protocol. In addition, peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll-Hypaque (Pharmacia) from the patients at the same time points. These cells (1×10^6 cells/mL/well; 24-well plate) were stimulated with concanavalin A (5 mg/mL) and supernatants were collected after 24 hours and frozen for later analysis of IL-1B, IL-2 and IL-4 as described above.

Flow cytometric analysis: Blood was collected by venipuncture into EDTA blood collection tubes and the immunophenotyping of the PBMC by flow cytometry was performed by the whole blood lysis method as previously described.[19] The flow cytometry was performed with a Becton Dickson FACScan and analysed with Lysys II or Paint-A-Gate^{Plus} software. The lymphocytes within the PBMC preparation were gated by forward angle and side light scatter. The purity of the lymphocyte gate and assessment of lymphocytes not within this gate is determined with antibodies to CD14 and CD45. The specific monoclonal antibodies employed for lymphocyte subset analyses were used in the following combinations: CD4/CD8/CD3 (T-cell subsets); CD4/CD29 (memory helper T cells); CD4/CD45RA (naïve helper T cells); CD5/CD10 (B1 and B2 cells). Lymphocyte immunophenotype counts were calculated by multiplying the percentage of the subpopulation obtained by FACS by the total absolute lymphocyte count obtained by coulter counter analysis and laboratory differential cell count. Both samples were obtained at the same blood draw.

Pharmacokinetics: PKCs of MTX were measured at each study visit when clinical, laboratory and immune parameters were also obtained. Patients received MTX at 8:00 after an overnight fast. Blood samples were collected prior to the 8:00 dose and at the 0.5, 1, 2, 3, 4, 6, 8 and 24 hours after the dose. Urine was collected for 24 hours for MTX and creatinine analysis. Serum and urine creatinine concentrations were determined by the clinical laboratory. Blood and urine were analysed by fluorescence polarization immunoassay (FPIA) for MTX concentration. Areas under the serum concentration versus time curve (AUC) were calculated by the trapezoidal rule through 8 hours. Area under the curve from 8 to 24 hours was determined using the log-trapezoidal rule or Simpson's approximation. The area under the curve from the last measured concentration to infinity ($AUC_{24\ oo}$) was determined by dividing the final concentration by the terminal elimination rate constant. AUC from time 0 to infinity ($AUC_{o\ 24}$) was the sum of the $AUC_{0\ 24\ oo}$ plus $AUC_{24\ oo}$. The terminal elimination rate constant was determined using the non-linear curve-fitting program RSTRIP. Renal clearance was determined by dividing the urine MTX by the $AUC_{0\ 24}$. Creatinine clearance was determined using standard formula. Systemic clearance (clearance/F, where F=oral bioavailability) was determined as dose divided by $AUC_{O\ oo}$. As the dose of MTX was adjusted to clinical response throughout the investigation, the PKC studies were conducted with different MTX doses throughout the study.

Statistics: Kolmogorov-Amirnoff tests were conducted to determine whether the data for key variables follows Gaussian (normal) distributions. None of these tests were statistically significant (α=0.05), which indicated that the data were not significantly non-Gaussian. The sample sizes in most of the analyses were large enough to eliminate concerns about using parametric statistical tests. A number of one-sample t-tests were performed on the data to determine whether average changes from baseline or average changes from previous visits were statistically significant for different clinical, laboratory and PKC variables. A Bonferroni correction was applied to all of the values.

The initial correlation analyses were a series of pairwise Pearson correlations to examine the extent to which variables in the data were correlated with one another. Variables involving changes from baseline or changes from previous visits were correlated with each other; variables involving direct measurements were independently correlated with each other.

A series of stepwise multiple linear regression models were performed. In these analyses, the dependent variables were the changes in clinical measurements between visits while the independent variables were the change in PKC variables between visits. Stepwise regression was used to limit the final models to only those independent variables that added significantly to the explanatory power of the models.

Chronological setting of investigation: The study was planned and completed at the Albany Medical College in the late 1990s and has never before been published in any form in any journal. Thus, access to biological agents was not available. Similarly, outcome measures such as a Disease Activity Scale in 28 joints (DAS28) were not in vogue and C reactive protein (CRP) measures were not performed.

RESULTS

Clinical results

As expected, significant improvements in multiple clinical and laboratory parameters were observed on MTX therapy (table 2).

Laboratory

Haemoglobin increased from baseline and achieved significance at p<0.001 after 18 months of MTX therapy (table 2). Other laboratory changes from baseline are also seen in table 2.

Cytokines

The timing of sampling around MTX ingestion was of particular concern. Therefore, differences between samples obtained immediately prior to and 24 hours following a sequential MTX dose (pre and post) from baseline through month 36 were calculated for each immune parameter. Only serum IL-6 levels decreased significantly between the presamplings and postsamplings across all study counts (20.1 (21.5) to 14.2 (15.2), p=0.0003 after correction); none of the other differences observed for immune moieties between prevalues and postvalues approached statistical significance (p>0.20). We therefore used only the prevalues (ie, those obtained just prior to the weekly MTX dose (see the Methods section)).

Changes from baseline values of the serum cytokines and lymphocyte subsets in patients receiving MTX are

Table 2 Mean (SD) change with time in clinical and laboratory values in patients with RA treated with MTX (n=17)

	Months in study									
	Baseline (SD)	**Values**	**6**	**7**	**8**	**12**	**18**	**24**	**30**	**36**
Tender joints	19.2	(8.9)	−10** (9)	−11	−13**	−15+	−10*	−9*	−6	−14**
			(8)	(12)	(10)	(10)	(13)	(12)	(12)	
Swollen joints	18.6	(8.2)	−8+	−9+	−11+	−12+	−9*	−11+	−9+	−11+
			(7)	(7)	(7)	(7)	(10)	(6)	(7)	(6)
AM stiffness (in minutes)	283.3	(365.8)	−223*	−198	−336*	−148*	−169	−181	−128	−187
			(350)	(429)	(343)	(231)	(338)	(343)	(232)	(444)
Hrs to fatigue	6.3	(2.5)	3.1	3.7	2.6	2.3	4.0	0.2	−0.4	–
			(5.1)	(5.0)	(4.0)	(–)	(–)	(1.7)	(2.3)	
Pt. pain (0–5)	2.4	(0.62)	−0.5	−0.8**	−0.8*	−0.9*	−0.9*	−0.9**	−0.8**	−0.6
			(0.9)	(1.0)	(1.0)	(0.95)	(1.0)	(0.9)	(0.8)	(1.0)
Pt. global (0–5)	2.4	(0.62)	−0.7*	−0.9+	−0.8+	−1.1+	−0.8*	−0.8**	−0.7**	−0.6
			(0.9)	(0.8)	(0.7)	(0.9)	(0.9)	(0.9)	(0.8)	(1.2)
Haemoglobin, g/dL	12.6	(2.0)	0.7*	0.1	0.8*	0.8*	1.3+	0.6	0.9**	0.7
		(1.0)	(1.3)	(1.0)	(1.2)	(0.6)	(1.6)	(1.0)	(1.6)	
Platelets 10^3/mm^3	390.3	(130.5)	−57**	−65**	−73+	−64**	−71**	−71*	−106+	−100*
			(57)	(70)	(56)	(75)	(40)	(106)	(90)	(106)
AST (IU)	18.2	(6.3)	4.8	5.0	5.5	10.0*	2.9	−2.0	1.7	−0.5
			(14.6)	(20.2)	(18.7)	(14.5)	(14.0)	(8.3)	(13.0)	(8.7)
ESR, mm/hour	68	(32)	−35.5+	−34.9+	−35.0+	−31.9**	−11.3	−32.9**	−32.5+	−41.8**
			(23.1)	(29.8)	(20.9)	(27.5)	(78.0)	(35.4)	(30.8)	(35.0)
WCC, 10^3/mm^3	9.0	(2.5)	−1.9+	−1.8**	−1.7+	−1.1	−1.9*	−1.2	−3.2+	−2.7+
			(1.5)	(1.8)	(1.3)	(2.3)	(1.9)	(2.1)	(2.9)	(1.6)
RF (nephelometry)	420.9	(598.6)	−19.3	−116.3	−148.0	−265.7	−119.8	−230.8	−49.3	−384.1
		(174.5)	(410.3)	(354.5)	(570.9)	(867.7)	(691.7)	(803.8)	(732.8)	
Weekly MTX Dose (mg)	7.5	(0.0)	13.1	13.8	13.3	13.6	13.8	14.5	15.6	15.5

*p<0.05.
**p<0.01.
+p<0.001.
AST, aspartate aminotransferase; ESR, erythrocyte sedimentation rate; MTX, methotrexate; RA, rheumatoid arthritis; RF, rheumatoid factor; WCC, white cell count.

Table 3 Mean (SD) change from baseline in cytokine values, and absolute lymphocyte counts with time in patients with RA treated with methotrexate (n=17)

Months in study										
Cytokine (pg/mL)	**Baseline**		**6**	**7**	**8**	**12**	**18**	**24**	**30**	**36**
IL-1BP[t]	188.2	(173.2)	130 (353)	−94 (165)	12 (134)	92 (174)	15 (13)	102 (102)	160 (98)	225 (178)
IL-1BS[H]	72.2	(94.2)	−34+ (51)	−41** (50)	−29** (33)	−19 (48)	−39* (33)	−26* (40)	−32 (49)	−72 (94)
IL-2P	300.0	(197.5)	183 (314)	330* (424)	193 (352)	503** (442)	285* (268)	540* (572)	289** (232)	398* (388)
IL-2S	55.3	(11.4)	32 (23)	12 (23)	16 (32)	7 (60)	22 (32)	12 (6)	−9 (65)	−31 (35)
IL-4P	26.7	(36.9)	−9 (23)	−4 (51)	10 (35)	9* (9)	11 (5)	21 (21)	6 (7)	17 (10)
IL-4S	6.2	(8.2)	−4 (14)	−4 (11)	−12 (12)	2 (3)	−5 (11)	−7 (12)	−0.4 (2)	–
IL-6S	44.1	(25.3)	−33+ (21)	−28** (27)	−28* (35)	−25** (27)	−37** (25)	−37+ (26)	−46+ (25)	−2.8 (32)
IL-8S	457.4	(722.1)	−424* (663)	−302 (736)	−419 (669)	−481 (881)	−826* (760)	−411* (642)	−344 (627)	−111 (487)
	Baseline		**6**	**7**	**8**	**12**	**24**	**30**	**36**	
CD4	645	(519)	310 (446)	149 (798)	−35 (443)	−25 (493)	−136 (581)	144 (687)	–	
CD8	282	(182) (140)	−23 (137)	12 (186)	−10 (186)	−69 (156)	18 (109)	−26	–	
CD4/CD8	255	(485) (335)	−183 (187)	−75 (135)	69 (795)	−203 (81)	−26 (410)	−274	–	
CD4/CD29	426	(288) (111)	−16 (214)	23 (187)	−6.3 (322)	−170 (261)	−16 (102)	−194++	–	
CD4/CD45RA	531	(315) (255)	−44 (352)	−207 (319)	84 (221)	58 (395)	−58 (253)	−110	–	
CD5/CD19	99	(173)	−10 (42)	–	−110 (114)	982 (–)	−7.5 (13)	−73 (114)	–	

t supernatants from peripheral blood mononuclear cells.
H S=serum.
*p<0.05.
**p<0.01.
***Values obtained by multiplying total absolute lymphocyte counts by the percentage of each subpopulation as measured by FACS analysis (see the Methods section).
+p<0.001.
++p<0.05.
IL, interleukin; RA, rheumatoid arthritis.

seen in table 3. Significant changes over time were observed in the levels of IL-6, IL-8 and IL-1B. Each measurement of serum IL-6 through 30 months of treatment was significantly decreased from baseline. Serum IL-1B and IL-8 levels also consistently decreased after MTX treatment. Serum IL-2 and IL-4 levels did not significantly change after MTX treatment; however, the ex vivo ability of MTX to modify IL-2 production was observed in that Con A-stimulation of lymphocytes from the MTX-treated patients did produce significantly higher levels of IL-2 at 7, 12, 18, 24, 30 and 36 months.

Lymphocyte populations

We observed few changes in cell populations. Only the CD4/CD29 at week 30 reduced in a statistically significant manner (p=0.013; table 3).

Correlation of clinical and laboratory values with cytokines

Consistently significant correlations were observed between serial measurements of IL-6 in sera and multiple simultaneously assessed clinical parameters (table 4). As would be expected if IL-6 was related to disease activity, serum IL-6 levels strongly correlated with swollen joints (r=0.45, p<0.0001), physician global assessment of disease activity (r=0.47, p<0.0001), patient global assessment of disease (r=0.32, p=0.001), tender joints (r=0.32, p=0.002), patient evaluation of pain (r=0.29, p=0.003) and morning stiffness (r=0.27, p=0.007). In addition, an inverse correlation was observed between IL-6 and the interval to the first onset of fatigue (r=−0.31, p=0.043). Positive correlations were also observed between IL-8 in the sera and multiple measures of disease activity including tender and swollen joints and both physician and patient

Table 4 Pearson correlations of clinical, laboratory and lymphocyte subset values with cytokines in patients with rheumatoid arthritis on MTX therapy

	Tender joints	Swollen joints	AM stiffness	Patient global assessment	Physician global assessment	Patient evaluation of pain	Interval to fatigue	Adverse events*
IL1-B serum	–†	–	–	–	–	–	–	r=0.38 p=0.002
IL1-B supernatants	–	–	–	–	–	–	–	–
IL-2 serum (N=105)	–	–	–	–	–	r=–0.19 p=0.05	–	
IL-2 supernatants	p=–0.24 p=0.02	–	–	–	r=–0.27 p=0.014	–	–	–
IL-4 serum	–	–	–	–	–	–	–	–
IL-4 supernatants (N=105)	–	–	–	–	r=–0.32 p=0.00	r=–0.31 p=0.00	r=–0.67 p=0.034	–
IL-6 serum (N=68)	r=0.32 p=0.002	r=0.45 p<0.0001	r=0.27 p=0.007	r=0.32 p=0.001	r=–0.47 p<0.0001	r= 0.29 p=0.003	r=–0.31 p=0.043	–
IL-8 serum (N=67)	r=0.24 p=0.020	r=0.25 p=0.013	r=0.25 p=0.023	r=0.26 p=0.010	r=0.25 p=0.015	r=0.24 p=0.021	–	–

	WCC	Neutrophils	Lymphocytes	Rheumatoid factor titer	ESR	Haemoglobin
IL-1B (N=105) serum	–	–	–	–	r=23 p=0.021	–
IL-1B (N=105) supernatants	–	–	–	–	–	–
IL-2 (N=105) serum	–	–	–	r=35 p=0.05	–	–
IL-2 (N=105) supernatants	r=0.24 p=0.013	r=–0.22 p=0.05	r=0.22 p=–0.05	–	–	–
IL-4 (N=105) serum	r=0.30 p=0.002	–	r=0.44 p=0.016	–	–	–
IL-4 (N=84) supernatants	–	–	r=0.33 p=0.005	–	–	–
IL-6 (N=103) serum	r=0.37 p<0.0001	r=0.37 p<0.0001	r=0.24 p=0.023	r=0.21 p=0.05	–	–
IL-8 (N=92) serum	r=0.33 p=0.001	r=0.34 p=0.001	–	–	–	–

	Tender joints	Swollen joints	AM stiffness	Patient global assessment	Physician global assessment	Patient evaluation of pain	Interval to fatigue
CD4 (n=53)	–	–	–	–	–	–	–
CD8 (n=53)	r=0.27 p=0.05	–	–	–	r=0.30 p=0.03	–	–
CD4/CD8 (n=53)	r=0.36 p=0.008		–	r=0.27 p=0.05	r=0.31 p=0.02	–	–
CD4/CD29 (n=53)	r=0.27 p=0.05	–	–	–	r=0.36 p=0.009	r=0.26 p=0.059	r=0.26 p=0.058
CD4/CD45 RA (n=53)	–	–	–	–	–	–	–
CD5/CD19 (n-28)	–	–	–	–	–	–	–

*Indicates the total number of investigator-related episodes of toxicity reported at the time of a clinical evaluation which were judged to be related to MTX.
†Indicates a non-significant correlation.
ESR, erythrocyte sedimentation rate; IL, interleukin; MTX, methotrexate; RA, rheumatoid arthritis; WCC, white cell count.

assessment of global arthritis activity. The correlations with IL-8 were somewhat weaker than those seen with IL-6."

We observed an inverse correlation between IL-2 in the sera and patient evaluation of pain (r=−0.19, p=0.05). Most of the correlations of both serum and supernatant IL-2 and IL-4 and disease activity were inverse, consistent with their expression reflecting some restoration of immune homeostasis.

Other correlations between serum cytokines and laboratory values are seen in table 4.

Correlations of clinical and laboratory values with lymphocyte phenotypic markers

Both tender joint count and physician global assessment of disease activity correlated with CD8 (r=0.27, p=0.05 and r=0.30, p=0.03, respectively), CD4/CD8 (r=0.36, p=0.008 and r=0.31, p=0.02, respectively) and CD4/CD29 (r=0.27, p=0.05 and r=0.36, p=0.009, respectively) counts (table 4). CD4/CD8 cell numbers also correlated with patient global assessment of disease activity (r=0.27, p=0.05). No correlations were observed between CD4 cells and clinical disease activity. As expected, positive correlations were observed between absolute lymphocyte counts at each visit and lymphocyte phenotypic markers (table 5).

An inverse correlation was observed between CD4/CD45 and rheumatoid factor (RF) (r=−0.67, p=0.007), while a trend towards a positive correlation was seen between RF and CD4/CD29 (r=0.50, p=0.056) and CD4/CD8 (r=0.42, p=0.002). CD4/CD8 cells also exhibited a correlation with platelet count (r=0.33, p=0.016). Interestingly, both CD4/CD29 and CD4/CD45 RA cells correlated with total WCC (r=0.39, p=0.005 and r=0.28, p=0.05).

Correlation of mMTX PKCs with clinical, immune and laboratory parameters

Correlations between repeat MTX PKC measurements and clinical, immune and laboratory parameters are seen in table 6. A significant inverse correlation was observed between the change in AUC between the visits and the number of swollen joints (r=−0.63, p=0.007). A regression analysis using change in tender joints between visits as a dependent variable and the change between visits in all PKC variables revealed an inverse correlation with the change in AUC (r=0.42, r^2=18, p=0.0005). A regression analysis using change in swollen joints between visits showed an inverse correlation with change in the AUC (r=0.30, r^2=0.9, p=0.015).

Mean weekly MTX dose correlated directly with IL-2 concentrations in sera (r=0.20, p=0.038) and inversely with IL-6 in sera (r=−0.30, p=0.016), platelet count (r=−0.17, p=0.017 and CD8 cells (r=−0.48, p=0.009)). Direct correlations of AUC were observed only for IL-2 in supernatants from stimulated PBMCs (r=0.23, p=0.045) while an inverse correlation was observed with CD8 cells (r=−0.41, p=0.028).

Correlation of cytokines with each other

Cross correlations of prospectively and simultaneously measured cytokines can be seen in online supplementary table S7.

DISCUSSION

We have demonstrated significant changes in the concentrations of several cytokines in sera and arising from ex vivo PBMC stimulation along with changes in lymphocyte phenotypic markers from a population of consecutive patients with active RA beginning therapy with MTX. By obtaining serial measures of these immune parameters along with simultaneous assessments of clinical and laboratory function over a period of 36 months of treatment, we have been able to derive correlations between these immune measures and simultaneously assessed MTX PKCs. We were less interested in composite efficacy outcomes such as the American College of Rheumatology (ACR) composite disease activity score or disease activity score (DAS), and more interested in individual clinical measures and their reaction to both PKCs

Table 5 Pearson correlations of lymphocyte subsets with laboratory values in patients with rheumatoid arthritis on methotrexate

	WCC	Neutrophils	Lymphocytes	Rheumatoid factor	Platelet count	ESR
CD4 (n=52)	–	–	r=0.82 p=0.001	–	–	
CD8 (n=52)	–	r=0.66 p=0.01	r=0.79 p=0.001	–	–	
CD4/CD8 (n=52)	–	–	–	r=0.33 p=0.016	–	
CD4/CD29 (n=52)	r=0.39 p=0.005	–	–	r=0.50 p=0.056	–	–
CD4/CD45 (n=52)	r=0.28 p=0.05	–	r=0.73 p=0.005	r=−0.67 p=0.007	–	–
CD5/CD19 (n=28)	–	–	–	–	–	–

ESR, erythrocyte sedimentation rate; WCC, white cell count.

Table 6 Pearson correlations of methotrexate pharmacokinetic parameters with immune parameters and laboratory values in patients with rheumatoid arthritis

	IL-1B serum	IL-1B supernatant	IL-2	IL-2 supernatants	IL-6 serum	IL-8 serum	Serum	WCC	Platelets	CD4	CD8	CD4, CD8
AUC			r=0.23 p=0.045							r=−0.41 p=0.028		
CMAX							r=−0.17 p=0.016	r=−0.17 p=0.017	r=0.34 p=0.072	r=−0.48 p=0.009	r=−0.32 p=0.09	
TMAX t*												
Weekly MTX dose (mean)				r=0.20 p=0.038	r=−0.30 p=0.002							

*t=Bioavailability.
AUC, area under the curve; CMax, maximal serum concentration; IL, interleukin; MTX, methotrexate; TMax, time from dosing to maximal serum concentration; WCC, white cell count.

of MTX and the multiple cytokines and lymphocyte immunophenotypes reported. In this way, we could distinguish the associations of both MTX PKCs and the immune parameters with the individual, and disparate, clinical measures reported. We believe that this approach is preferable to associations with composite measures for deriving meaningful clinical insights. To our knowledge, this is the first investigation to combine prospective sequential measures of clinical outcomes along with MTX PKC and immune measures of disease activity over periods which are significantly longer than any randomised controlled trial.

We report highly significant decreases in serum IL-1B, IL-6 and IL-8 on start of MTX. These changes were persistent over 36 months. The highly significant decreases in IL-6 and IL-8 correlated strongly with the changes in multiple targeted and specific measures of disease activity including both swollen and tender joints as well as patient and physician assessment of global disease activity. Given the known systemic biological impact of IL-6 especially, it is likely that these findings may account for at least some of the beneficial effects of MTX in patients with RA.

Decreases from baseline measures in IL-6 and other cytokines have been reported in patients with RA on gold therapy,[20] hydroxychloroquine[21] and corticosteroids[22] as well as MTX.[23 24] Barrerra *et al*[23] reported serial measurement of serum IL-6 along with tumour necrosis factor (TNF)-α and sIL-2R over a period of 48 weeks in a combined cohort of patients with RA on azathioprine and MTX. Correlations were sought with clinical outcomes and some were found although decreases in sIL-2R 'were associated with clinical improvements'. Crilly *et al*[24] reported significant decreases in IL-6 after 12 weeks of MTX, which were not seen at 24 weeks, perhaps because the mean weekly MTX dose in that study was only 6.5 mg/week. The functions of IL-6 have been extensively reviewed[25–28] and include the stimulation of hepatocyte synthesis of acute phase reactants, B-cell differentiation[27] and stimulation of osteoclastogenesis.[28] IL-6 may also be involved in the cartilage degradation seen in RA,[29 30] and is protective for hepatocyte injury.[31] Thus our data have important functional implications.

The consistent and highly significant decreases of IL-6 that we observed with MTX treatment are striking. IL-6 correlations with disease activity and both total WCC and absolute neutrophil counts were stronger than for those of the other measured cytokines, suggesting a central role in disease pathogenesis, perhaps manifest at least in regulating bone marrow function and circulatory properties of the measured leucocyte subsets. Recent studies identifying a STAT3 transcriptional profile in CD4T cells in early RA are consistent with these observations.[32] IL-6 may be derived from several cellular sources including T cells and monocytes. The significant positive correlations between serum levels of IL-6 and both IL-8 and IL-1B and the lack of significant effects of MTX on T-cell-derived cytokines IL-2 and IL-4 in sera suggest a

common MTX effect on monocytes/macrophage-derived cytokines.

In spite of the significant and consistent decreases in serum IL-1B from baseline values seen with our patients with RA receiving MTX, no correlations of IL-1B with clinical markers of disease were observed (table 4), suggesting that at least the clinical outcomes we measured are not tightly linked to the presence of circulating IL-1. However, IL-1B in serum was the only cytokine that correlated with patients reporting adverse events to MTX (table 4). The significance of this observation is presently unclear.

Memory T cells may be important in early RA synovitis[33] although parallel, not incompatible models for pannus formation have been proposed.[34] Early RA synovial biopsies are enriched for clonal selection consistent with local antigen-driven T-cell expansion.[34] While most investigations of the effect of MTX on circulating blood lymphocytes have reported no consistent effect,[35 36] alterations in CD8 cells in blood from patients with RA on MTX have been reported.[37 38] Patients with recent onset RA have an increase in CD8+ lymphocytes in blood and synovial fluid.[38] Both peripheral blood and synovial fluid from patients with RA exhibited a decrease in CD8+/CD45RA+ suppressor effector cells and normal percentages of CD4+/CD29+ helper inducer or memory cells.[39] Notably CD8 cells recirculate from lymph nodes to the circulation and as such may be a population whose circulatory frequency is such as to allow them to report on such functional changes after initiation of MTX therapy. Whether this confers functional primacy is unclear at this stage.

We observed no significant changes from baseline in lymphocyte subsets with the exception of a decrease in the CD4+/CD29+ population after 30 months of MTX. We did, however, observe several correlations with serial measurements of lymphocyte surface phenotypes with both clinical and laboratory parameters of disease activity (tables 4 and 5). It is of interest that cells with the CD4 immunophenotype exhibited no correlations with disease activity while circulating blood CD8, CD4/CD8 and CD4/CD29 helper inducer or memory cells exhibited correlations with several parameters of disease including tender joints. The majority of lymphocytes in the synovial fluid of patients with RA are known to be of the CD4/CD29 immunophenotype,[39] and we observed some correlations between circulating cells of this lineage and markers of disease activity (tables 4 and 5). It is possible that MTX has a greater effect on these memory helper T cells in synovium than the generally non-significant decreases which we observed in blood (table 4). It should be noted that in our hands, the CD4/CD8 phenotype is the result of a serum factor influence that occurs in about 45% of the patients with RA tested and the <2% of the 'healthy' controls.[40] This phenomenon has been suggested to be due to an artifactual staining from serum immunoglobulin.[41] Whether the CD4/CD8 double-positive phenotype results from immunoglobin or some other serum factor, the level of this confounding factor must be modified by MTX. The absence of more significant correlations of lymphoid subset changes with disease status may be due to the specific marker analysis employed or restriction of analysis to peripheral blood. Numerous lymphocyte subsets exist in different states of maturation and activation. Since a minor lymphoid population could be responsible for disease maintenance, a general screen with a limited number of markers could miss the population of importance. Changes in synovial T-cell populations may also not parallel those observed in blood.

We also performed detailed and repeated MTX PKC measurements and examined correlations with laboratory and immune parameters (table 6). We believe that this is the first time that MTX PKC measures have been repeated over these very long treatment intervals with simultaneous measures of disease activity, as well as clinical, cytokine, lymphocyte immunophenotype and laboratory measures. We found that production of IL-2 from PBMC supernatants obtained at the time of the PK measures correlated with the AUC for MTX, while weekly MTX dose correlated with serum levels of IL-2. The increases from baseline in IL-2 from serum in stimulated PBMCs during MTX treatment were observed at multiple time points (table 3) but did not correlate with most measures of disease activity (table 4). IL-2 deficiency in serum and synovial fluid of patients with RA has been noted for some time.[42 43] It is possible that the mechanism of the MTX-induced increase in IL-2 reflects MTX-induced alterations in polyamine production.[44 45] Expansion of IL-2-dependent Treg subsets is an intriguing hypothesis that arises and deserves further consideration as this might account for robust durable response over time as homeostatic regulatory responses are recovered in patients on MTX. Emerging Treg populations have been reported also for TNFi, for example, etanercept, adalimumab.[46]

All other correlations between MTX PKCs and immune parameters were inverse as would be expected if increasing the weekly dose resulted in a decrease in the value being measured. Inverse correlations were observed between AUC and CD8 cells and MTX Cmax and CD8 and CD4/CD8 lymphocytes as well as total WCC and platelet counts. We observed variable increases in the production of IL-2 and inconsistent effects on the number of CD4 and CD8 cells. The alterations in lymphocyte phenotypic markers were generally not significant when compared with baseline values but the direction of change in response to MTX is evident by the clinical correlations observed (tables 4 and 5).

In summary, we have described significant alterations in prospectively measured serum and PBMC-derived cytokines, as well as lymphocyte phenotypic markers, from blood in patients with RA on MTX along with simultaneously measured clinical and PKC parameters. We have performed these measures prospectively, at regular 6-month intervals, over a period of 36 months. We have demonstrated for the first time that an increase in the MTX AUC per se produces significant decreases in swollen and tender joint counts. We believe that the

scope, duration and breadth of the measurements are unique. While the studies were actually performed just prior to the biological era, we believe that the results actually have become increasingly compelling.

Owing to the unique and notable design of the study in sequential patients starting MTX, we could derive correlations between all of these measures and individual clinical parameters of disease. The weaknesses include the fact that we could of course have included many other measures of immune function and that a total of only 17 patients were included. We however believe that it is unlikely that the measures we report are skewed from what would be expected in other patients starting MTX, but we acknowledge that our observations should be replicated. It should also be noted that most measures of PK and immune function have typically included smaller numbers in the range which we report.

In addition, the serum cytokine measured may not be reflective of those in synovium, or the totality of the implicate immune response in RA. Nevertheless, serum measures of cytokines are typically reported in association with IL-6 and Janus kinase (JAK) inhibitors and are an accepted surrogate for the mechanism of action of these agents.[47] It is possible that the alterations in cytokine production and lymphocyte immunophenotypes may be secondary events, which occur when disease improves. We do not therefore easily infer causality in our analyses and their interpretation. A control group was simply not feasible over the time course of this trial. However, the compelling relationship between the immune changes reported and simultaneous PK measures strongly suggest that the findings are related to MTX intervention and are not simply a surrogate for general disease improvement. The significant decreases in serum IL-6 observed with MTX may explain further increases in transaminase enzymes when the drug is combined with either IL-6, or JAK, inhibitors.[48 49] While a single prior publications of the effects of MTX on lymphocyte immunophenotypes[50] has been previously published, it was of only 16 weeks duration and did not have the benefit of simultaneous measurements of PK measures. We believe that in an era of expanded attention to biomarkers of disease, our findings, which correlate with PK parameters and disease activity over 3 years, should help illuminate the field.

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