A Novel Predictor of Clinical Response to Methotrexate in Patients with Rheumatoid Arthritis: A Pilot Study of *in Vitro* T Cell Cytokine Suppression

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ABSTRACT. ***Objective.*** Methotrexate (MTX) is an important drug for treatment of rheumatoid arthritis; however, there is variation in the clinical response. MTX inhibits T cell cytokine production, with significant interindividual variability in the dose required. We investigated if the variability in clinical response was related to variability in the *in vitro* assay.

Methods. Patients with disease modifying antirheumatic drug-naive, active RA [1982 American College of Rheumatology (ACR) criteria] seen from September 2005 through January 2006 were enrolled. MTX was started at 10 mg/week and increased monthly by 2.5 mg/week. Baseline whole-blood cultures were set up with anti-CD3, anti-CD28, and increasing doses of MTX. Supernatants were harvested at 96 hours and tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and interleukin 10 (IL-10) concentrations were estimated by ELISA. The dose of MTX (ID_{50}) required for 50% suppression of production of cytokines and the change in Disease Activity Score-28 (ΔDAS) at 4 months were noted.

Results. T cell stimulation resulted in significant increase in cytokine release, and addition of MTX led to a dose-dependent suppression of all 3 cytokines. There was significant negative correlation of ΔDAS with ID_{50} values for TNF-α ($R = –0.62$, $p < 0.01$) and IFN-γ ($R = –0.43$, $p = 0.04$). At 4 months, EULAR moderate and ACR 20% responses were achieved by 13 and 16 patients, respectively. EULAR moderate response could be predicted using ROC curves for TNF-α (sensitivity 93%, specificity 86%) and IFN-γ (60% specificity, 71% sensitivity). ACR response was correctly predicted in 14 of 16 ACR 20% responders and in all ACR 50% and ACR 70% responders.

Conclusion. An *in vitro* TNF-α suppression assay may help predict clinical response to MTX in RA. (First Release May 1 2008; J Rheumatol 2008;35:975–8)

Key Indexing Terms:
DRUG RESPONSE DISEASE MODIFYING ANTIRHEUMATIC DRUGS CYTOKINES
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Patients with rheumatoid arthritis (RA) have a 7-fold increase in disability and higher mortality compared to a normal population[1,2]. Maximal joint damage occurs early and the use of disease modifying antirheumatic drugs (DMARD) slows progression[3]. Methotrexate (MTX), the cornerstone of DMARD therapy in RA, retards radiological damage[4]. However, 30% of patients fail to respond and require additional therapy[5]. Thus substantial delay occurs in controlling disease activity in this subset of patients; a method to predict treatment failure at baseline itself would be useful. General indicators of poor response to DMARD have been identified, but do not help in individual treatment decisions[6,7].

MTX affects DNA synthesis, adenosine synthesis, and production of cytokines and other inflammatory mediators[8]. Genetic polymorphisms in the enzymes of the folate pathway predict MTX toxicity well, but not the clinical response to it[8,9]. MTX inhibits T cell cytokine production, with significant interindividual variability[10]. Is this interindividual variability in *in vitro* cytokine suppression related to variability in clinical response to MTX? We correlated the results of baseline *in vitro* cytokine suppression using MTX to the clinical response in patients with DMARD-naive RA.

MATERIALS AND METHODS

Consecutive patients with RA [1982 American College of Rheumatology (ACR) criteria] seen from September 2005 through January 2006 were enrolled if they had active disease, defined as a 28-joint Disease Activity Score (DAS28) > 3.2. Those with prior DMARD or corticosteroid treatment and contraindications to MTX were excluded. The study was approved by the institutional ethics committee and consent was given by all patients.

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At first visit, after clinical examination and baseline laboratory tests, a stable dose of nonsteroidal antiinflammatory drug (NSAID) was started. Two weeks later, if the laboratory results revealed no contraindications to MTX treatment, blood was withdrawn for whole-blood culture and MTX (10 mg/wk) and folic acid (10 mg/wk) were started. Physician and patient global assessment, Health Assessment Questionnaire (HAQ), and DAS28 score were assessed at baseline and on each monthly visit thereafter. Corticosteroids were not permitted. The MTX dose was increased every month by 2.5 mg until remission of disease (DAS28 ≤ 2.6) or a dose of 17.5 mg was reached at 3 months. The European League Against Rheumatism (EULAR) and ACR response criteria were assessed at 4 months[11,12]. The clinical examiner was blinded from the cytokine assay results.

Cytokine suppression assay. Heparinized blood (2 ml) was diluted 1:5 in complete RPMI media containing 25 mM Hepes (SRL, India) supplemented with glutamine and antibiotic, antimycotic solution (Sigma, St. Louis, MO, USA). Cultures (1 ml each) were set up in 24-well polystyrene flat-bottom multidishes (Nunclon, Denmark). T cells were stimulated with 1 µg/well of anti-CD3 and anti-CD28 monoclonal antibodies (eBioscience, San Diego, CA, USA). Seven concentrations of MTX (Sigma) in doubling dilutions from 1000 to 15.6 ng per well were used for the inhibition assay. After 96 hours[10], plates were centrifuged and supernatants harvested and stored at –70°C until analysis. At the doses of MTX used, cell viability by MTT reduction assay and trypan blue assay was > 95% and similar to control cultures.

Appropriately diluted supernatants were tested in commercial enzyme immunoassays for tumor necrosis factor-α (TNF-α; R&D Systems, Minneapolis, MN, USA), interferon-γ (IFN-γ), and interleukin 10 (IL-10; Becton Dickinson, Mountain View, CA, USA). The sensitivity of assays was 15.0 pg/ml for TNF-α and IFN-γ and 7.8 pg/ml for IL-10.

Cytokine production was plotted on a log-linear graph against the corresponding dose of MTX; and the inhibitory dose 50 (ID_{50}), the dose required to suppress cytokine production by 50%, was calculated based on the curve generated. The reproducibility of the assay was verified by estimating the intrapatient variability in values of ID_{50} when the test was repeated 24 h later. The intrapatient assay variability calculated in 4 patients and 2 normal controls was 1%–5%.

The change in DAS28 (ΔDAS) at 4 months was correlated to ID_{50} values of MTX. Using receiver-operating characteristic (ROC) curves, a cutoff value for ID_{50} that gave optimum specificity and sensitivity in identifying EULAR moderate responders was identified. These cutoffs were used for prediction of ACR and EULAR responses. EULAR moderate response refers to a reduction in DAS28 of > 1.2 with the baseline DAS28 being > 5.1, or a reduction of > 0.6 if the baseline DAS28 is > 3.2 and < 5.1[11]. Spearman's rank correlation and chi-square tests were used, and all analyses were done using SPSS 13® for Windows software.

RESULTS

Thirty-one patients were enrolled, of which 6 were excluded (2 contamination of cultures, 1 took corticosteroids, 2 lost to followup, 1 developed pulmonary tuberculosis). Clinical characteristics of the 25 patients (18 female, 20 seropositive) and the basal and stimulated cytokine levels are shown in Table 1. T cell stimulation resulted in significant increase in cytokine release, and addition of MTX led to a dose-dependent suppression of all 3 cytokines. The MTX ID_{50} values varied significantly (Figure 1A, 1B) with median (interquartile range; IQR) values of 180 (131, 300) ng/ml for TNF-α, 148 (10, 253) ng/ml for IFN-γ, and 129 (61, 173) ng/ml for IL-10.

At 4 months, 13 patients achieved moderate response, 4 had good response, and 3 were in remission (EULAR).

Table 1. Baseline characteristics of patients.

Characteristic	Median (interquartile range)
Age, yrs	44.5 (36.5, 53.5)
Duration of disease, yrs	3 (1.1, 5.5)
Tender joint count	12 (7.5, 26)
Swollen joint count	7 (4, 10.5)
Erythrocyte sedimentation rate, mm/h	68 (46, 96)
Patient's general health VAS (0–100)	65 (60, 70)
DAS28	6.5 (5.5, 7.2)
HAQ	1.5 (1, 1.9)
TNF-α level, pg/ml	
Baseline	330 (60–650)
Stimulated	1725 (718–3701)
IFN-γ level, pg/ml	
Baseline	26 (1.6–117)
Stimulated	14,434 (304.5–833,149)
IL-10 level, pg/ml	
Baseline	26 (17–122)
Stimulated	92 (48–361)

DAS28: 28-Joint Disease Activity Score; HAQ: Health Assessment Questionnaire; VAS: visual analog scale.

ACR20, ACR50, and ACR70 responses were observed in 16, 8, and 3 patients, respectively. The median MTX dose was 17.5 (IQR 15, 17.5) mg/week at 4 months.

There was significant negative correlation of ΔDAS with ID_{50} for TNF ($R = –0.62$, $p < 0.01$; Figure 1A) and IFN-γ ($R = –0.43$, $p = 0.04$; Figure 1B) but not with IL-10. Using a ROC curve, a TNF-α ID_{50} cutoff value of 224 ng/ml gave the best sensitivity and specificity values for predicting EULAR moderate response. This cutoff predicted patients having moderate EULAR response with 93% sensitivity and 86% specificity (area under curve 0.898). Patients with TNF-α ID_{50} values above and below the cutoff had distinct clinical courses, with a majority of patients with lower ID_{50} having a better response at 4 months (Figure 1C). Out of 16 patients who achieved more than ACR20 response, 14 patients had TNF-α ID_{50} lower than the cutoff (Table 2). The positive (PPV) and negative (NPV) predictive values of the cutoff for achieving the ACR20 response were 87.5% and 77.78% (OR 49, 95% CI 3.8–637.8). Similarly, ACR50 and ACR70 responses were achieved by 8 and 3 patients, respectively; all had MTX ID_{50} of TNF-α lower than the cutoff.

Similarly, an IFN-γ ID_{50} cutoff value of 148 ng/ml identified patients having moderate EULAR response with 60% sensitivity and 71% specificity (area under curve 0.694). Using this cutoff, the clinical courses of patients were not as distinct as observed using the TNF-α cutoff (Figure 1D). ACR20, ACR50, and ACR70 responses in the 2 groups were not statistically significant (Table 2). The PPV and NPV for achieving ACR20 response were 72.72% and 36.36%, respectively.

DISCUSSION

Our data show that there is significant interindividual variability in MTX-induced suppression of TNF-α, IFN-γ, and

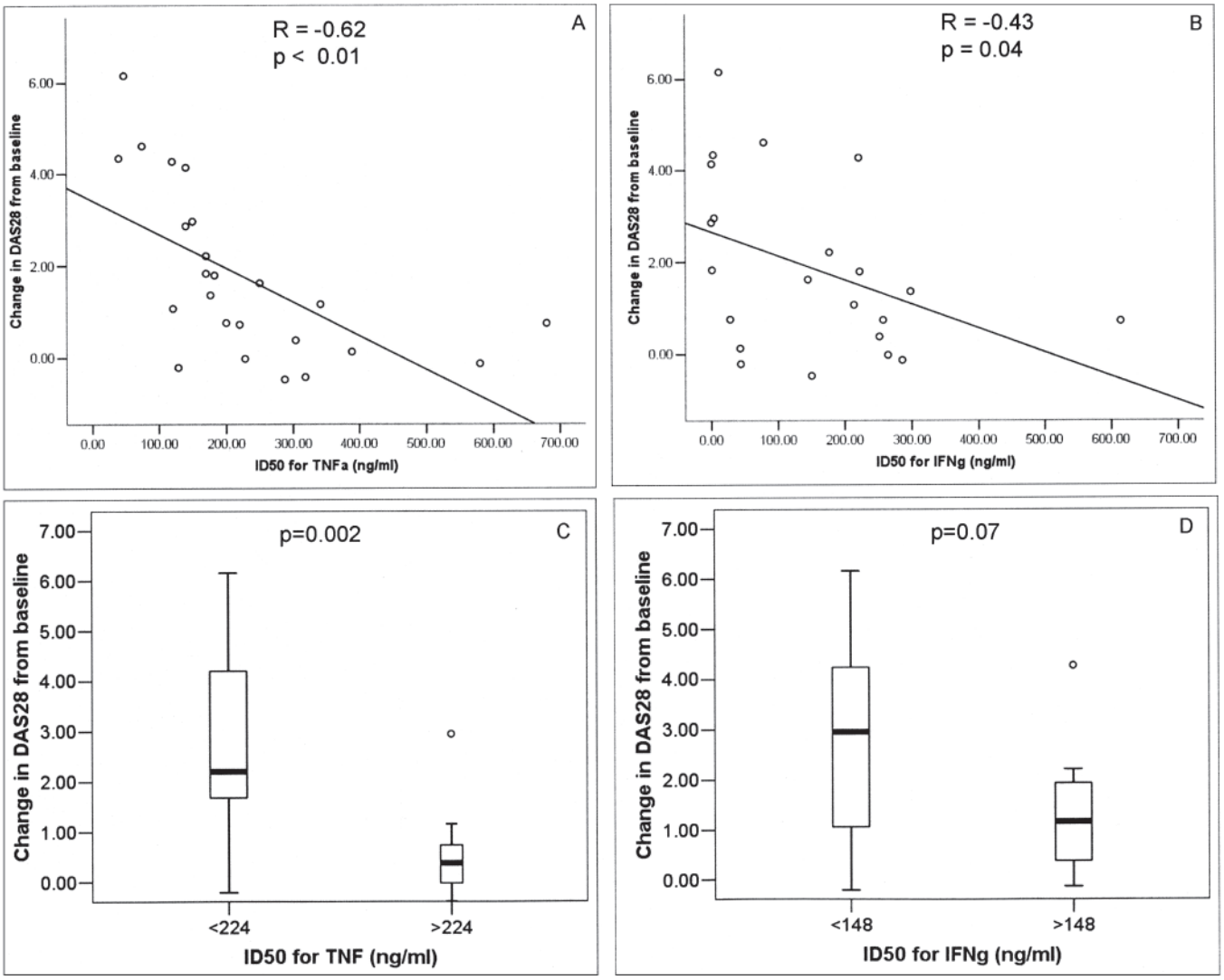

Figure 1. Data show significant variability in ID_{50} values for TNF-α (A) and IFN-γ (B). A significant inverse correlation can be seen between the ID_{50} values of both TNF-α and IFN-γ and the change in DAS28 (ΔDAS). The mean reduction in DAS28 in patients with an ID_{50} above the cutoff value (224 ng/ml) was significantly less than in those with lower ID_{50} (C). The mean reduction in DAS28 in patients with ID_{50} values for IFN-γ above and below the cutoff value 148 ng/ml was not significantly different (D).

IL-10 production in whole-blood cultures. This interindividual variability correlated significantly with clinical response to MTX. Thus, *in vitro* measurement of the ID_{50} of MTX is a useful predictor of clinical response to this drug.

Despite the extensive clinical use of MTX, identifying clinical response predictors has not been easy. It has been shown in murine models of arthritis and humans that MTX inhibits TNF-α production mainly through its effect on T cells, not macrophages[10,13]. Further, patients who are treated with MTX show a gradual decline in the frequency of TNF-α-producing T cells[14].

This novel finding of a strong relationship between the *in vitro* and clinical responses suggests that T cell cytokine inhibition is an important mechanism of action of MTX. Patients in whom a higher dose of MTX was required for *in vitro* cytokine suppression had a poorer clinical response to this drug. Of the 3 cytokines tested, suppression of TNF-α best discriminated the responders and nonresponders to MTX. This is in agreement with observations that anti-TNF therapy results in excellent clinical response in RA. In view of the pivotal role of TNF-α in the pathogenesis of RA, the *in vitro* effect of MTX on TNF-α production is possibly determining the sensitivity of patients' T cells to MTX. Cell viability assays showed no significant cell death and the effect seen was most likely due to suppression of T cell metabolism[14].

Another important issue that has to be considered is the cell count in each well at the beginning. As our aim was to devise a test that can be used in routine clinical practice, we tried to keep the assay as simple as possible. If the number

Table 2. ACR responses at 4 months in patients with high and low methotrexate ID_{50} values for TNF-α and IFN-γ.

ACR Response	ID_{50} TNF-α (ng/ml) < 224 (n = 16)	> 224 (n = 9)	p	ID_{50} IFN-γ (ng/ml) < 148 (n = 11)	> 148 (n = 11)	p
Nonresponders	2	7	0.002	3	4	NS
> ACR20	14	2		8	7	
> ACR50	8	0	0.02	5	3	NS
> ACR70	3	0	NS	3	0	NS

ACR: American College of Rheumatology; ID_{50}: 50% inhibitory dose; NS: nonsignificant.

of cells is significantly different, it is possible that the wells with more cells would have higher cytokine production. The variation in basal and stimulated cytokine production among patients could be related to this or due to cytokine gene polymorphism. However, as each patient serves as a control for him/herself, and the percentage suppression rather than absolute reduction in cytokine was considered, this variability would not affect the ID_{50}. The other issue is intraindividual variability. However, our limited data on this show that it was not significant. We did not test for circadian variation, which could occur because of a circadian rhythm of endogenous steroids, but samples from all patients were obtained in the morning.

The mean time to initial response with MTX is 9.5 weeks[15], thus assessment at 4 months is sufficient to identify patients who would respond to MTX. With the paradigm shift in the treatment of RA toward aggressive and early treatment, most rheumatologists would decide by 3 months about MTX response.

We are trying to validate this assay, in a larger cohort, and are also studying cytokine-producing cells before and after MTX. With a larger sample size it would also be possible to study the efficacy of the same assay in predicting EULAR good response and remission.

Oral MTX (10 mg/week) results in blood concentrations of 50–100 ng/ml, and higher levels would be expected with doses up to 17.5 mg/week[8]. Hence the plasma MTX levels achieved are comparable to the ID_{50} values observed in our study. This is further corroboration of the clinical–*in vitro* response link, although the correlation between serum levels and the clinical response to MTX is not strong[5].

A multiple gene polymorphism score predicted response to MTX but it underperformed and was more complicated compared to our study[9]. Patients who attained higher red blood cell (RBC) polyglutamated (PG) MTX levels at 3 months and any reduction in RBC folate PG levels at 4 months of MTX administration had better clinical response at 6–8 months[9]. However, this method cannot be used at baseline. The simple one-point assay we described is sensitive and specific in identifying the response to MTX in individual patients at baseline.

In vitro suppression of TNF-α is a novel and efficient assay to predict the clinical response to MTX in patients with RA. Validation of the assay in a larger cohort is needed to determine its role in clinical decision-making.

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