

Combination therapy with cyclosporine and methotrexate in patients with early rheumatoid arthritis soon inhibits TNF production without decreasing TNF mRNA levels. An *in vivo* and *in vitro* study

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Abstract

Objective

To evaluate the ability of two different combination therapies with prednisone (PDN), methotrexate (MTX) and cyclosporine (CSA) to modulate both TNF transcription and production in early rheumatoid arthritis (RA).

Methods

24 patients with early RA received a step-down bridge therapy with MTX and PDN (group A). Twelve patients out of the 24 randomly received also CSA (group B). Blood samples and peripheral blood mononuclear cells (PBMC) were collected at different times. TNF levels were measured both in sera and in PBMC supernatants. TNF mRNA was assessed by use of RT-PCR.

Results

10 patients in group A and 9 in group B improved. At baseline, RA patients serum TNF levels were increased compared to controls ($p < 0.001$) and did not correlate with clinical and serological parameters. These levels decreased within the first month of therapy in both groups, the lower levels being observed in the sera of CSA treated patients. After 30 days of therapy, TNF levels in group B supernatants were significantly lower than those observed in group A, both after 24 and 48 hours of PHA stimulation ($p < 0.03$ and $p < 0.05$ respectively). TNF mRNA levels never differed between patients and controls, independently of both the clinical picture and the assigned therapy.

Conclusion

The addition of CSA to a treatment regimen of PDN + MTX lowers TNF production *in vitro* without decreasing TNF mRNA expression. This effect could help to induce early immunosuppressive and therapeutic effects during RA.

Key words

TNF, rheumatoid arthritis, drug therapy.

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Introduction

An imbalance in the production of competing cytokines plays an important role in the pathogenesis of chronic synovitis characteristic of rheumatoid arthritis (RA) (1). In RA, it has been shown that an over-production of IL1 and TNF can promote inflammation and tissue destruction. Furthermore, anti-inflammatory cytokines and the shedding of soluble receptors of TNF and IL1, which display cytokine inhibitory activity, are insufficient in RA joints to continuously suppress the effects of pro-inflammatory cytokines. This may explain, on the one hand, the typical alternate remissions and relapses of the disease (1,2) and, on the other hand, the evidence that although markers of inflammation such as joint swelling may improve over 5 years, markers of damage such as radiologic progression may show worsening over the same period in the same patients (3).

Although cytokines other than TNF may be able to directly damage different structures in involved joints, it has been reported that the production of many pro-inflammatory cytokines is controlled by TNF, suggesting its pivotal role in the pathogenesis of RA and its potential role as a new therapeutic target (4). In fact, chimeric anti-TNF monoclonal antibody (cA2) (5), as well as the use of recombinant soluble receptor (p75)-Fc fusion protein (p75) (6), improve the clinical and laboratory parameters of disease activity. Unfortunately, the therapeutic effects of a single dose of these substances last 8-10 weeks or less and are followed by relapses. This is a general trend observed using any monotherapy in RA and thus continual "coverage" with combination therapy, as in transplantation, is required for rheumatologists to control RA.

In recent years several immunosuppressive and anti-inflammatory drugs [disease modifying anti-rheumatic drugs (DMARDs)] have been used in combination therapy in RA patients, with evidence of clinical improvement. This evidence, together with the importance

of TNF as a target in RA, suggest that drugs affecting anti-TNF activity may induce clinical improvement. This consideration suggests a potential new role for active DMARDs in therapeutic strategies, using both biological response modifiers and organic substances via their TNF inhibition, thus decreasing the number of disease relapses.

We sought to evaluate the effect of two different combination therapies with prednisone (PDN), cyclosporine (CSA) and methotrexate (MTX) on: (i) circulating levels of TNF *in vivo*; and (ii) the production and transcription of this cytokine by peripheral blood mononuclear cells (PBMC) *in vitro* in early RA patients. Furthermore, we analyzed possible correlations of these findings with clinical and laboratory parameters of clinical response and disease activity.

Patients and methods

Patient groups and monitoring

Twenty-four patients (20 to 60 years of age) who: (a) met the revised criteria of the American Rheumatism Association for RA (7); (b) had a disease duration of less than 2 years; and (c) never received any DMARD therapy, were enrolled, after giving their informed consent, in a controlled, randomized, without placebo study. They were placed on a step-down bridge therapy for 6 months, with a starting dose of 15 mg/week of MTX and 25 mg daily of PDN in the first month (group A). Twelve patients out of the 24 also randomly received 3.5 mg/Kg/day of CSA (group B). After the first month, PDN was reduced to 12.5 mg daily for 15 days and progressively tapered off. After 2 months CSA was decreased to 2.5/Kg/day in group B. The weekly dosage of MTX was not changed. No patient needed supplementation with any other NSAIDs during the study period. This study design was chosen because the primary objective of our study was to examine cytokine production under different conditions in early RA patients rather than the statistical power of the demographic data provided. Furthermore, it has recently been reported

that when studying different combinations of drugs, the appropriate comparison may not be to a placebo but between the different combinations themselves in order to assess the most effective and least toxic (8), mirroring the oncologic experience.

Disease was defined as active by the presence of at least 3 of the following criteria: >6 joints that were tender or painful on motion, >3 swollen joints, increase in the Westergren erythrocyte sedimentation rate (ESR) >28, morning stiffness >45 minutes in duration, both the patient's and physician's analog scale for the global assessment of disease activity >50. The total blood count, urinalysis, and serum determinations of: creatinine, blood urea nitrogen, electrolytes, total bilirubin, aspartate aminotransferase, alkaline phosphatase and uric acid were monitored every 15 days in the first 2 months of treatment and then monthly. ESR and rheumatoid factor were measured at baseline and at the end of the 6-month study period. CSA was discontinued if the serum creatinine levels increased by 30% or more from the baseline level until the level decreased and CSA could be re-administered. MTX was discontinued if the serum aminotransferase concentration exceeded twice the upper limit of normal, the white cell count fell below 3000/mm³, the platelet count fell below 100,000/mm³ and a significant red cell macrocytosis was evident (MCV > 96 fl). MTX therapy was re-administered when the values became normal.

Patients were excluded from the study if they had hypertension, abnormal hepatic or renal functioning, a platelet count below 100,000/mm³, leukopenia (total white blood cells < 3000/mm³), red cell macrocytosis (MCV > 96 fl), a history of cancer, or concomitant therapy with experimental drugs during the month before enrollment. Women of reproductive age were required to take appropriate contraceptive measures.

Twenty-four sex- and age-matched healthy subjects, with the same demographic profile as the studied population served as controls.

Definition of clinical improvement

Clinical assessment included the same parameters used to define active RA for study inclusion, namely: (i) both the tender and swollen joint counts (68 and 66, respectively); (ii) the patient's and physician's global assessment of disease activity, using a visual analog scale (0 mm = no disease; 100 mm = very severe disease); (iii) ESR values; and (iv) duration of morning stiffness, were considered. Improvement was defined as >20%, >50%, >70% improvement in both the tender and swollen joint counts respectively, plus >20%, >50%, >70% improvement respectively in 3 of the other variables (ACR20, ACR50, ACR70, respectively), according to the preliminary definition of improvement in RA (9).

Blood samples

Blood samples were collected from patients before treatment, after 30 days of treatment with PDN plus MTX (group A) or PDN plus MTX and CSA (group B), and during the maintenance therapy (about 60 and 180 days from the beginning of the study). Sera were recovered and aliquoted into vials, stored frozen at -80°C and defrosted immediately before assays. To minimize interassay variations, all samples from each patient were measured in a single assay.

Cell cultures

Heparinized venous blood was obtained from all patients and controls and peripheral blood mononuclear cells (PBMC) were isolated by centrifugation on a Ficoll-Hypaque gradient and counted. Their viability, as assessed by trypan blue dye exclusion, was routinely greater than 95%. PBMC, at a density of 2 x 10⁶ cells/ml (in RPMI 1640, with 10% heat-inactivated fetal calf serum supplemented with 1% L-glutamine, 1100 U/ml penicillin, 200 mg/ml streptomycin and 100 mg/ml gentamycin), were incubated in the presence of the medium alone or medium plus phytohaemoagglutinin (PHA, Biochrom KG, Berlin) at a final concentration of 10 mg/ml, in 96-well culture plates and cultured at 37°C in a 5%

CO₂ atmosphere. Supernatants of unstimulated and stimulated cells were harvested after 24 hrs and 48 hrs of culture.

Assay for TNF in supernatants and blood samples

Supernatants and sera were harvested and TNF was measured by ELISA test kits (Predicta TNF, Genzyme, Boston, MA). The assay was based on the dual immunometric sandwich principle and was performed according to the manufacturer's instructions. 15 mg/ml of immunoglobulin in the samples do not interfere with the ELISA kit. Moreover, the addition of purified rheumatoid factors to the test samples did not alter the levels of TNF detected.

Assay for TNF RT-PCR

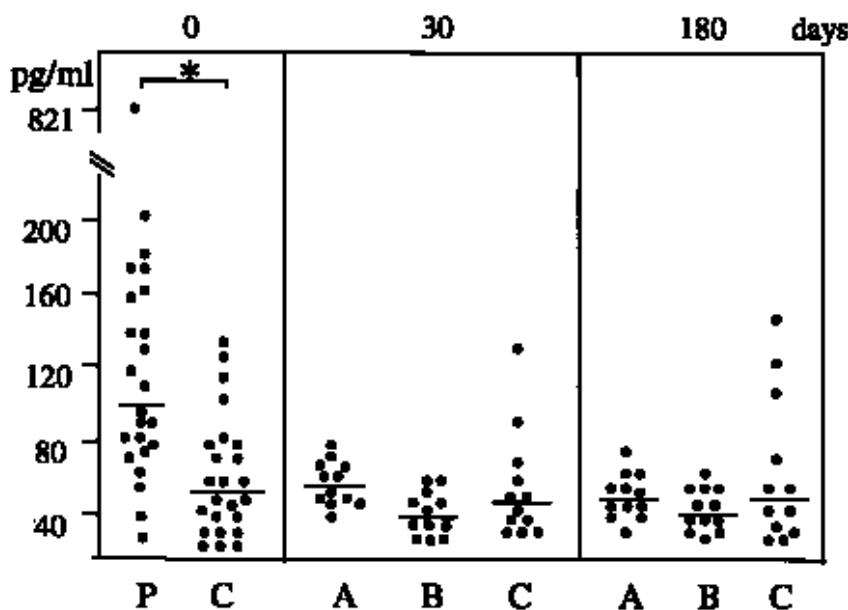
After 24 h and 48 h of stimulated and unstimulated culture, PBMC of patients and controls were gently removed, washed in ice-cold PBS and RNAs were isolated in isothiocyanate as described (10). RT-PCR was performed by a first step of reverse transcription from DNase I-treated total RNA, followed by PCR, using a Perkin Elmer Gene Amp RNA PCR Kit (Perkin Elmer Cetus Corporation, Norwalk, CT). The amount of starting material and the number of cycles were selected, so that the amplified product signal was related quantitatively to the input RNA; i.e. samples of the PCR reactions were taken at multiple points throughout the amplification, allowing the analysis of the product during the exponential phase of DNA amplification, for appropriate quantitation. Negative controls included RT-PCR without reverse transcription or without RNA. Amplification of β -actin with the 5' (AGCGGGAAATCGTGCGTG) and 3' (CAGGGTACATGGTGGTGCC) primers, giving a 280 bp DNA fragment, was used as an internal control for both reverse transcription and PCR and as a measure of the amount of input RNA (11). Amplification of the TNF cDNA was performed using the 5' (GAGCT-GAGAGATAACCAGCTGGTG) and 3' (CAGATAGATGGGCTCATACCAGGG) primers, which amplify a 237

Table I. Baseline characteristics of RA patients and outcome measures in the 2 groups.

	Group A		Group B	
Age	39 ± 7		41 ± 6	
Disease duration (yrs)	1.6 ± 4		1.8 ± 3	
Female	9/12		8/13	
	Day 0	Day 30	Day 60	Day 180
Tender joint count				
Group A	18.1 ± 1.3	8.7 ± 1.6°	8.5 ± 1.4°	9.7 ± 2.1°
Group B	20.0 ± 2.6	9.5 ± 2.4°	8.8 ± 1.1°	8.6 ± 1.5°
Swollen joint count				
Group A	15.6 ± 1.5	6.5 ± 1.4°	6.0 ± 1.3°	7.5 ± 1.3°
Group B	15.1 ± 3.1	5.8 ± 2.1°	6.1 ± 0.8°	6.9 ± 1°
Physician global assesment				
Group A	41.7 ± 6.2	26.3 ± 3.5°	28.2 ± 2.7	24.5 ± 2.7°
Group B	43.2 ± 4.3	22.5 ± 6.2°	21 ± 0.9°	25 ± 1.9°
Patient global assesment				
Group A	52.1 ± 5.1§	28 ± 4.2§	27.5 ± 2.2§	25.5 ± 3.5§
Group B	49.2 ± 3.2§	25.1 ± 3.2§	24 ± 2.9§	27.4 ± 4§
ESR (mm/hr)				
Group A	31 ± 16	25 ± 2	27 ± 3	26 ± 5
Group B	34 ± 13	21 ± 4	24 ± 5	25 ± 4
Morning stiffness				
Group A	90 ± 21	25 ± 11*	27 ± 10*	29 ± 11*
Group B	80 ± 17	29 ± 4*	26 ± 15*	29 ± 6*

The range of possible scores for each of the outcome measures is described in Materials and methods.
 ° = $p < 0.01$; § = $p < 0.007$; * = $p < 0.0001$.

bp DNA fragment included in the coding region of the gene. Amplifications were performed in a thermocycler (GeneAmp PCR system 9600, Perkin Elmer 9600, Norwalk, CT) as follows: 94°C, 3 min, followed by 30 cycles (95°C 1 min, 60°C 1 min, 72°C 2 min), and then 72°C 5 min.

**Fig. 1.** TNF levels in patients (P) and controls (C) before therapy, and in group A (MTX+PDN) and group B (MTX+PDN+CSA) after 30 and 180 days of therapy. * $p < 0.001$

Statistical analysis

Unless otherwise specified, data are expressed as the median and range. Since the variables studied were not normally distributed, the non-parametric Wilcoxon's 2-tailed test (normal approximation) was used for statistical analysis of the data. Changes in continuous variables were compared by the t-test. Correlations between serum TNF levels and any of the parameters used to define active RA were evaluated using Spearman's rank correlation coefficient for each datum.

Results

Response to treatment

The response to therapy, as determined by the various outcome variables, is shown in Table I. Two patients from group A (1 F, 1 M) and 3 patients from group B (3 F) withdrew prior to the 60th day (1 due to toxicity and 1 due to lack of efficacy in group A and 2 due to toxicity and 1 due to lack of efficacy in group B). Ten patients in group A (83%) and nine (74%) in group B met the preliminary criteria for ACR20 improvement in RA. In both groups 4 patients (33%) met the ACR50 criteria; 1 patient in group A (8%) and none in group B met the ACR70. Both groups showed a pronounced improvement in their clinical picture, without significant differences between the two groups, which persisted through day 180. In fact, none of variables differed significantly between day 30 and day 180.

The average daily dosage of CSA in group B decreased from 2.6 ± 0.4 mg/kg body weight at baseline to 2.1 ± 0.3 mg/kg body weight at six months. The patient in group A who stopped MTX showed a red cell macrocytosis (MCV >104 fl), resistant to both discontinuation of MTX and B group vitamin supplementation; in group B, one patient stopped MTX and CSA for a concomitant infectious disease, and another for continuous dyspepsia and nausea during treatment.

The mean serum creatinine level increased from baseline to six months by 0.02 ± 0.06 mg per deciliter in the MTX group and by 0.18 ± 0.31 mg per de-

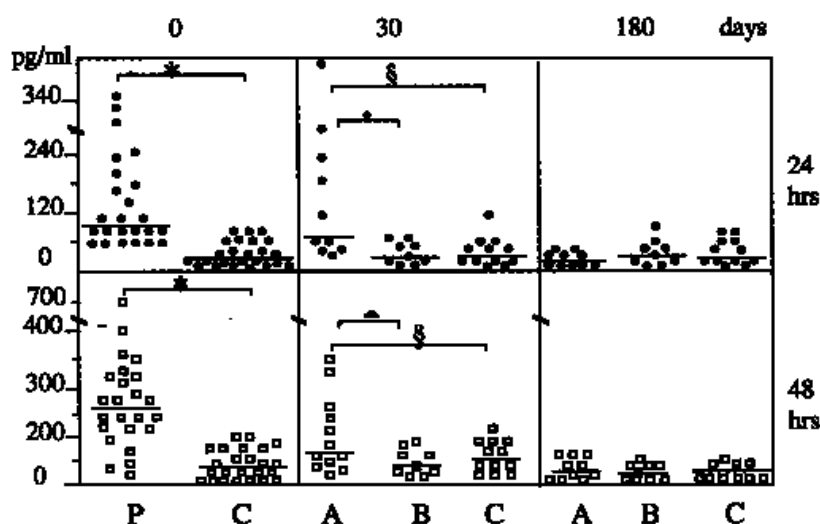


Fig. 2. TNF levels in 24 and 48 hour PHA stimulated PBMC supernatants of patients (P) and controls (C) before therapy, and in 24 and 48 hour PHA stimulated PBMC from group A (MTX+PDN) and group B (MTX+PDN+CSA) patients after 30 and 180 days of therapy.
* $p < 0.001$; § $p < 0.02$; p < 0.03 ; p < 0.05 .

ciliter in the CSA plus MTX group ($p < 0.02$). The mean diastolic blood pressure remained stable in the MTX group, from 77.3 ± 2 to 77.8 ± 1.9 mm Hg at six months; on the contrary, in the CSA plus MTX group it rose from 78.0 ± 2.1 mm Hg to 81.5 ± 3.1 mmHg. The differences between the two groups were not statistically significant.

Other adverse effects included hypertrichosis, paresthesia, diarrhoea, mouth ulcers but did not need discontinuation of therapy, improving over time.

Serum TNF levels

Increased serum TNF levels were observed at baseline in our patients compared to healthy controls (98, 20-821 pg/ml vs 41, 22-138 pg/ml respectively,

$p < 0.001$). As shown in Figure 1, levels strongly decreased within the first month of therapy in both groups, falling within the normal range (42, 32-77 pg/ml in group A and 31, 24-53 pg/ml in group B vs 35, 26-110 pg/ml in normal controls), the lower levels being observed in the sera of patients treated with CSA, although this difference was not statistically significant. Similar findings were observed after 180 days (35, 26-73 pg/ml in group A and 29, 21-50 pg/ml in group B vs 32, 24-141 pg/ml in normal controls). At baseline, serum TNF concentrations showed no significant correlations with any of the clinical and serological parameters studied (data not shown).

TNF levels in cell culture supernatants

Supernatants of both 24 and 48 h PHA-stimulated cultures of active RA patients showed significantly increased titres of TNF when compared to healthy controls (24 h: 98, 57-351 pg/ml vs 32, 12-79 pg/ml respectively, $p < 0.001$; 48 h: 312, 46-730 pg/ml vs 90, 22-212 pg/ml respectively, $p < 0.001$).

Interestingly, after 30 days of therapy, no differences were observed in TNF levels in the stimulated culture supernatants of group B patients when compared to controls (24 h: 22, 9-75 pg/ml vs 27, 5-126 pg/ml respectively; 48 h: 90, 17-212 pg/ml vs 104, 19-241 pg/ml respectively); on the contrary, TNF levels in the supernatants of group A patients were significantly higher than those observed in controls (24h: 106, 39-408 pg/ml vs 27, 5-126 pg/ml respectively, $p < 0.02$; 48 h: 120, 34-412 pg/ml vs 104, 19-241 pg/ml, respectively, $p < 0.02$). Furthermore, TNF levels in supernatants of group B patients were significantly lower than those observed in group A patients, after both 24 and 48 hours of PHA stimulation (24 h: 22, 9-75 pg/ml vs 106, 39-408 pg/ml, respectively, $p < 0.03$; 48 h: 90, 17-212 pg/ml vs 120, 34-412 pg/ml U/ml, respectively $p < 0.05$).

After 180 days of therapy, TNF levels

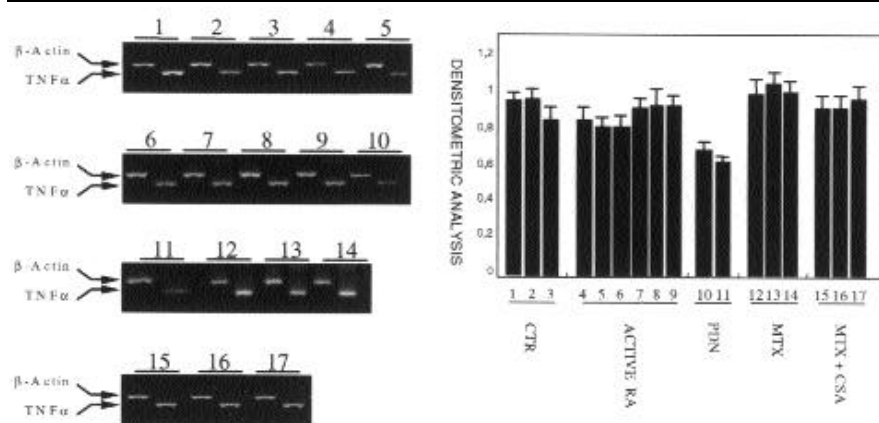


Fig. 3. RT-PCR analysis of TNF mRNA from PBMC of 3 healthy controls and 6 RA responder patients, followed during the study; before treatment (lanes 4, 5, 6, 7, 8, 9) and after 3 months of assigned therapy (lanes 12,13,14 - group A; lanes 15,16,17 - group B). Lanes 10 and 11 show the levels of TNF mRNA during the steroid treatment. As was expected, the values were significantly decreased ($p < 0.05$ vs active patients, data not shown). The left part of the picture shows the EtBr stained PCR products on agarose gel. No differences were observed in the amounts of TNF mRNAs among controls, active patients and both groups A and B. The histogram in the right side of the picture shows the ratio between the optical densities of TNF and -Actin. Results are means of 5 experiments with PBMC from each patient and control. All values are close to one, indicating that treatments did not modulate the TNF mRNA intracellular levels.

in the supernatants of groups A and B were similar to those of controls after both 24 and 48 hours of PHA stimulation (24 h: group A 36, 28-71 pg/ml, group B 27, 20-42 pg/ml vs 32, 24-68 pg/ml of controls; 48 h: group A 46, 19-110 pg/ml, group B 37, 19-105 pg/ml vs 42, 25-102 pg/ml of controls), the lowest levels observed in the group co-treated with CSA, although this trend did not reach statistical significance (Fig. 2).

Intracellular levels of TNF mRNA

Messenger RNA encoding TNF was detected in the cultured PBMC of all the patients and controls studied. Although the supernatants of some cultures (see results) showed a significant decrease in TNF levels, levels of intracellular TNF mRNA never differed between patients and controls, independently of both the clinical picture and the assigned therapy, showing normal amounts of specific mRNA. In Figure 3, an experiment involving 48 h stimulated cells from 3 healthy controls, 3 active RA patients, and 2 patients from each group (both during PDN therapy and successively without PDN) is shown. Throughout these experiments mRNA expression of the housekeeping β -actin gene was normal.

Discussion

The inflammatory reaction in RA is a complex process including humoral and cellular components, different mediators such as amplifying cytokines, adhesion molecules, complement components, metalloproteinases, all linked in a network probably modulated by TNF and contributing to joint damage, disability and constitutional symptoms (1). Neither currently available DMARDs nor biological response modifiers seem to be capable of controlling or suppressing this process (12). Thus, it may be hypothesized that the control of TNF activity by combination therapies using biological response modifiers and chemical compounds may become the treatment of choice in early aggressive RA. The administration of very expensive biological re-

sponse modifiers and the reported side effects of this treatment must be factored into the cost/benefit ratio of this regimen. In this light, how DMARDs may stand, concerning their possible synergy with biological response modifiers and the consequent cost reduction and increase in tolerability, opens interesting new perspectives for the future. In the present study, we compared the clinical efficacy of two different six-month step-down bridge therapies (PDN plus MTX vs PDN plus MTX and CSA) and their ability to control TNF production, both *in vivo* and *in vitro*. It has been shown that both CSA (13) and MTX (14) can control the symptoms of RA as single drugs and that the two drugs together are more effective than either one alone (15), probably because of their specific mechanisms which interfere with different steps of inflammation: MTX through macrophage and monocyte inhibition (16), CSA through the blockade of interleukin (IL) 2 synthesis by T lymphocytes and their subsequent activation (17). Thus, the association of these two drugs, interfering with the complex mechanism leading to TNF production in RA, may justify their administration in association with anti-TNF therapy.

The clinical response did not differ between the two groups studied and remained stable up to the 180th day. The beneficial effects were seen in all outcome variables, included ESR, which seems less sensitive as an improvement measure during CSA therapy. In our study, the percentage of patients who met the preliminary criteria of the American College of Rheumatology for improvement in RA did not differ between the two groups and, as far as the ACR20 criteria are concerned, was higher than in other reported studies. This finding may be related to many variables: the selection of patients (early disease and no previous therapy), the use of PDN for two months, and the design of the study in which Cy + MTX were begun at the same time, rather than using a step-up strategy with full response to MTX and then adding CSA. This phenomenon suggests that

some advantages of combination DMARD therapy may heavily depend on the study design (Pincus, personal communication). In group B, the tolerance to CSA was good. The two major side effects, hypertension and worsening of renal function, are dose dependent (18) and were appropriately managed by following the safety guidelines and using a low CSA dosage (19).

In order to assess the effects of the two therapeutic regimens on TNF production, we studied serially the circulating levels of TNF in patients with active RA and, during the follow-up, we evaluated both the TNF levels in the supernatants of stimulated and unstimulated PBMC of our patients and controls and the levels of TNF mRNA in the same cells. Before therapy, we observed significantly higher TNF serum levels in active patients, without any correlation with the activity criteria. Several studies on RA report conflicting results concerning the levels of TNF, their correlation with disease activity and the modulation of circulating levels of cytokines by MTX and CSA (20, 21). These discrepancies may be due to the presence of soluble TNF receptors in biological fluids which may bind the cytokine, thus interfering with the assay (22), or to different TNF bioactivity in different diseases, as well as to the possibility that TNF producing cells may be confined in specific target organs and release it to the circulation only in response to specific stimuli (23).

After the first month of therapy the serum levels of TNF were significantly decreased in both groups; the lowest cytokine levels (although these values did not reach statistical significance) were observed in patients treated with both MTX and CSA. This *in vivo* observation may be related to the decreased levels of TNF observed in the supernatants of stimulated PBMC cultures of patients treated with MTX and CSA. Our data indicate that *in vitro* combined treatment with MTX and CSA results in an early and significant decrease in TNF release in culture. This finding shows that although MTX

alone and MTX plus CSA had the same clinical efficacy in early RA, the association of these two drugs is more potent and rapid in decreasing *in vitro* TNF production.

In our patients, the TNF molecular analysis showed that normal amounts of specific mRNA can be detected in cultured cells independently of both the assigned therapy and cytokine levels in supernatants. In fact, stimulated cells from CSA treated patients secreted lower levels of TNF but showed normal amounts of TNF mRNA, similar to those observed in the other groups. It is well known that unstimulated TNF producing cells transcript a polyadenylate form of TNF mRNA that is accumulated, after deadenylation, in the cytoplasm, as a translationally silent form (24). This mechanism generates an available pool of specific mRNA. Activatory stimuli may induce re-adenylation and translation of this cytoplasmic TNF mRNA, in order to secrete a functional cytokine (25). Although our experiments do not identify which of these steps is affected by CSA to reduce TNF production, this finding mirrors some previously reported data on CSA mediated TNF inhibition (26, 27). Our study suggests that the *in vivo* activity of CSA on the regulation of the TNF gene might involve the re-adenylation process of cytosolic mRNA, and/or induce a partial blockade of the translation process as well as of the cytokine secretion. Therefore, this inhibitory mechanism differs from the pre-transcriptional mechanism observed for CSA mediated IL2 suppression (17). In humans, our study mirrors at molecular levels, as recently observed in experimental collagen-induced arthritis, where an inhibitory effect by CSA on TNF producing cells has been shown, and that a combination therapy with low-dose CSA plus low-dose cA2 caused a significant reduction in disease severity and good protection against joint erosions in affected mice (28). Furthermore, our study confirms a previous paper showing decreased serum TNF levels in RA patients, with partial response to MTX therapy, after

CSA supplementation (29).

Recently, the efficacy of combination therapy with anti TNF monoclonal antibody and low dose weekly MTX has been reported to reduce the disease activity in RA patients who were responsive to low-dose MTX alone but retained features of active disease (30). The major problems during therapy with anti-TNF monoclonal antibody are their immunogenicity (31, 32) and the development of IgM class anti-DNA antibodies (1). The production of antiglobulin antibodies reduces the effectiveness of repeated infusions of monoclonal antibodies and induces allergic reactions. The presence of anti-DNA antibodies suggests that a subset of RA patients might develop a lupus-like syndrome after long-term anti-TNF therapy, similarly to NZB/W F1 mice in which anti-TNF treatment caused rapid recurrence of severe disease (33). Thus, the presence of CSA in a combination therapy which is capable of decreasing TNF production, and of controlling IL2 production, lymphocyte activation, B-T cell cooperation and antibody formation, could be helpful on the one hand to control the natural history of RA and on the other hand to prevent immunogenicity and anti-DNA antibody production.

In conclusion, the results of this study indicate that both combination therapies tested can control the clinical features of early RA in a short-term follow-up, and that the presence of CSA in the treatment lowers the TNF levels with a more rapid effect, showing a trend *in vivo* and an early and significant decrease of TNF release in the PBMC supernatants *in vitro*, without decreasing TNF mRNA expression. MTX treatment alone did not modify the TNF release in PBMC supernatants after 30 days. These findings, at the cellular and molecular levels, indicate a role for low-dose CSA plus low-dose MTX to induce early immunosuppressive effects during RA and suggest that a longer follow-up is needed to assess whether different effects on *in vitro* TNF release may mirror different outcomes of the disease.

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