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EFFECT OF METHOTREXATE ALONE OR IN COMBINATION WITH SULPHASALAZINE ON THE PRODUCTION AND CIRCULATING CONCENTRATIONS OF CYTOKINES AND THEIR ANTAGONISTS. LONGITUDINAL EVALUATION IN PATIENTS WITH RHEUMATOID ARTHRITIS

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SUMMARY

In a recent study from our group, the combination of methotrexate and sulphasalazine (MTX + SASP) seemed superior to MTX alone in the treatment of rheumatoid arthritis (RA). To assess the impact of these therapies on the cytokine cascade, the *in vitro* production and circulating concentrations of several cytokines and endogenous cytokine antagonists were measured in 30 healthy controls and longitudinally in a subset of 26 patients enrolled in this study. Compared to controls, RA patients had significantly higher circulating concentrations of interleukin-6 (IL-6), soluble receptors for tumour necrosis factor (sTNFR), soluble receptors for interleukin-2 (sIL-2R) and interleukin-1 receptor antagonists (IL-1RA), and their peripheral blood mononuclear cells (PBMNC) showed a higher spontaneous production of interleukin-1 β (IL-1 β), tumour necrosis factor α (TNF α) and IL-1RA (both secreted and cell-associated) and a higher stimulated production of cell-associated TNF α , IL-1RA and (to a lesser extent) IL-1 β . Treatment with MTX alone ($n = 12$) or combined with SASP ($n = 14$), resulted in significant reductions of circulating IL-6 and sIL-2R but did not alter IL-1 β , TNF α or IL-1RA concentrations. Decreases in circulating levels of sTNFR and in the *in vitro* production of cell-associated IL-1 β and IL-1RA after stimulation were only observed in patients treated with MTX + SASP. The concentrations of IL-1RA and sTNFR in the circulation exceeded moderately those of IL-1 β and TNF α but this is probably insufficient to block IL-1 and TNF α activity. In conclusion, therapy with MTX alone or with SASP modulates IL-6 and sIL-2R concentrations in RA. Decreased production of IL-1 β and IL-1RA and circulating sTNFR levels were only observed during therapy with MTX + SASP. Whether this relates to the better clinical effect observed with the combination therapy remains to be investigated. Circulating levels of IL-6, sIL-2R and sTNFR seem useful markers of disease activity in RA.

KEY WORDS: Cytokines, Methotrexate, Sulphasalazine, Combination therapy.

THE involvement of cytokine networks in the pathogenesis of rheumatoid arthritis (RA) is proved beyond discussion [1, 2]. Proinflammatory cytokines such as interleukin-1 (IL-1) and tumour necrosis factor α (TNF α) [3, 4] are present in the rheumatoid synovial membrane and implicated in cell proliferation, synthesis of prostaglandins, metalloproteinases and interleukin-6 (IL-6) [5], proteoglycan breakdown and bone resorption [1]. Intra-articular injection of IL-1 [6] and TNF [7] result in joint inflammation and blocking IL-1 and TNF ameliorates several animal models of arthritis [8, 9]. IL-6, the major inducer of acute-phase response [10], is also present in RA synovial tissue [2] and may amplify the inflammatory process by its action on B- and T-cells [1]. However, in contrast to IL-1 and TNF, IL-6 does not enhance prostaglandins or collagenase production and may counteract inflammation by enhancing the synthesis of tissue inhibitor of metalloproteinase-1 [11] and reducing IL-1 and TNF pro-

duction [12]. The role of these cytokines in human arthritis is supported by preliminary studies showing improvement during IL-1 [13], TNF [14], and IL-6 [15] blockade in RA patients.

The activity of cytokines in biological fluids is subject to modulation by natural inhibitors and soluble cytokine receptors. The IL-1 receptor antagonist (IL-1RA), a competitive inhibitor of IL-1 which binds to the IL-1 receptors without inducing a biological response [16] is present in the rheumatoid synovial membrane [17]. High concentrations of soluble receptors for IL-2 (sIL-2R) [18-20] and TNF (sTNFR) [19, 21] have been detected in plasma and synovial fluid of RA patients. The sIL-2R, a soluble form of the α chain IL-2 receptor, is a marker of lymphocyte activation *in vivo* [22] and may be useful to monitor disease activity in RA [18]. The two types of sTNFR (p55 and p75) are shed from the cellular membrane and bind to TNF in solution acting as endogenous TNF antagonists [23].

Methotrexate (MTX) and sulphasalazine (SASP) are currently used anti-rheumatic drugs. Although their mechanism of action remains unclear, several reports suggest that these drugs may differ in their effects on circulating cytokines and cytokine production. *In vitro* studies with MTX have shown inhibitory effects on

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IL-1 [24] and IL-6 but not TNF bioactivity [25] while SASP has been reported to decrease IL-1 [26, 27] and TNF [27] production and to inhibit the binding of TNF α to its receptor [28]. Furthermore in RA patients, decreases in synovial levels of IL-1 β [29], in the production of IL-1 by peripheral blood mononuclear cells (PBMNC) [30, 31] and in circulating concentrations of IL-6, p55 sTNFR [19] and sIL-2R [19, 20] have been reported during MTX, whereas treatment with SASP has been associated with reductions in IL-1 α , IL-1 β and TNF α [32], but not in sIL-2R [33] or IL-6 [32] concentrations, though the latter was not corroborated in another study [34].

To date, the effect of combination therapy with MTX and SASP on cytokines has not been assessed. Therefore, after a recent study in our centre showed that the combination of MTX and SASP was superior to MTX alone in the treatment of active RA [35], we analysed the effects of these therapies on circulating levels and on the *in vitro* production of several cytokines and their endogenous antagonists.

PATIENTS

Twenty-six patients with active RA enrolled in a 24-week, randomized, open trial of MTX *vs* MTX in combination with SASP [35] and 30 healthy controls (laboratory staff) were studied. Cytokine concentrations were assessed at baseline and after 4, 8 and 24 weeks. Fourteen additional patients enrolled in the original clinical study [35] were excluded from cytokine measurements since they were controlled in another centre. Active RA was defined as a disease activity score (DAS) > 3. The DAS is a validated score composed of the Ritchie Articular Index, number of swollen joints, erythrocyte sedimentation rate (ESR) and patient well-being on a visual analogue scale [36]. Twelve patients were allocated to therapy with MTX alone (7.5 mg weekly in a single dose) and 14 patients to the combination MTX + SASP (2000 mg daily in two doses). If no clinical improvement was observed after 16 weeks, the MTX dose was increased to 15 mg weekly. Non-steroidal anti-inflammatory drugs (NSAIDs) were kept at a stable dose and no corticosteroids were administered either at study entry or during the study.

METHODS

Circulating cytokine concentrations

Blood samples used to assess circulating cytokines (IL-1 β , TNF α , IL-1RA) and soluble receptors (sIL-2R, sTNFR) were drawn in Vacutainer tubes (Becton & Dickinson, Rutherford, NJ, USA), containing 48 μ l EDTA-K₃ and 250 μ l aprotinin (10 000 kallikrein inactivating units/ml, Bayer, Mijdrecht, The Netherlands). IL-6 was measured in serum from a Vacutainer tube without additives. Samples were centrifuged 10 min at 2250 g and 5 min at 15 000 g to remove the platelets and frozen at -80°C until assay.

Cytokine production by PBMNC

The production of IL-1 β , TNF α and IL-1RA was assessed. sTNFR production was not measured since PBMNC do not seem to release these receptors either spontaneously or after LPS stimulation [31]. PBMNC were isolated from heparinized blood by Percoll (Pharmacia, Upsala, Sweden) centrifugation ($\delta = 1.075$ g/ml) at 4°C and washed twice in saline. PBMNC were suspended at a concentration of 5×10^6 cells/ml in RPMI complete medium [RPMI 1640 medium with 20 mM HEPES (Flow, UK) supplemented with 2 mM L-glutamine (Gibco, New York, USA), 1 mM pyruvic acid (Sigma, St Louis, MO, USA), 50 μ g/ml gentamicin and 5% heat-inactivated pooled human serum]. Aliquots of 100 μ l (5×10^5 cells) were added to 96-well round-bottomed microtitre plates (Greiner, Feirickenhausen, Germany). An equal volume of RPMI complete medium with or without lipopolisaccharide (LPS) (50 ng/ml final concentration) was added. The plates were incubated at 37°C in a humidified, 5% CO₂ atmosphere for 24 h. Thereafter, the supernatants containing secreted cytokines, were centrifuged for 5 min at 13 000 g. The remaining cell fractions were resuspended in 200 μ l RPMI complete medium/well and exposed to three freeze-thaw cycles to lyse the cells and obtain material to measure cell-associated cytokines. The samples were stored at -80°C. The RPMI culture medium, Percoll and saline used in this study were sterile and pyrogen-free.

Cytokine assays

IL-1 β , TNF α [31] and IL-1RA [37] were assessed by radioimmunoassays (RIA) with an average sensitivity of 40, 60 and 150 pg/ml, respectively. The RIAs for IL-1 β and IL-1RA are specific, i.e. the coexistence of IL-1 β and IL-1RA in a given sample does not alter the results (unpublished results and [37]). IL-6 was measured by a B9 hybridoma bioassay with a sensitivity of 0.3 U/ml. As previously reported, this assay is not influenced by treatment with MTX or SASP [38]. sTNFR (p55 and p75) were measured by enzyme-linked binding assay (ELIBA) (kind gift of Dr H. Gallati, Hoffmann la Roche, Basel, Switzerland) with a sensitivity of 100 pg/ml. The assays for TNF α and sTNFR measure total concentrations (both free levels and complexes of TNF and sTNFR) as previously reported [31]. sIL-2R was measured using an enzyme-linked immunosorbent assay (ELISA) (Innotest hIL-2Rs, Innogenetics, Antwerp, Belgium), according to the manufacturer's instructions. The average sensitivity of this ELISA is 390 pg/ml. To minimize interassay variations, all samples were measured in a single assay.

STATISTICAL ANALYSIS

Baseline variables were compared using the Student *t*-test or Wilcoxon rank sum test. Closed testing procedures [39] were used to compare cytokine measurements during the study with pretreatment values. Differences in cytokine concentrations between groups during the 24-week follow-up were analysed by a

TABLE I
Clinical and laboratory parameters at baseline (mean \pm s.d.), and changes after 24 weeks of treatment (mean and 95% confidence intervals)

Variable	Baseline		Changes week 24 vs baseline				P
	MTX (n = 12)	MTX + SASP (n = 14)		MTX	MTX + SASP		
Age (yr)	52.2 \pm 13.6	58.9 \pm 10.6					
Sex (female/male)	9/3	12/2					
Disease duration (yr)	4.9 \pm 4.8	4.6 \pm 4.6					
Previous DMARDs (number)	0.75 \pm 1.16	0.79 \pm 1.01					
Duration SASP use (months)	23.9 \pm 14.3	20.1 \pm 13.8					
RF positive (number)	10	11					
Ritchie articular index	16.6 \pm 5.3	18.4 \pm 7.1	7.4	(-11 to -3.8)	-15.4	(-18.5 to -12.2)	<0.005
DAS	5.1 \pm 0.6	5.3 \pm 0.9	-1.1	(-1.8 to -0.5)	-2.8	(-3.3 to -2.4)	<0.001
Number swollen joints	23.7 \pm 6.7	22.3 \pm 7.1	-3.8	(-6.2 to -1.3)	-13.5	(-17.5 to -9.5)	<0.001
Number painful joints	21.9 \pm 8.2	26.2 \pm 6.2*	-10.3	(-15.1 to -5.4)	-22.6	(-25.8 to -19.4)	<0.001
VAS wellbeing (mm)	43.4 \pm 14.9	60.4 \pm 25.4	-9.2	(-25.1 to 6.8)	-33.5	(-46.6 to -20.4)	<0.05
VAS pain (mm)	49.9 \pm 19.0	61.0 \pm 19.5	-16.9	(-31.9 to -1.9)	-39.2	(-50.4 to -28)	<0.05
Morning stiffness (min)	107 \pm 93	101 \pm 89	-30	(-105 to 45)	-88	(-131 to -46)	
Grip strength right/left hand (kPa)	19.1/17.8 \pm 10.5/9.5	22.4/18.4 \pm 19.8/10.4	6.0/6.6	(0.6 to 11.4/0 to 13.1)	14.2/17.9	(9.3 to 19.2/9.2 to 26.7)	<0.05
ESR (mm)	37.5 \pm 25.3	36.9 \pm 18.4	-14.2	(-22 to -6.3)	-22	(-31 to -13.4)	
Haemoglobin (mmol/l)	7.2 \pm 1.0	7.3 \pm 0.8	0.37	(0 to 0.7)	0.14	(-0.2 to 0.4)	
MCV (fl)	86.8 \pm 8.7	89.8 \pm 6.6	0.08	(-3.1 to 3.2)	3.09	(1.5 to 4.7)	<0.05
Platelets ($\times 10^9/l$)	361 \pm 123	300 \pm 45	-32	(-85 to 21)	-35	(-54 to -16)	
CRP (mg/l)	63 \pm 50	77 \pm 67	-40	(-60 to -20)	-53	(-91 to -16)	

DAS, disease activity score; MTX, methotrexate; SASP, sulphasalazine; DMARDs, disease-modifying anti-rheumatic drugs; VAS, visual analogue scale; RF, rheumatoid factor; ESR, erythrocyte sedimentation rate; MCV, mean corpuscular volume; CRP, C-reactive protein.

P values refer to significant differences between therapy groups.

*Except in the number of painful joints ($P < 0.05$) no significant difference was observed at baseline.

distribution-free test for curve analysis [40]. Spearman's rank correlation coefficient was used to evaluate correlations between variables at study entry, and between changes in variables after 24 weeks. A two-sided *P* value of 0.05 was considered to be significant. The clinical trial was an open study [35], but cytokine measurements were performed in a modified single-blind fashion.

RESULTS

Clinical results

No significant differences in pretreatment characteristics were observed between both treatment groups except for the number of painful joints which was higher in the group treated with combination therapy (Table I). After 24 weeks of treatment, improvement was observed in both treatment groups. However, the combination therapy proved to be superior to MTX alone as reflected by the more pronounced changes in clinical variables including the Ritchie Articular Index, DAS, number of swollen and painful joints, general well-being, pain score, grip strength (Table I) and the number of patients where the MTX dose was increased after 16 weeks because of lack of improvement (*n* = 8, all but one in the MTX group). Toxicities occurred with similar frequency in both treatment groups and were observed in seven patients treated with MTX alone (gastrointestinal *n* = 5, increase in transaminases *n* = 2, headache *n* = 1, stomatitis *n* = 1) and in six patients treated with MTX + SASP (gastrointestinal *n* = 2, increase in transaminases *n* = 5). One patient in the combination group dropped out at week 4 because

of the development of an 'overlap syndrome' with leucopenia, lung abnormalities, skin lesions and anti-dsDNA antibodies. The results of this small open study suggest that the combination MTX + SASP is clinically superior and not more toxic than MTX alone [35]. This is currently being evaluated in large double-blind trials comparing MTX + SASP versus the individual components.

Baseline comparisons in cytokine concentrations

Compared to healthy controls, the circulating concentrations of IL-6, soluble TNF and IL-2 receptors and IL-1RA and the spontaneous production of secreted and cell-associated IL-1 β , TNF α and IL-1RA by PBMNC were significantly higher in RA patients (Table II). The production of cell-associated cytokines after *in vitro* stimulation was also higher in RA patients and this difference was significant for TNF α and IL-1RA. In contrast, the concentrations of IL-1 β and TNF α in the circulation and the secreted amount of IL-1 β , TNF α and IL-1RA after PBMNC stimulation were similar in patients and controls (Table II). No differences in pretreatment levels of circulating cytokines or cytokine production by PBMNC were observed between treatment groups (data not shown). In RA patients, circulating IL-1RA concentrations were higher (median 4-fold) than those of IL-1 β and the ratio sTNFR to TNF α was 20–30 and 40–70 for p55 and p75, respectively. The spontaneous production of IL-1RA *in vitro* exceeded that of IL-1 β by 10–20-fold in the secreted fraction and 3–6-fold in the cell-associated fraction, respectively. In contrast, the

TABLE II

Circulating cytokines and cytokine production by PBMNC in healthy controls and RA patients at baseline. Median and (25, 75 percentile)

Variable	Controls (<i>n</i> = 30)		Patients (<i>n</i> = 26)		<i>P</i>
<i>Circulating concentrations</i>					
IL-1 β (pg/ml)	65	(ND–90)	77.5	(70–85)	NS
TNF α (pg/ml)	105	(85–115)	92.5	(75–105)	NS
IL-6 (U/ml)	1.6	(1.2–2.2)	29.4	(11.5–38.9)	<0.001
p55 (pg/ml)	950	(630–1100)	2110	(1730–2700)	<0.001
p75 (pg/ml)	3035	(2710–3290)	4785	(3720–7150)	<0.001
sIL-2R (pg/ml)	1825	(1300–2838)	4625	(3150–7200)	<0.001
IL-1RA (pg/ml)	220	(205–285)	312	(230–520)	0.005
<i>Production by PBMNC:</i>					
<i>Secreted without stimulation</i>					
IL-1 β (pg/ml)	100	(65–165)	375	(155–1940)	<0.001
TNF α (pg/ml)	72.5	(ND–150)	152.5	(65–565)	0.01
IL-1RA (pg/ml)	2880	(2420–4070)	5305	(2480–7200)	0.02
<i>Cell-associated without stimulation</i>					
IL-1 β (pg/ml)	257	(185–510)	743	(485–1840)	<0.001
TNF α (pg/ml)	ND	ND	72.5	(35–135)	<0.001
IL-1RA (pg/ml)	1690	(1250–2200)	4810	(1270–6690)	0.005
<i>Secreted after stimulation</i>					
IL-1 β (pg/ml)	10 650	(6970–14 100)	10 500	(4080–15 400)	NS
TNF α (pg/ml)	2535	(1930–3590)	3445	(1570–5500)	NS
IL-1RA (pg/ml)	7745	(5360–10 600)	8990	(5760–13 200)	NS
<i>Cell-associated after stimulation</i>					
IL-1 β (pg/ml)	4985	(3080–6470)	7690	(3520–13 200)	NS
TNF α (pg/ml)	195	(120–230)	290	(185–390)	0.02
IL-1RA (pg/ml)	4860	(2750–6780)	7895	(3430–14 000)	0.02

PBMNC, peripheral blood mononuclear cells; ND, not detectable, NS, not significant.

median concentrations of IL-1RA and IL-1 β produced after PBMNC stimulation were similar (Table II).

Effects of treatment in cytokine concentrations

Significant decreases in IL-6 and sIL-2R (Fig. 1A and B) occurred in both treatment groups. The baseline concentrations of sTNFR in the circulation (Fig. 1C and D) and the production of cell-associated IL-1 β and IL-1RA (Fig. 2A and B) after PBMNC stimulation tended to be higher (not significant) in patients treated

with MTX + SASP, and significant decreases during follow-up were only observed in this treatment group. The secreted amount of IL-1 β after PBMNC stimulation decreased after 4 weeks, especially in the group treated with MTX alone, however, the median values at week 24 were not significantly different from baseline (Fig. 2C). Neither the circulating levels of IL-1RA (Fig. 1E), IL-1 β , and TNF α nor the spontaneous production or the secretion of these cytokines after PBMNC stimulation (data not shown) showed

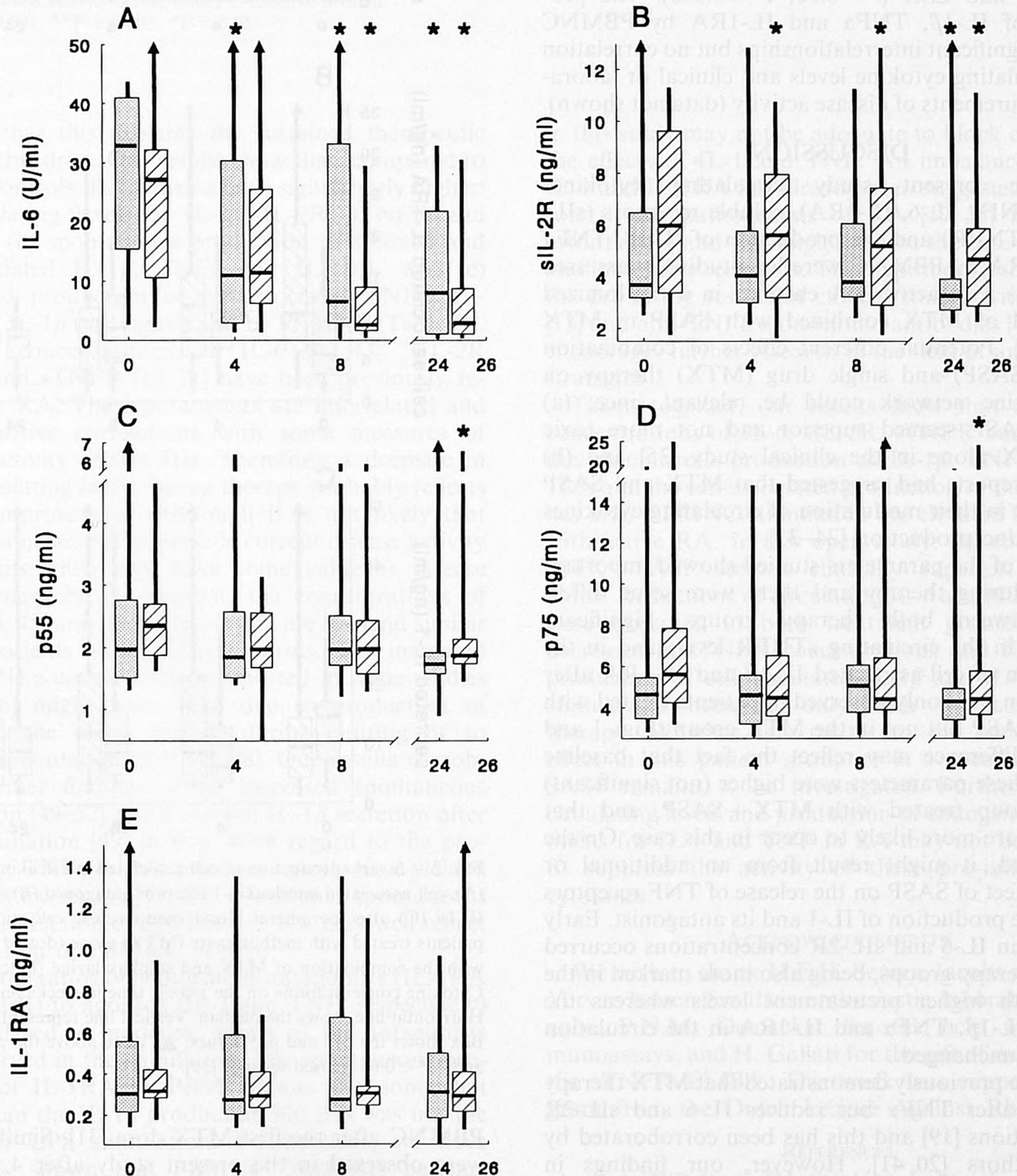


FIG. 1.—Circulating concentrations of interleukin-6 (A), soluble receptors for interleukin-2 (B), soluble tumour necrosis factor receptors (C, D) and interleukin-1 receptor antagonist (E) in patients treated with methotrexate (MTX) alone (dotted boxes) and with the combination of MTX and sulphasalazine (hatched boxes). Cytokine concentrations on the y-axis, time in weeks on the x-axis. Horizontal line shows the median. Vertical line represents the range. Box shows the p75 and p25 values. ▲, value above the y-axis range and * $P < 0.05$ (closed testing) [39].

significant changes during the study. Longitudinal comparison of circulating cytokines and cytokine production, corrected for pretreatment values, did not show significant differences between treatment groups.

Interrelationships between cytokines and other parameters

sTNFR and sIL-2R showed positive correlations with the DAS, number of swollen joints, C-reactive protein (CRP) and IL-6 and negative correlations with haemoglobin concentrations (Table III). IL-6 measurements also correlated with baseline CRP ($r = 0.49$, $P < 0.05$) and ESR ($r = 0.58$, $P < 0.005$). The production of IL-1 β , TNF α and IL-1RA by PBMC showed significant interrelationships but no correlation with circulating cytokine levels and clinical or laboratory measurements of disease activity (data not shown).

DISCUSSION

In the present study circulating cytokines (IL-1 β , TNF α , IL-6, IL-1RA), soluble receptors (sIL-2R and sTNFR) and the production of IL-1 β , TNF α and IL-1RA by PBMC were longitudinally assessed in patients with active RA enrolled in a randomized open trial of MTX combined with SASP *vs* MTX alone [35]. Potential different effects of combination (MTX + SASP) and single drug (MTX) therapy on the cytokine network could be relevant since: (a) MTX + SASP seemed superior and not more toxic than MTX alone in the clinical study [35] and (b) previous reports had suggested that MTX and SASP may differ in their modulation of circulating cytokines and cytokine production [24–33].

Several of the parameters studied showed important changes during therapy and there were some differences between both therapy groups. Significant decreases in the circulating sTNFR levels and in the production of cell-associated IL-1 β and IL-1RA after stimulation were only observed in patients treated with MTX + SASP but not in the MTX group (Figs 1 and 2). This difference may reflect the fact that baseline levels of these parameters were higher (not significant) in the group treated with MTX + SASP, and that decreases are more likely to occur in this case. On the other hand, it might result from an additional or specific effect of SASP on the release of TNF receptors and on the production of IL-1 and its antagonist. Early decreases in IL-6 and sIL-2R concentrations occurred in both therapy groups, being also more marked in the group with higher pretreatment levels whereas the levels of IL-1 β , TNF α and IL-1RA in the circulation remained unchanged.

We have previously demonstrated that MTX therapy does not alter TNF α but reduces IL-6 and sIL-2R concentrations [19] and this has been corroborated by other authors [20, 41]. However, our findings in patients receiving combination therapy do not support the claim that SASP reduces circulating levels of IL-1 β and TNF α as reported by Danis *et al.* [32].

In patients with active RA, we have observed reductions in the secretion of IL-1 β by stimulated

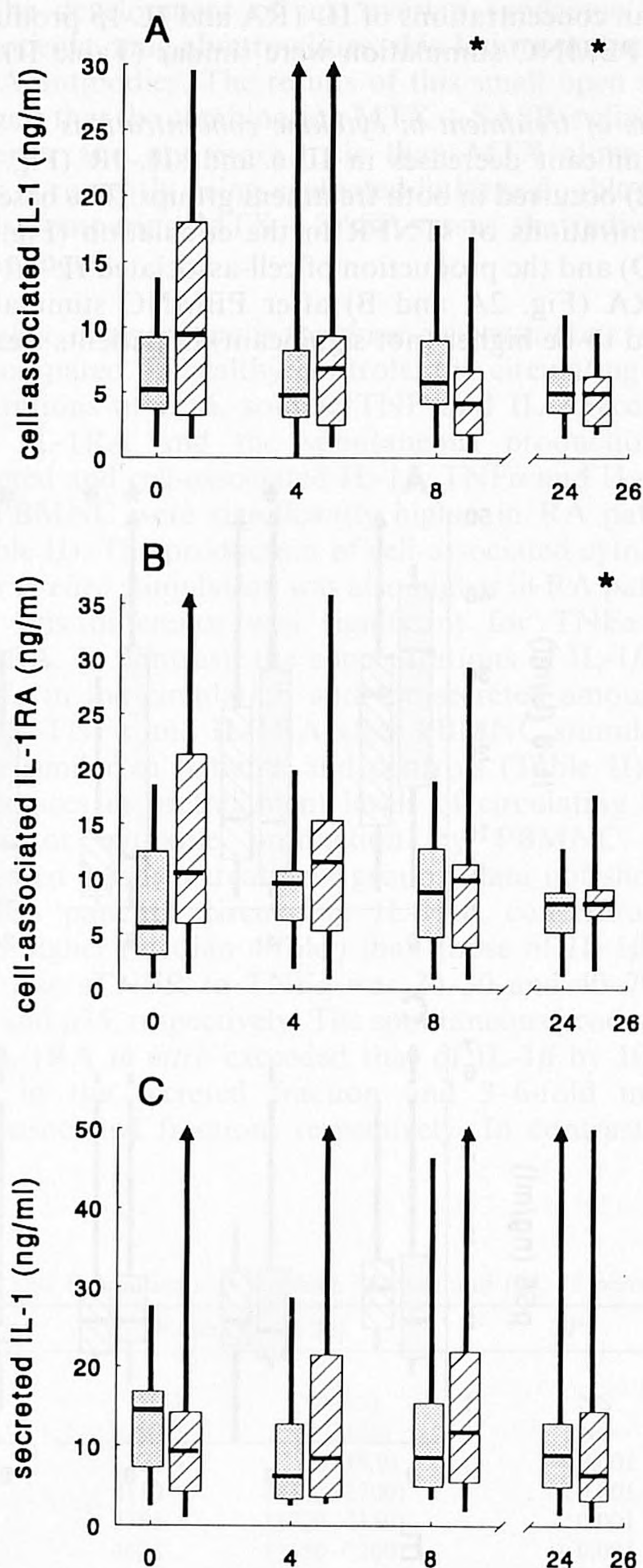


FIG. 2.—*In vitro* production of cell-associated interleukin-1 β (IL-1 β) (A), cell-associated interleukin-1 receptor antagonist (B) and secreted IL-1 β (C) after peripheral blood mononuclear cell stimulation in patients treated with methotrexate (MTX) alone (dotted boxes) and with the combination of MTX and sulphasalazine (hatched boxes). Cytokine concentrations on the y-axis, time in weeks on the x-axis. Horizontal line shows the median. Vertical line represents the range. Box shows the p75 and p25 values. \blacktriangle , value above the y-axis range and * $P < 0.05$ (closed testing) [39].

PBMC after the first MTX dose [31]. Similar effects were observed in the present study after 4 weeks of MTX therapy. This effect was not progressive over the time, and also not observed in the group treated with combination therapy which had lower IL-1 β secretion at baseline. Therefore, although MTX may decrease IL-1 β secretion in some patients in the short term, it is

TABLE III

Correlation between circulating concentrations of sTNFR and sIL-2R and several cytokines and clinical and laboratory parameters. Spearman correlation coefficient between absolute values at entry and between changes *vs* baseline after 24 weeks

	p55		sIL-2R		p75	
	Entry	24 weeks	Entry	24 weeks	Entry	24 weeks
DAS	0.61**	0.66***	0.48*	0.45*	0.61**	0.43*
Swollen joints	0.67***	0.64**	0.56**	0.56**	0.59**	0.44*
CRP	0.45*	0.59**	0.35	0.52*		
Haemoglobin	-0.46*	-0.44*	-0.40*	-0.47*		
p75	0.81***	0.68***	0.67***	0.68***		
sIL-2R	0.65***	0.63**				
IL-6	0.53**	0.52*				
IL-1RA	0.61**	0.63**				

DAS, disease activity score; CRP, C-reactive protein.

*** $P < 0.001$, ** $P < 0.005$, * $P < 0.05$.

unlikely that this explains the sustained therapeutic effect of this drug. Our results show that, compared to healthy controls, RA patients have significantly higher: (a) circulating levels of IL-6, sIL-2R, sTNFR and IL-1RA, (b) spontaneous production of secreted and cell-associated IL-1 β , TNF α and IL-1RA, and (c) stimulated production of cell-associated TNF α , IL-1RA and IL-1 β (not significant) by PBMNC (Table II). Elevated concentrations of IL-6 [42, 43], sIL-2R [18–20] and sTNFR [19, 21] have been previously reported in RA. These parameters are interrelated and show positive correlations with some measures of disease activity (Table III). Therefore, a decrease in their circulating levels during therapy probably reflects clinical improvement. Although it is not likely that these parameters will supersede current disease activity assessments, they may have some value as disease activity markers. In contrast, the concentrations of IL-1 β [44, 45] and TNF α in plasma are low and similar in RA patients and healthy controls. The increased serum TNF α concentrations reported in some studies [19, 42, 46] might have been due to production of TNF α in the blood sample during clotting or to endotoxin contamination [47, 48]. Our results corroborate former findings of an increased spontaneous production [49–52] and a normal IL-1 β secretion after LPS stimulation [49] in RA. With regard to the production of TNF α in RA, previous reports have yielded contradictory results [51, 53]. The higher spontaneous *in vitro* production of cytokines in RA may well reflect an activation of PBMNC *in vivo*.

When the ratios of endogenous antagonists (IL-1RA and sTNFR) to agonists (IL-1 and TNF, respectively) were analysed, a moderate excess of the antagonists was observed in the circulation. The spontaneous production of IL-1RA by PBMNC was also somewhat higher than the IL-1 β production but this was not the case after LPS stimulation. Furthermore, we have previously shown that PBMNC from RA patients do not release sTNFR either spontaneously or after stimulation [31]. Since 10–500- and 30–300-fold higher levels of IL-1RA [16, 17] and sTNFR [54], respectively are required for 50% suppression of IL-1 and TNF bioactivity, the concentrations of antagonists measured

in this study may not be adequate to block completely the effects of IL-1 and TNF. An imbalance between IL-1 and IL-1RA has already been suggested to play a role in the pathogenesis of RA [17, 55] and Lyme arthritis [56] and this might also be the case for TNF α and its soluble receptors. This hypothesis, sustained by the promising results of clinical studies with IL-1RA [13] and anti-TNF α monoclonal antibodies [14] in RA, encourages further research in the field of anticytokine strategies.

Taken together, our results show that circulating concentrations of IL-6, sIL-2R, sTNFR and IL-1RA, the spontaneous production of IL-1 β , TNF α and IL-1RA and the cell-associated production of these cytokines after PBMNC stimulation are elevated in patients with active RA. In this open study, the combination MTX + SASP seemed clinically superior to MTX alone. Reductions of circulating IL-6 and sIL-2R levels were observed in both treatment groups, whereas decreases in sTNFR concentrations and the *in vitro* production of cell-associated IL-1 β and IL-1RA after stimulation occurred only with the combination therapy. Whether this relates to the better clinical effect in this patient group or is a specific effect of SASP remains to be investigated. Furthermore, the circulating levels and production of endogenous antagonists for IL-1 and TNF in RA may not be sufficient to suppress the activity of these pro-inflammatory cytokines.

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