Clin Exp Immunol 1996; **103**:357–367

Expression of interferon-gamma (IFN-γ), IL-10, IL-12 and transforming growth factor-beta (TGF-β) mRNA in synovial fluid cells from patients in the early and late phases of rheumatoid arthritis (RA)

A. BUCHT*†, P. LARSSON†, L. WEISBROT¶, C. THORNE‡, P. PISA§, G. SMEDEGÅRD†, E. C. KEYSTONE¶ & A. GRÖNBERG† **Department of Rheumatology, Karolinska Hospital, Stockholm, and †Department of Pharmacology, Pharmacia Pharmaceuticals, Uppsala, Sweden, ‡Department of Rheumatology and Internal Medicine, York County Hospital, Canada, §Department of Haematology, Karolinska Hospital, Stockholm, Sweden, and ¶The Rheumatic Disease Unit of the Wellesley Hospital, University of Toronto, Toronto, Canada*

(Accepted for publication 6 November 1995)

SUMMARY

The expression of immunoregulatory cytokines was investigated in freshly isolated synovial fluid mononuclear cells (SFMC) and peripheral blood mononuclear cells (PBMC) from patients with RA, using a quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay. IFN-γ, TGF-β, IL-10 and IL-12 (p40) transcripts were detected in SFMC of patients with early disease (<1 year duration) as well as in patients with long standing arthritis (>1 year). The expression of IFN-γ, IL-10 and IL-12 mRNA was increased in SFMC compared with RA PBMC. In addition, the expression was higher in RA SFMC than in PBMC from healthy control individuals. Immunoassay analysis of the secreted IL-12 heterodimer demonstrated increased levels in RA SF compared with levels found in serum from RA patients and control individuals. High levels of TGF-β mRNA were found in SFMC, but a significantly decreased TGF-β/β_2-microglobulin (β_2-M) ratio was found compared with PBMC from both patients and control individuals. IL-4 mRNA could not be detected, either in SFMC or in PBMC. Cytokine expression in RA PBMC did not differ from control PBMC, with the exception of a decreased TGF-β/β_2-M ratio in RA patients with early disease. Our findings of IFN-γ mRNA and IL-12, but undetectable levels of IL-4 mRNA, suggest that the synovitis is characterized by a type 1 immune response. The presence of TGF-β and IL-10 mRNA indicates that immunosuppressive cytokines may also operate in the inflamed joint, although their level of expression may not be sufficient for down-modulation of immune activation.

Keywords rheumatoid arthritis interferon-gamma transforming growth factor-beta IL-4 IL-10 IL-12

INTRODUCTION

Cytokines are implicated as important mediators of inflammation and joint destruction in RA. Pro-inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α) and IL-1 may induce the production of proteoglycan- and collagen-degrading enzymes, resulting in cartilage destruction [1]. TNF-α and IL-1, in association with IL-6, exert systemic effects such as fever, fatigue and induction of acute-phase proteins. Treatment of RA patients with anti-TNF-α antibodies induce marked improvement in both clinical and laboratory disease measures, supporting a pathogenic role for TNF-α in arthritis [2].

The production of TNF-α and IL-1 is down-regulated *in vitro* by IL-4, IL-10 and prostaglandin E_2 (PGE_2), whereas IFN-γ stimulates TNF-α and IL-1 expression [3–7]. This suggests that a type 1 immune response (predominant cellular immunity) rather than a type 2 response (predominant humoral immunity) would enhance the inflammatory cascade in RA. The type 1 response (mediated by IL-2, IFN-γ and IL-12) and type 2 response (mediated by IL-4, IL-5, IL-6, IL-10 and IL-13) are reciprocally down-regulatory [8,9]. For example, IL-10 inhibits lymphocyte IFN-γ production, primarily by suppressing the synthesis of the IFN-γ-inducing factor IL-12, in accessory cells [10,11]. Immune responses are further modulated by the multifunctional cytokine, TGF-β. The immunosuppressive effects of TGF-β include inhibition of T and B cell function, cytokine production, antibody formation and natural killer (NK) cell activity [12]. In collagen type II arthritis in mice

Correspondence: Anders Bucht, Department of Rheumatology, Karolinska Hospital, S-171 76 Stockholm, Sweden.

(CIA), TGF-β or anti-TNF-α antibodies protect against CIA, whereas TNF-α or anti-TGF-β antibodies increase CIA incidence, indicating opposite effects of these cytokines *in vivo* [13]. However, local administration of TGF-β into the synovial tissue (ST) of rats induces chemotaxis of polymorphonuclear leucocytes, suggesting that TGF-β may under certain circumstances exert pro-inflammatory activities [14].

Several cytokines such as IL-1, IL-6, IL-8, TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF) and TGF-β have been detected in the rheumatoid joint [3,15–27]. Recently, IL-10 mRNA and protein expression was also demonstrated in the synovial membrane [4,28] and peripheral blood [PB] of RA patients [29]. Although the synovial compartment is highly infiltrated by activated T cells [30,31], it has often proven difficult to detect T cell-derived cytokines such as IL-4 [25,28] and IFN-γ. Conflicting data have been presented regarding IFN-γ expression in RA patients. Undetectable levels of IFN-γ transcripts in synovial fluid (SF) and ST have been reported [16,35], and immunoassay analyses have shown only low levels of protein secretion in SF [15,22,32,33]. However, other studies have demonstrated IFN-γ mRNA expression in freshly isolated SF and ST [28,34].

In the present study, mRNA expression of immunoregulatory cytokines was analysed in freshly isolated SF mononuclear cells (SFMC) and PB mononuclear cells (PBMC) from RA patients and compared with unstimulated and phytohaemagglutinin (PHA)-stimulated PBMC from healthy control individuals. For this purpose, we utilized a quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay for IFN-γ, TGF-β, IL-4, IL-10 and IL-12 as well as markers for total mRNA (β_2-microglobulin (β_2-M)) and T cell-derived mRNA (T cell receptor α-chain). IL-12 was further studied by immunoassay analysis of the secreted protein in SF and serum.

PATIENTS AND METHODS

Patients

Twenty-two patients with RA, according to the 1987 American Rheumatism Association (ARA) criteria [36], were included in the study. Eleven patients were defined as early RA (disease duration < 1 year, defined from the onset of symptoms). The other 11 patients were defined as late RA (duration range 3–39 years). None of the patients was receiving disease-modifying antirheumatic drugs for at least 2 months before sampling, but all patients were taking non-steroidal antiinflammatory drugs. Paired samples of PB and SF were obtained from 17 patients (11 early and six late RA). From five patients, only PB was taken (see Table 1 for patient characteristics). The number of tender joints and erythrocyte sedimentation rate (ESR) (Westergren) were not significantly different between the two patient groups, whereas the number of effused joints was significantly higher in the group of early RA patients ($P = 0{\cdot}005$). PB was also obtained from a control group consisting of eight healthy adult individuals.

Table 1. Patient characteristics and clinical analysis

Sex	Age (years)	ESR	No. tender joints	No. effused joints	Disease duration (years)	RF status	No. ARA criteria	Sample
Early RA								
F	54	34	21	17	0·1	NA	5	PB + SF
F	67	55	5	3	0·1	Pos.	5	PB + SF
F	83	32	16	8	0·1	Neg.	4	PB + SF
F	52	70	13	6	0·2	Neg.	4	PB + SF
F	54	85	18	10	0·2	Pos.	$\geqslant 5$	PB + SF
F	71	59	12	4	0·2	Pos.	5	PB + SF
M	48	58	17	10	0·2	Pos.	4	PB + SF
M	69	30	12	17	0·25	Pos.	4	PB + SF
M	80	25	27	17	0·4	Neg.	4	PB + SF
F	25	NA	0	3	0·5	NA	4	PB + SF
M	58	34	26	4	0·6	Pos.	5	PB + SF
Late RA								
F	32	74	29	6	7	Pos.	$\geqslant 5$	PB + SF
F	56	NA	1	0	7	Pos.	$\geqslant 5$	PB + SF
F	60	NA	5	5	8	Neg.	$\geqslant 5$	PB + SF
F	60	38	2	2	9	Pos.	$\geqslant 5$	PB + SF
M	37	22	4	5	18	Neg.	$\geqslant 5$	PB + SF
F	66	21	0	0	36	Pos.	$\geqslant 5$	PB + SF
F	35	24	13	1	3	Pos.	$\geqslant 5$	PB
F	38	12	8	4	14	Pos.	$\geqslant 5$	PB
F	59	72	33	4	15	Pos.	$\geqslant 5$	PB
F	42	29	28	2	25	Neg.	$\geqslant 5$	PB
F	72	54	11	0	39	Pos.	$\geqslant 5$	PB

NA, Not analysed; PB, peripheral blood; SF, synovial fluid.

Preparation of PBMC and SFMC

Immediately after sampling, mononuclear cells were isolated by density centrifugation on a Ficoll–Paque gradient (Pharmacia, Uppsala, Sweden) and subsequently washed twice with PBS. Cell suspensions were stored at −70°C or in liquid nitrogen until the preparation of RNA. PBMC from one healthy donor was subjected to PHA (Difco, Detroit, MI)-induced activation *in vitro*. Two million cells suspended in RPMI 1640 medium + 10% fetal calf serum (FCS) were cultured with or without PHA (2 μg/ml) for 3 h and 6 h, followed by centrifugation (200 *g*, 10 min, 4°C) and storage at −70°C.

RNA preparation and cDNA synthesis

Total cytoplasmic RNA was prepared from 2–5 × 10^6 cells, essentially according to the guanidinium thiocyanate extraction method [37]. After purification, RNA concentration was determined by measuring optical density (OD) at 260 nm. Negative controls were included in each RNA preparation (no cells added to the extraction solution). First strand cDNA synthesis was performed in a 20 μl reaction mix containing denatured RNA (heating to 70°C for 5 min followed by fast cooling to 4°C), 1 × RT buffer (BRL, Bethesda, MD), 1 mM dNTP (dATP, dCTP, dGTP and dTTP; Pharmacia), 5 ng/μl random hexamer primer (pd(N)6; Pharmacia), 5 mM DTT (BRL), 2 U/μl human placenta RNAse inhibitor (RNAguard; Pharmacia) and 10 U/μl reverse transcriptase (Superscript; BRL). The reaction was carried out at 42°C for 60 min. After synthesis, samples were diluted to 0·01 μg/μl (based on the RNA concentration) and stored at −70°C until the PCR amplification.

PCR amplification and Southern hybridization

PCR amplification of cDNA was performed using oligonucleotide primers specific for β_2-M (5′ GAATGGCTATGTGTCTGGGT 3′ and 5′ CATCTTCAAACCTCCATGATG 3′) [38], T cell receptor α constant chain (TCR Cα) [39], IFN-γ (5′ TCTGCATCGTTTTGGGTTCTC 3′ and 5′ TCAGCTTTTCGAAGTCATCTC 3′) [40], TGF-β1 (5′ GCCCTGGACACCAACTATTGC 3′ and 5′ GAAGTTGGCATGGTAGCCCTT 3′) [41], IL-4 (5′ CCTCTGTTCTTCCTGCTAGCA 3′ and 5′ GCCGTTTCAGGAATCGGATCA 3′) [42], IL-10 (5′ ACAGCTCAGCACTGCTCTGT 3′ and 5′ AGTTCACATGCGCCTTGATG 3′) [43] and IL-12 p40 (5′ CAGCAGTTGGTCATCTCTTG 3′ and 5′ CCAGCAGGTGAAACGTCCA 3′) [44]. cDNA (5 μl) was added to a reaction mixture with a final concentration of: 1 × PCR buffer (Perkin-Elmer, Norwalk, CT), 0·2 mM of each dNTP, 0·5 μM of each primer and 0·025 U/μl Taq polymerase (AmpliTaq; Perkin-Elmer). Primer–dimer formation was avoided by incubating primers at 75°C before initiation of the PCR reaction ('hot start'). The reaction mixture (final volume 20 μl) was overlayed with 20 μl mineral oil. The PCR profile used was: denaturation at 94°C for 30 s, annealing at 58°C for 60 s, and extension at 72°C for 60 s (Hybaid OmniGene Thermal cycler, London, UK). Negative controls (see above) were included in each experiment to ensure that the reagents were free of contamination. The amplification was run for 23–35 cycles (optimized for each primer pair).

The identity of the PCR products was confirmed by Southern hybridization using internal sequence-specific oligonucleotide probes: β_2-M (5′ CATCAATCCGACATTGAAGTTGA 3′), TCR Cα (5′ CAGAATCCTTACTTTGTGACA 3′), IFN-γ (5′ GCAGGTCATTCAGATGTAGCG 3′), TGF-β1 (5′ GAGAAGAACTGCTGCGTGCGG 3′), IL-4 (5′ TCTGTGCACCGAGTTGACCGTA 3′), IL-10 (5′ CTGGGTCTTGGTTCTCAGCTT 3′) and IL-12 p40 (5′ CTGGCATCTCCCCTCGTGGC 3′). Probes were digoxigenin-labelled either by 3′ end labelling (Boehringer, Mannheim, Germany) or by direct incorporation during oligonucleotide synthesis (British Biotechnology, Abingdon, UK). Southern blotting and hybridization were performed essentially as described previously [45]. Fifty pmol probe/ml hybridization solution were added to each filter (2 ml/40 cm^2 filter) followed by incubation at 42°C for 16–20 h with agitation. After hybridization, the filters were washed in 2 × SSC for 2 × 5 min. High stringency washings were performed in 0·1 × SSC for 2 × 5 min at 5°C below the melting temperature of the respective probe (35–47°C). Colour development of blotted PCR fragment was performed using a standardized protocol as described [45].

Quantification

PCR products (5 μl) were electrophoresed in agarose gels (1·5–2%) at 6 V/cm for 25 min. After electrophoresis, the gels were stained with ethidium bromide for 30 min, followed by washing in water for 3 × 15 min. The gels were photographed in UV light by a polaroid camera. Signal intensity was measured by scanning photographs in an CCD-72 camera (DAGE-MTI, Michigan City, IN) connected to a computerized image analysis system (labview run time 2; National Instruments Corp., Austin, TX). Each PCR reaction contained a dilution series of standard samples with a known amount of cDNA (PCR products purified by ion exchange chromatography or plasmids with inserted cDNA). To avoid systematic errors in the PCR reaction, unknown samples and standard samples were added to the reaction block in a randomized order. Standard curves for each cytokine, β_2-M and TCR Cα were obtained by plotting signal intensity (expressed as spot volume) against the log number of cDNA copies added to the PCR reaction (see Fig. 1). Typically, the standard curves yielded a sigmoid shape, reaching a plateau at high cDNA concentrations. The standard curves were created individually for each PCR reaction using a mathematical sigmoid curve fit program. The number of cytokine cDNA copies in each sample was divided by the number of β_2-M or TCR Cα copies (after correction for dilution factors), giving a ratio of cytokine/β_2-M or cytokine/TCR Cα. β_2-M and TCR Cα functioned as markers for the total amount of mRNA and T cell-derived mRNA, respectively. To increase the accuracy of the measurement, mean values of at least triplicate analyses were calculated.

IL-12 immunoassay

The concentration of secreted IL-12 heterodimer was analysed using a specific immunoassay (R&D Systems, Minneapolis, MN) in SF of 14 patients (nine early RA and five late RA) as well as in serum of 15 patients (nine early RA and six late RA) and seven healthy control individuals. A sigmoid shaped standard curve was performed by simultaneously analysing a dilution series of standard samples according to the manufacturer's protocol. Mean values of duplicated measurements were calculated. Samples below the detection limit for the assay were regarded as negative and given the value 0.

Statistical analysis

Non-parametric statistical methods (two-tailed) were utilized

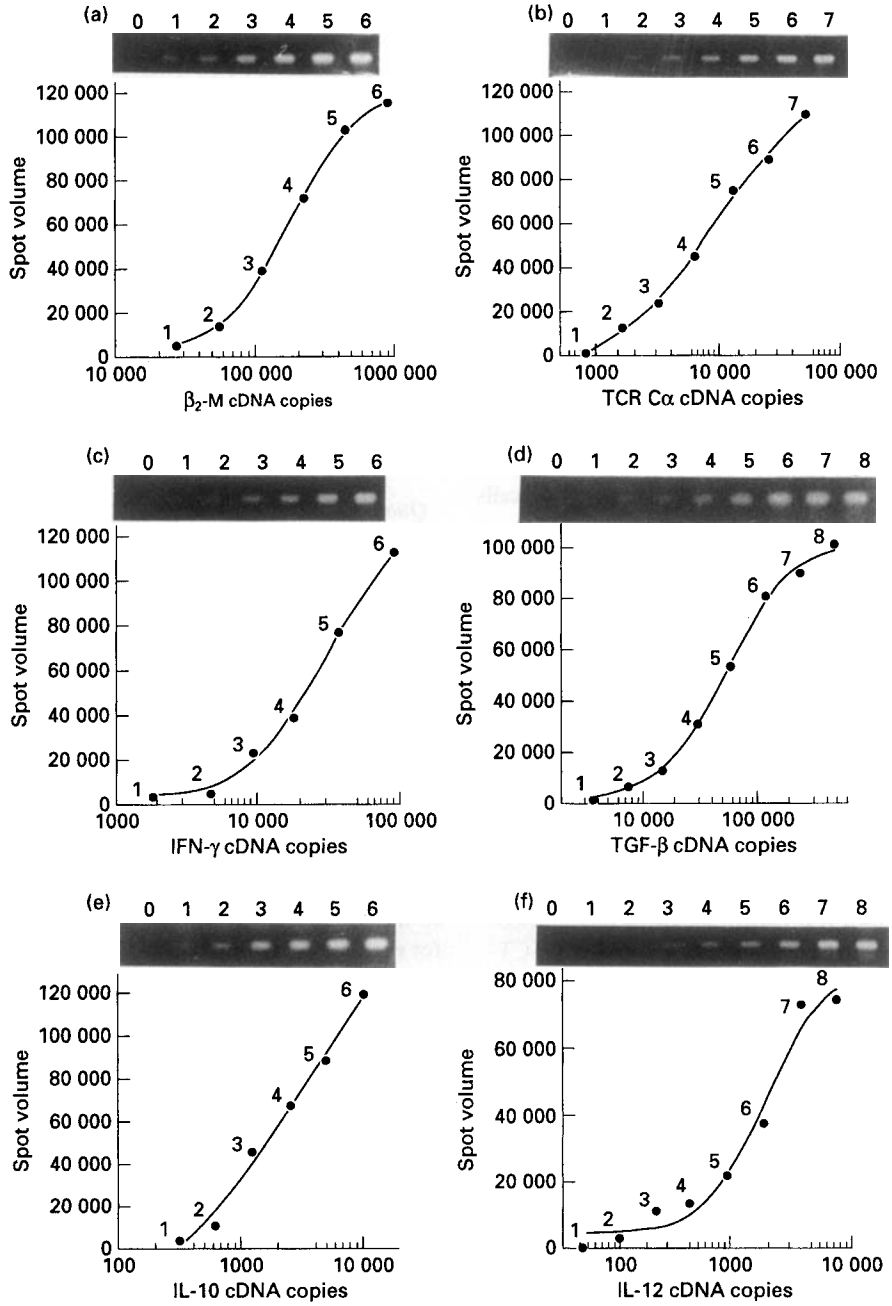


Fig. 1. Amplification of serial dilutions of a known amount of target cDNA. Polymerase chain reaction (PCR) products were visualized by ethidium bromide staining. Negative controls were included in each experiment (lane 0). Signal intensity was measured and plotted against the log number of target cDNA copies added to the PCR reaction. The sigmoid shaped standard curve is shown below. (a) β_2-microglobulin (β_2-M), amplified for 23 cycles, yielding a 256 bp fragment. (b) T cell receptor (TCR) Cα, 25 cycles, 593 bp. (c) IFN-γ, 28 cycles, 321 bp. (d) TGF-β, 26 cycles, 129 bp. (e) IL-10, 30 cycles, 329 bp. (f) IL-12, 30 cycles, 420 bp.

for calculation of significance. The Mann–Whitney *U*-test was used when comparing two populations (Fig. 2 and Table 3). Wilcoxon signed rank test was employed for paired comparisons (Fig. 3). A χ^2 test was used when evaluating frequency measurements (Table 2 and Table 3).

RESULTS

Quantitative PCR assay

In order to analyse cytokine mRNA expression, a quantitative RT-PCR assay was used. Since the level of mRNA expression and the amplification efficiency of different primer pairs were highly variable, the assay was individually optimized for each transcript to be analysed. Theoretically, the PCR reaction yields an exponential increase in target cDNA until the accumulating PCR product limits the amplification efficiency. In our assay system, this plateau phase was defined by amplifying series of diluted samples with known amounts of the particular cDNA. Unknown samples were carefully titrated (by dilution) to give a signal corresponding to the growth phase of the

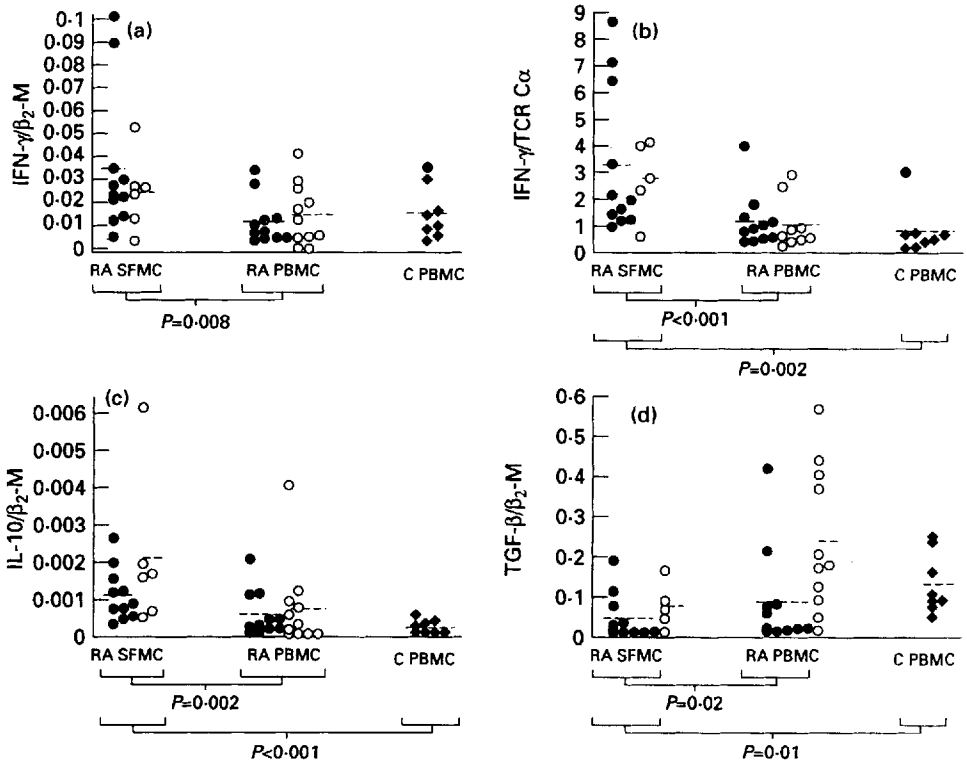


Fig. 2. Cytokine mRNA expression in RA synovial fluid mononuclear cells (SFMC), RA peripheral blood (PB) MC and control PBMC (C). The RA patient population was divided into early RA (●) and late RA (○). Arithmetic mean values are indicated by dashed lines. The groups of early and late RA were combined in the statistical analysis (Mann–Whitney *U*-test). *P* values are shown below each figure. (a) IFN-γ/β_2-microglobulin (β_2-M) ratio. (b) IFN-γ/T cell receptor (TCR) Cα ratio. (c) IL-10/β_2-M ratio. (d) TGF-β/β_2-M ratio. Due to shortage of material, one SFMC sample was excluded from the TGF-β analysis. Three samples (one RA SFMC and two RA PBMC) were exluded from the calculation of IFN-γ/TCR Cα ratio due to weak TCR Cα expression.

standard curve (Fig. 1). Since signal intensity is dependent on the number of amplification cycles, the number of cycles needed for a detectable signal reflects the amount of target cDNA present in the original sample. Typically, β_2-M was amplified for 23 cycles, TCR Cα for 25 cycles, TGF-β for 26 cycles, IFN-γ for 28 cycles, IL-10 and IL-12 for 30 cycles. By increasing the number of cycles to 32, low abundant transcripts could be detected. Samples with no detectable signal after 32 cycles were regarded as negative and given the value 0. The detection limit of the IL-4 assay was further decreased by increasing the number of cycles to 35.

The size of the amplified fragments corresponded to the expected number of bases between the primers (see legend to Fig. 1). The identity of the PCR products was further confirmed by Southern hybridization using sequence-specific probes. PHA stimulation of PBMC functioned as a positive control, demonstrating that cytokine mRNA production induced by *in vitro* activation was easily detected in our assay

Table 2. IL-12 mRNA expression. The number of samples with detectable signal after 30 (++) or 32 cycles (+) is indicated

	RA SFMC ($n = 14$)	RA PBMC ($n = 21$)	Control PBMC ($n = 7$)
++	5	1	1
+	9	5	3
–	0	15	3

Due to shortage of material, five samples were excluded (three RA synovial fluid mononuclear cells (SFMC), one RA peripheral blood (PB) MC and one control PBMC).

++, Detectable signal after 30 cycles; +, undetectable signal after 30 cycles, detectable signal after 32 cycles; –, undetectable signal after 32 cycles.

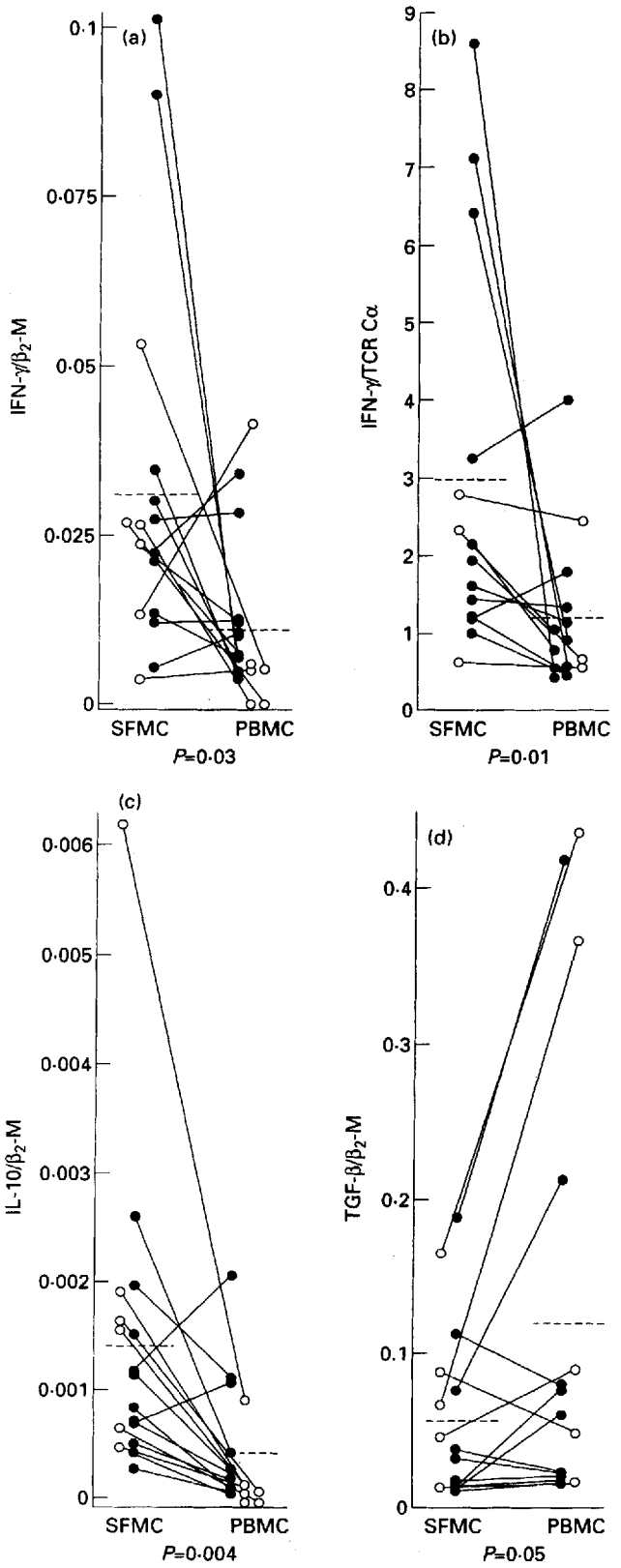
χ^2 test: RA SFMC *versus* RA PBMC, $P < 0{\cdot}001$; RA SFMC *versus* control PBMC, $P = 0{\cdot}03$.

Table 3. Immunoassay analysis of IL-12 in synovial fluid (SF) and serum

	No. of samples with detectable IL-12/no. of samples analysed	Mean ± s.d. (pg/ml)
RA SF	10/14*	14 ± 19†
RA serum	5/15	3·9 ± 9·0
Control serum	2/7	1·5 ± 2·8

* χ^2 test: RA SF *versus* RA serum, $P = 0{\cdot}04$; RA SF *versus* control serum, $P = 0{\cdot}06$.

† Mann–Whitney *U*-test: RA SF *versus* RA serum, $P = 0{\cdot}05$; RA SF *versus* control serum, $P = 0{\cdot}06$.



(Fig. 4). The levels of β_2-M and TCR Cα mRNA were unaffected by mitogen stimulation. Therefore, these transcripts were selected as markers for constitutively expressed mRNA and T cell-derived mRNA, respectively. The relative amount of cytokine cDNA present in each sample was calculated by dividing the number of cDNA copies by the number of β_2-M cDNA copies. Since IFN-γ is an important T cell-derived cytokine, an additional ratio of IFN-γ/TCR Cα was calculated.

Comparison of RA SFMC cytokine mRNA expression with PBMC from patients and control individuals

Detectable levels of IFN-γ mRNA were found in all SFMC samples (17/17) as well as in most samples from PBMC (20/22 RA patients and 8/8 control individuals). Quantitative analysis demonstrated a significantly increased IFN-γ/β_2-M ratio in RA SFMC compared with RA PBMC, indicating that the local synovial inflammation was associated with a specific increase in IFN-γ mRNA (Fig. 2a). In addition, the IFN-γ/TCR Cα ratio was increased in RA SFMC, suggesting that the IFN-γ expression was not due to an increased number of T cells, but rather to an elevated production by the SF T cell population (Fig. 2b). Comparing synovial IFN-γ mRNA expression with that from control PBMC demonstrated a significantly increased IFN-γ/TCR Cα ratio in SFMC (P = 0·002), whereas the IFN-γ/β_2-M ratio was not statistically different between the two groups (P = 0·14). No significant difference was found between RA PBMC and control PBMC.

IL-10 mRNA could be detected in all SFMC samples as well as in most PBMC samples from patients (18/22) and control individuals (6/8). The IL-10/β_2-M ratio was significantly elevated in RA SFMC compared with RA PBMC and control PBMC, indicating that the local synovial inflammation was associated with a relative increase in IL-10 mRNA expression (Fig. 2c). Some RA patients demonstrated elevated levels of IL-10 transcripts in PBMC, but the mean expression was not different from that in control PBMC (P = 0·13).

Although TGF-β mRNA was detected in all SFMC samples analysed, the level of expression was not increased compared with patient and control PBMC. Instead, a significantly decreased TGF-β/β_2-M ratio was observed in SFMC relative to RA and control PBMC (Fig. 2d). However, the absolute values of TGF-β transcripts (expressed as cDNA copies/ng added RNA) were not significantly different in PBMC and SFMC. Therefore, we cannot exclude the possibility that the decreased ratio in SFMC was partly due to increased β_2-M expression.

Since most samples from PB expressed low or undetectable levels of IL-12 mRNA after 30 or 32 cycles, the PBMC

Fig. 3. Paired analysis of cytokine mRNA expression in synovial fluid mononuclear cells (SFMC) and peripheral blood (PB) MC of RA patients. The RA patient population was divided into early RA (●) and late RA (○). Arithmetic mean values (dashed lines) and analysis of significance were calculated on the combined groups of early and late RA (Wilcoxon signed rank test). P values are shown below each figure. (a) IFN-γ/β_2-microglobulin (β_2-M) ratio. (b) IFN-γ/T cell receptor (TCR) Cα ratio. (c) IL-10/β_2-M ratio. (d) TGF-β/β_2-M ratio. Three samples were exluded from the IFN-γ/TCR Cα analysis and one sample from the TGF-β analysis (see legend to Fig. 2).

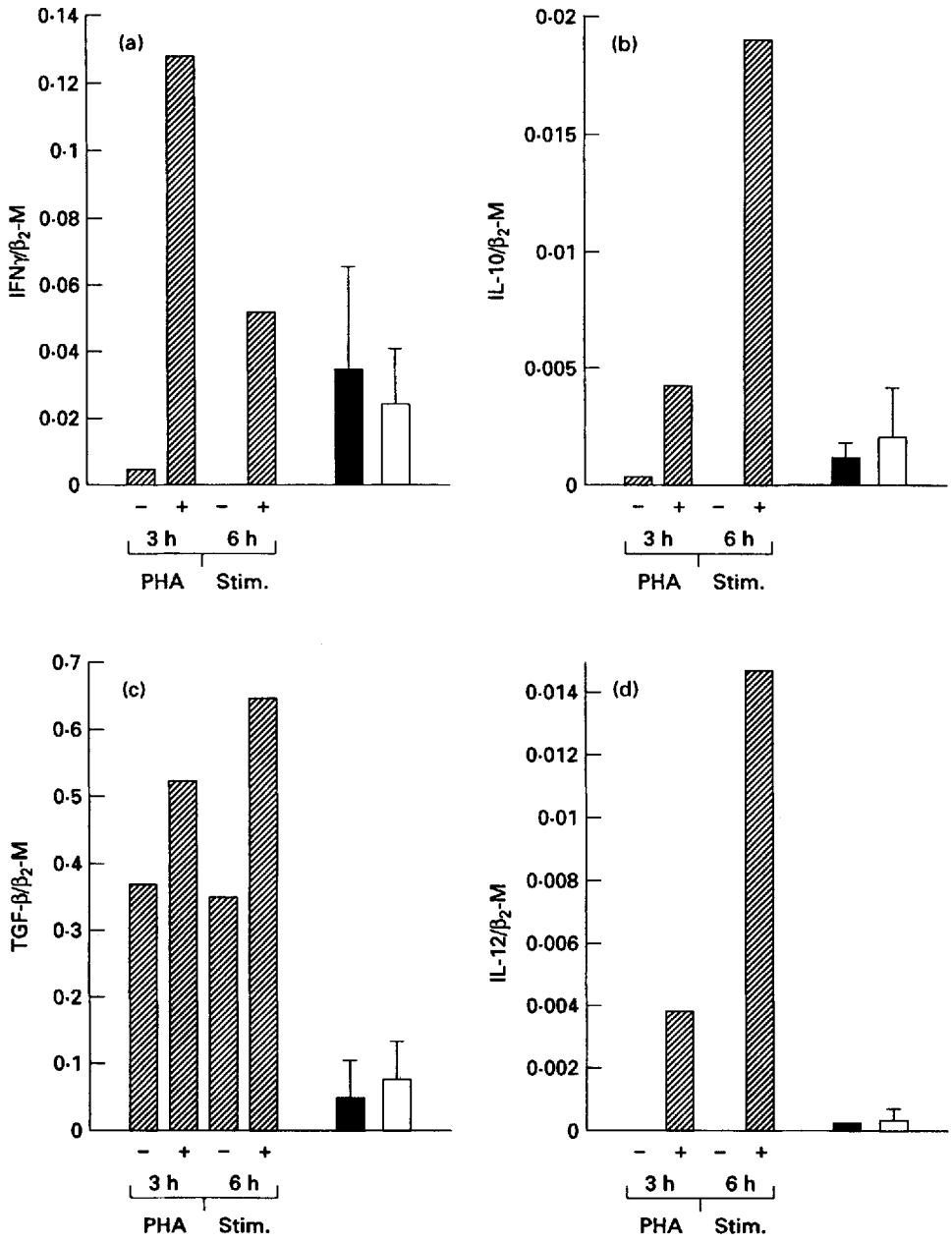


Fig. 4. Comparison of cytokine expression in freshly isolated synovial fluid mononuclear cells (SFMC) with phytohaemagglutinin (PHA)-stimulated peripheral blood (PB) MC from a healthy individual. Cells were cultured with (+) or without (–) PHA for 3 h and 6 h. The arithmetic mean values for the SFMC samples are shown (■, early RA; □, late RA). Error bars indicate s.d. for the analysed population. (a) IFN-γ/β_2-microglobulin (β_2-M) ratio. (b) TGF-β/β_2-M ratio. (c) IL-10/β_2-M ratio. (d) IL-12/β_2-M ratio.

expression of this cytokine could not be accurately quantified. Therefore, a qualitative/semiquantitative evaluation was carried out (Table 2). After 30 cycles of amplification detectable signals were found in 5/14 RA SFMC but only in 1/21 RA PBMC and 1/7 control PBMC. By increasing the number of cycles to 32, all SFMC samples demonstrated detectable signals (14/14), whereas signals were detectable in only 6/21 RA PBMC samples and 4/7 control samples. χ^2 analysis demonstrated a statistically significantly higher proportion of detectable IL-12 transcripts in SFMC compared with patient and control PBMC.

We were unable to detect IL-4 mRNA expression in any sample from RA SFMC, RA PBMC or control PBMC, even though the number of PCR cycles was increased to 35. Although the detection limit for the IL-4 assay was further decreased by subjecting the PCR products to the more sensitive Southern hybridization, no signals were detected. Since the positive control (*in vitro* stimulation by PHA) demonstrated detectable signals, we cannot explain the results by an error in the experimental system. The sensitivity of the IL-4 assay (Southern hybridization after 35 cycles) was determined by analysing standard samples with known amounts of IL-4

cDNA, yielding a detection limit down to a few cDNA copies (data not shown). Thus, we conclude that the IL-4 mRNA expression in our SFMC and PBMC samples was either extremely low or absent.

Paired comparison of cytokine expression between RA SFMC and PBMC

The gene transcription associated with the local synovitis was further analysed by comparing cytokine expression in SFMC with PBMC from the same patient (Fig. 3). Most patients expressed higher levels of IFN-γ and IL-10 mRNA in SFMC than in PBMC, as shown by the ratios of IFN-γ/β_2-M (11 out of 17 patients), IFN-γ/TCR Cα (12 out of 14) and IL-$10/\beta_2$-M (15 out of 17), whereas the TGF-β/β_2-M ratio was higher in only six out of 16 patients. Similar to the unpaired comparisons (Fig. 2), paired analysis demonstrated a significantly increased expression of IFN-γ and IL-10, but not of TGF-β in SFMC. Patients with high TGF-β expression in PBMC tended to also have high levels in SFMC, while patients with low expression in PBMC were also low in SFMC. Thus, TGF-β levels in SFMC were apparently more influenced by systemic expression than by that associated with local synovial inflammation.

Immunoassay of secreted IL-12 protein

Detectable levels of secreted IL-12 heterodimer were found in the SF of most patients (10 out of 14), whereas only a few serum samples were positive (five out of 15 patients and two out of seven healthy control individuals) (Table 3). Comparing the mean concentrations demonstrated significantly increased IL-12 levels in the SF compared with patient and control serum.

Comparison of cytokine expression in early and late RA

The RA patient population was divided into those with early disease ($<$ 1 year duration) and long-standing RA ($>$ 1 year). A trend toward higher IFN-γ/β_2-M and IFN-γ/TCR Cα ratios was found in SFMC of early RA, whereas the opposite was found for the IL-$10/\beta_2$-M and TGF-β/β_2-M ratios (Fig. 2). However, the differences were not statistically significant. The expression of IFN-γ and IL-10 mRNA in PBMC was similar in the two patient groups, whereas the TGF-β/β_2-M ratio was decreased in early RA compared with long-standing RA ($P = 0{\cdot}02$). We were not able to demonstrate a difference in IL-12 expression between early and late RA, neither by mRNA analysis of the p40 chain, nor by immunoassay of the secreted heterodimer (data not shown).

Five early RA patients had a very high number of swollen joints (10–17), indicating very active disease. This disease activity parameter was, however, not correlated with increased expression of any cytokine analysed in this study.

Comparison of RA SFMC cytokine mRNA expression with that in PHA-stimulated PBMC

The level of cytokine mRNA expression induced by PHA stimulation of PBMC from a healthy individual was quantified (after 3 h and 6 h incubation) and compared with cytokine mRNA expression in freshly isolated SFMC (Fig. 4). Similarly to freshly isolated PBMC, *in vitro* cultures without PHA expressed low or undetectable levels of IFN-γ, IL-10 and IL-12, demonstrating that the *in vitro* cultures *per se* did not induce cytokine expression. Addition of PHA induced a dramatic increase in cytokine mRNA expression at both time points. Substantial levels of TGF-β transcripts were expressed in cultured PBMC without PHA (Fig. 4c) as well as in freshly isolated PBMC (Fig. 2d), indicating that TGF-β is constitutively produced in normal PBMC. TCR Cα and β_2-M were expressed at high levels in unstimulated PBMC, and their expression was not altered by PHA (data not shown). The mean synovial IFN-γ/β_2-M ratio represented 27% (early RA) and 20% (late RA) of the maximal PHA induction (3 h), ranging from 3% to 79%. The corresponding numbers for IL-10 were 6% (early) and 11% (late) of 6 h PHA stimulation, ranging from 1% to 32%. The mean TGF-β production represented 7% (early) and 12% (late), ranging from 2% to 29%. The corresponding proportions for IL-12 were 1% (early) and 2% (late), ranging from 0·2% to 6%.

DISCUSSION

In the present study, gene transcripts of immunoregulatory cytokines were measured in freshly isolated SFMC and PBMC from RA patients. By using a quantitative RT-PCR assay, we showed that local synovial inflammation is associated with increased levels of IFN-γ, IL-10 and IL-12 (p40) mRNA. The finding of IFN-γ expression in SFMC is controversial, since previous studies have demonstrated only low levels of IFN-γ protein in SF of RA patients [15,22,32,33]. Conflicting data have been reported regarding the presence of IFN-γ transcripts in the rheumatoid joint. By using PCR amplification of purified mRNA, Chen *et al.* could not detect IFN-γ in purified T cell preparations from SF or ST [35], which is in concordance with results by Firestein *et al.* obtained by *in situ* hybridization of ST [16]. In contrast, Simon *et al.* recently demonstrated IFN-γ expression in ST by using PCR and *in situ* hybridization [28]. By using the Northern blot assay, Buchan *et al.* detected IFN-γ mRNA in freshly isolated SFMC and STMC from RA patients [34]. The divergent results are possibly due to differences in the sensitivity of the employed assays, of which PCR and *in situ* hybridization are the most sensitive, but may also be explained by differences in the selection of patients (e.g. disease activity, duration and medication). In the present study, patients with RA of early onset as well as patients with long standing disease were included. None of the patients was taking disease-modifying antirheumatic drugs at the time of sampling.

Our data demonstrated significantly increased IFN-γ/TCR Cα ratios in SFMC compared with patient and control PBMC, indicating that the synovial T cell population expresses elevated levels of IFN-γ transcripts. Since T cells predominate in the mononuclear fraction of SF and are preactivated *in vivo* [30,31,46], we suggest that IFN-γ is mainly produced by T cells. However, we cannot exclude the possibility that a fraction of the IFN-γ is derived from activated NK cells. The IFN-γ/β_2-M ratio was significantly increased in SFMC compared with corresponding samples from PBMC, but not when compared with control PBMC, possibly due to the extensive individual variation in the patient and control populations. Several previous studies based on immunoassays have shown only low levels of secreted IFN-γ in SF of RA patients [15,22,32,33]. The discrepancy between these data and our results of mRNA expression may indicate that the secretion is post-transcriptionally regulated, analogous to the recently reported post-transcriptional defect in atopic dermatitis [47]. Since the IFN-γ protein was not analysed in SF and serum samples obtained

from the patients included in this study, we could not determine whether the mRNA levels correlated with the production of functional polypeptides. However, preliminary results of IFN-γ secretion in another population of RA patients, using a novel and highly sensitive immunoassay, have shown increased levels of the secreted protein in SF compared with levels found in serum (A. Bucht and A. Grönberg, unpublished observation). Therefore, we also consider the possibility that previously used immunoassays might have underestimated the IFN-γ concentration in SF.

In addition to IFN-γ, the expression of IL-10 mRNA in SFMC was increased compared with PBMC from patients and control individuals. IL-10 mRNA and protein expression has previously been demonstrated in the synovial membrane [4,28]. Using a methodology similar to ours, Llorente *et al.* demonstrated increased production of IL-10 mRNA in RA PBMC compared with control PBMC [29]. Although some patients included in the present study had increased expression of IL-10 in PBMC, we were not able to demonstrate a statistically significant difference between patients and control individuals. The concordant over-expression of IFN-γ and IL-10 in the rheumatoid synovium is paradoxical, since IL-10 is known to down-regulate IFN-γ production *in vitro*, possibly by blocking IL-12 synthesis in accessory cells [10,11]. Katsikis *et al.* demonstrated that addition of exogenous IL-10 to RA synovial membrane cultures decreased the levels of TNF-α and IL-1, whereas the addition of neutralizing antibodies to IL-10 increased the secretion of TNF-α and IL-1, and to some extent IFN-γ, further demonstrating the immunoregulatory role of IL-10 [4].

Similar to IL-10, IL-4 exerts anti-inflammatory properties by inhibiting macrophage functions [48] and by down-regulating TNF-α and IL-1 production [5,6]. In addition, IL-4 promotes differentiation and expansion of B cells and the induction of Th2 cells (type 2 immune response) [8,9,42,49]. Although a highly sensitive assay was employed, we could not detect IL-4 mRNA, neither in SFMC nor in PBMC of RA patients. Our results therefore indicate that the synovial inflammation is characterized by a predominant type 1 immunity (strong cellular immune response), supporting previous findings of detectable expression of IFN-γ but not IL-4 mRNA in most synovial membranes from RA patients [28]. The expression of IFN-γ may enhance the inflammatory cascade by inducing macrophages to produce TNF-α. This scenario is supported by the up-regulation of secreted TNF-α by IFN-γ and the correlation of TNF-α levels with IFN-γ levels in rheumatoid SF [3,27].

Our finding that IL-12 p40 mRNA is present in rheumatoid SFMC further argues for a type 1 immune response, since IL-12 is an important costimulatory factor for the production of IFN-γ by T and NK cells [10,11,44,50,51]. Recent data from Ling *et al.* have shown that IL-12 mediates biological activity only when the p40 subunit is associated with the p35 subunit [52]. Therefore, we cannot from the mRNA data alone conclude that biologically active IL-12 is produced. However, we have also demonstrated elevated levels of the IL-12 protein in SF compared with the levels found in serum, further supporting that the biological functional heterodimer is produced and secreted in the rheumatoid joint.

To our knowledge this study represents the first comparative analysis of TGF-β mRNA levels in rheumatoid SFMC with PBMC from patients and control individuals. TGF-β protein and mRNA (β1) have previously been detected in rheumatoid ST and SF [16,24–26,53]. However, the transcription of TGF-β is not limited to inflammatory reactions, but is also found in normal unstimulated PB cells as shown by Northern blot analysis [41] and our own data on PBMC from healthy individuals. Although we detected high levels of TGF-β (β1) transcripts in rheumatoid SFMC, the expression was lower than in PBMC. Since TGF-β is expressed as a pro-cytokine which is processed after secretion, the mRNA expression may not necessarily reflect biologically active peptides [12]. TGF-β is a pleitropic cytokine, exerting both pro-inflammatory and immunosuppressive properties. Therefore, the net effect of TGF-β production is difficult to establish [12]. Injection of TGF-β into the synovial cavity of rats induces hyperplasia and recruitment of polymorphonuclear leucocytes [14]. However, systemically administered TGF-β protects against collagen II arthritis in mice (CIA), whereas anti-TGF-β antibodies enhance CIA [13]. This implies that local production of TGF-β in the joint may under certain circumstances promote inflammation, probably by chemotactic and fibrogenic effects, but systemically produced TGF-β exerts immunosuppressive activity.

In order to study the cytokine profile in different temporal phases of RA, the patient population was divided into RA patients with early disease (< 1 year duration) and long standing arthritis (> 1 year). No such comparison has to our knowledge been performed in RA patients or in animal models of arthritis. In experimental allergic encephalomyelitis, an autoimmune model resembling multiple sclerosis, expression of lymphotoxin (TNF-β) and IL-12 in the central nervous system coincides with the first appearance of immunohistochemically detectable inflammatory cells [54]. Production of pro-inflammatory cytokines such as IFN-γ and TNF-α is associated with the peak of disease, whereas immunoregulatory cytokines such as TGF-β, IL-10 and possibly IL-4 are produced during recovery [54,55]. Although the pathogenic mechanisms and the time course of an induced disease in animals are different from those of a chronic inflammatory disease in humans, it is of interest to compare the balance of pro-inflammatory and immunosuppressive cytokines in different temporal phases. From our data in RA patients, we could, however, not provide evidence for a time-dependent cytokine profile, although a tendency to higher IFN-γ expression was found in SF of early RA, whereas the opposite was found for IL-10 and TGF-β.

In conclusion, the over-expression of IFN-γ and IL-12 but undetectable levels of IL-4 indicate a strong bias toward a cellular immune response (type 1) in the rheumatoid SF. The presence of TGF-β and IL-10 indicates that the immune response is further modulated by additional regulatory cytokines. The multifunctional properties of TGF-β make it difficult to assess the role in the immune response. IL-10 is primarily an immunosuppressive cytokine, inhibiting IFN-γ, IL-12 and TNF-α expression. Thus, in this context IL-10 would counteract the inflammatory cascade.

REFERENCES

1 Arend WP, Dayer JM. Cytokines and cytokine inhibitors or antagonists in rheumatoid arthritis. Arthritis Rheum 1990; 3:305–15.

2 Elliott MJ, Maini RN, Feldmann M *et al.* Randomised double-blind comparison of chimeric monoclonal antibody to tumor necrosis factor α (cA2) vs. placebo in rheumatoid arthritis. Lancet 1994; **344**:1105–10.
3 Yocum DE, Esparza L, Dubry S, Benjamin JB, Volz R, Scuder P. Characteristics of tumor necrosis factor production in rheumatoid arthritis. Cell Immunol 1989; **122**:131–45.
4 Katsikis PD, Chu CQ, Brennan FM, Maini RN, Feldmann M. Immunoregulatory role of interleukin 10 in rheumatoid arthritis. J Exp Med 1994; **179**:1517–27.
5 Essner R, Rhoades K, McBride WH, Morton DL, Economou JS. IL4 down-regulates IL1 and TNF gene expression in human monocytes. J Immunol 1989; **142**:3857–61.
6 Donnelly RP, Fenton MJ, Finbloom DS, Gerrard TL. Differential regulation of IL1 production in human monocytes by IFNγ and IL4. J Immunol 1990; **145**:569–75.
7 Oswald IP, Wynn TA, Sher A, James SL. Interleukin 10 inhibits macrophage microbicidal activity by blocking the endogenous production of tumor necrosis factor α required as a costimulatory factor for interferon γ-induced activation. Proc Natl Acad Sci USA 1992; **89**:8676–80.
8 Clerici, M, Shearer GM. The Th1-Th2 hypothesis of HIV infection: new insights. Immunol Today 1994; **15**:575–81.
9 Maggi E, Parronchi P, Manetti R *et al.* Reciprocal regulatory effects of IFNγ and IL4 on the *in vitro* development of human Th1 and Th2 clones. J Immunol 1992; **148**:2142–7.
10 Tripp CS, Wolf SF, Unanue ER. Interleukin 12 and tumor necrosis factor α are costimulators of interferon γ production by natural killer cells in severe combined immunodeficiency mice with listeriosis and interleukin 10 is a physiologic antagonist. Proc Natl Acad Sci USA 1993; **90**:3725–9.
11 DÁndrea A, Aste-Amezaga M, Valiante NM, Ma X, Kubin M, Trinchieri G. Interleukin 10 (IL10) inhibits human lymphocyte interferon γ-production by suppressing natural killer cell stimulatory factor/IL12 synthesis in accessory cells. J Exp Med 1993; **178**:1041–8.
12 Fontana A, Constam DB, Frei K, Malipiero U, Pfister HW. Modulation of the immune response by transforming growth factor beta. Int Arch Allergy 1992; **99**:1–7.
13 Thorbecke GJ, Shah R, Leu CH, Kuruvilla AP, Hardison AM, Palladino MA. Involvement of endogenous tumor necrosis factor α and transforming growth factor β during induction of collagen type II arthritis in mice. Proc Natl Acad Sci USA 1992; **89**:7375–9.
14 Fava RA, Olsen NJ, Postlethwaite AE *et al.* Transforming growth factor β1 (TGFβ1) induced neutrophil recruitment to synovial tissues: implication for TGFβ-driven synovial inflammation and hyperplasia. J Exp Med 1991; **173**:1121–32.
15 Kahle P, Saal G, Schaudt K, Zacher J, Fritz P, Pawelec G. Determination of cytokines in synovial fluids: correlation with diagnosis and histomorphological characteristics of synovial tissue. Ann Rheum Dis 1992; **51**:731–4.
16 Firestein GS, Alvaro-Garcia JM, Maki R. Quantitative analysis of cytokine gene expression in rheumatoid arthritis. J Immunol 1990; **144**:3347–53.
17 Farahat MN, Yanni G, Poston R, Panayi GS. Cytokine expression in synovial membranes of patients with rheumatoid arthritis and osteoarthritis. Ann Rheum Dis 1993; **52**:870–5.
18 Wood NC, Symons JA, Dickens E, Duff GW. *In situ* hybridization of IL6 in rheumatoid arthritis. Clin Exp Immunol 1992; **87**:183–9.
19 Field M, Chu D, Feldmann M, Maini RN. Interleukin-6 localisation in the synovial membrane in rheumatoid arthritis. Rheumatol Int 1991; **11**:45–50.
20 Houssiau FA, Devogelaer JP, van Damme J, de Deuxchaisnes CN, van Snick J. Interleukin-6 in synovial fluid and serum of patients with rheumatoid arthritis and other inflammatory arthritides. Arthritis Rheum 1988; **31**:784–8.
21 Koch AE, Kunkel SL, Burrows JC, Evanoff HL, Haines GK, Pope RM, Streiter RM. Synovial tissue macrophage as a source of the chemotactic cytokine IL8. J Immunol 1991; **147**:2187–95.
22 Hopkins SJ, Meager A. Cytokines in synovial fluid: II. The presence of tumor necrosis factor and interferon. Clin Exp Immunol 1988; **73**:88–92.
23 Xu W, Firestein GS, Taetle R, Kaushansky K, Zvaifler NJ. Cytokines in chronic inflammatory arthritis. J Clin Invest 1989; **83**:876–82.
24 Fava R, Olsen N, Keski-Oja J, Moses H, Pincus T. Active and latent forms of transforming growth factor β activity in synovial effusions. J Exp Med 1989; **169**:291–6.
25 Miossec P, Naviliat M, DÁngeac AD, Sany J, Banchereau J, Low levels of interleukin-4 and high levels of transforming growth factor β in rheumatoid synovitis. Arthritis Rheum 1990; **33**:1180–7.
26 Lafyatis R, Thompson NL, Remmers EF *et al.* Transforming growth factor-β production by synovial tissues from rheumatoid arthritis patients and streptococcal cell wall arthritic rats. J Immunol 1989; **143**:1142–8.
27 Saxne T, Palladino MA, Heinegård D, Talal N, Wollheim F. Detection of tumor necrosis factor α but not tumor necrosis factor β in rheumatoid arthritis synovial fluid and serum. Arthritis Rheum 1988; **31**:1041–5.
28 Simon AK, Seipelt E, Sieper J. Divergent T-cell cytokine patterns in inflammatory arthritis. Proc Natl Acad Sci USA 1994; **91**:8562–6.
29 Llorente L, Richaud-Patin Y, Fior R *et al. In vivo* production of interleukin-10 by non-T cells in rheumatoid arthritis, Sjögren's syndrome, and systemic lupus erythematosus. Arthritis Rheum 1994; **37**:1647–55.
30 Potocnik AJ, Kinne R, Menninger H, Zacher J, Emmrich F, Kroczek RA. Expression of activation antigens on T cells in rheumatoid arthritis patients. Scand J Immunol 1990; **31**:213–24.
31 Iannone F, Corrigall VM, Kingsley GH, Panayi G. Evidence for the continuous recruitment and activation of T cells into the joints of patients with rheumatoid arthritis. Eur J Immunol 1994; **24**:2706–13.
32 Firestein GS, Zvaifler NJ. Peripheral blood and synovial fluid monocyte activation in inflammatory arthritis. II. Low levels of synovial fluid and synovial tissue interferon suggest that gamma-interferon is not the primary macrophage activating factor. Arthritis Rheum 1987; **30**:864–71.
33 Yamagata N, Kobayashi K, Kasama T *et al.* Multiple cytokine activities and loss of inteleukin 2 inhibitor in synovial fluids of patients with rheumatoid arthritis. J Rheumatol 1988; **15**:1623–7.
34 Buchan G, Barrett K, Fujita T, Taniguchi T, Maini R, Feldmann M. Detection of activated T cell products in the rheumatoid joint using cDNA probes to interleukin-2 (IL2), IL2 receptor and IFNγ. Clin Exp Immunol 1988; **71**:295–301.
35 Chen E, Keystone EC, Fish EN. Restricted cytokine expression in rheumatoid arthritis. Arthritis Rheum 1993; **36**:901–10.
36 Arnett FC, Edworthy SM, Bloch DA *et al.* The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988; **31**:315–24.
37 Chomczynski P, Sacchi N. Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987; **162**:156–9.
38 Suggs SV, Wallace B, Hirose T, Kawashima EH, Itakura K. Use of synthetic oligonucleotides as hybridization probes: isolation of cloned cDNA sequences for human β_2-microglobulin. Proc Natl Acad Sci USA 1981; **78**:6613–7.
39 Choi Y, Kotzin B, Herron L, Callahan J, Marrack P, Kappler J. Interaction of *Staphylococcus aureus* toxin 'superantigens' with human T cells. Proc Natl Acad Sci USA 1989; **86**:8941–5.
40 Gray PW, Goeddel DV. Structure of the human immune interferon gene. Nature 1982; **298**:859–63.
41 Derynck R, Jarrett JA, Chen EY *et al.* Human transforming growth factor-β complementary DNA sequence and expression in normal and transformed cells. Nature 1985; **316**:701–5.

42 Yokota T, Otsuka T, Mosman T *et al.* Isolation and characterization of a human interleukin cDNA clone, homologous to mouse B-cell stimulatory factor 1, that express B-cell- and T-cell-stimulating activities. Proc Natl Acad Sci USA 1986; **83**:5894–8.

43 Vieira P, deWaal-Malefyt R, Dang MN *et al.* Isolation and expression of human cytokine synthesis inhibitory factor cDNA clones: homology to Epstein–Barr virus open reading frame BCRF1. Proc Natl Acad Sci USA 1991; **88**:1172–6.

44 Wolf SF, Temple PA, Kobayashi M *et al.* Cloning of cDNA for natural killer cell stimulatory factor, a heterodimeric cytokine with multiple biologic effects on T and natural killer cells. J Immunol 1991; **146**:3074–81.

45 Bucht A, Söderström K, Esin S *et al.* Analysis of γδ V region usage in normal and diseased human intestinal biopsies and peripheral blood by polymerase chain reaction (PCR) and flow cytometry. Clin Exp Immunol 1995; **99**:57–64.

46 Réme T, Portier M, Frayssinoux F, Combe B, Miossec P, Favier F, Sany J. T cell receptor expression and activation of synovial lymphocyte subsets in patients with rheumatoid arthritis. Arthritis Rheum 1990; **33**:485–92.

47 Tang MLK, Varigos G, Kemp AS. Reduced interferon-gamma (IFN-γ) secretion with increased IFN-γ mRNA expression in atopic dermatitis: evidence for a post-transcriptional defect. Clin Exp Immunol 1994; **97**:483–90.

48 Oswald IP, Gazzinelli RT, Sher A, James SL. IL10 synergizes with IL4 and transforming growth factor-β to inhibit macrophage cytotoxic activity. J Immunol 1992; **148**:3578–82.

49 Abeshira-Amar O, Gibert M, Joliy M, Théze J, Jankovic D. IL4 plays a dominant role in the differential development of Th0 into Th1 and Th2 cells. J Immunol 1992; **148**:3820–9.

50 Schmitt E, Hoehn P, Huels C, Goedert S, Palm N, Rüde E, Germann T. T helper type 1 development requires the coordinate action of interleukin-12 and interferon-γ and is inhibited by transforming growth factor-β. Eur J Immunol 1994; **24**:793–8.

51 Kennedy MK, Picha KS, Shanebeck KD, Anderson DM, Grabstein KH. Interleukin-12 regulates the proliferation of Th1, but not Th2 or Th0, clones. Eur J Immunol 1994; **24**:2271–8.

52 Ling P, Gately MK, Gubler U *et al.* Human IL-12 p40 homodimer binds to the IL-12 receptor but does not mediate biologic activity. J Immunol 1995; **154**:116–27.

53 Wahl SM, Allen JB, Wong HL, Dougherthy SF, Ellingsworth LR. Antagonistic and agonistic effects of transforming growth factor-β and IL-1 in rheumatoid synovium. J Immunol 1990; **145**:2514–9.

54 Olsson T. Role of cytokines in multiple sclerosis and experimental autoimmune encephalomyelitis. Eur J Neurol 1994; **1**:7–19.

55 Khoury SJ, Hancock WW, Weiner H. Oral tolerance to myelic basic protein and natural recovery from experimental autoimmune encephalomyelitis are associated with downregulation of inflammatory cytokines and differential upregulation of transforming growth factor β, interleukin 4 and prostaglandin E expression in the brain. J Exp Med 1992; **176**:1355–64.