Expression of interleukin-1β, tumor necrosis factor α, interleukins-6, -10 and -4, and metalloproteases by freshly isolated mononuclear cells from early never-treated and non-acute treated rheumatoid arthritis patients

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Abstract

Objective

To determine IL-1β, TNFα, IL-6, IL-4, IL-10, MMP-1, MMP-3 and MMP-13 expression by freshly isolated peripheral blood (PBMC) and synovial fluid mononuclear cells (SFMC) in early, never-treated (ENT-RA) and non-acute, treated rheumatoid arthritis (NAT-RA) patients. To elucidate whether excessive or inadequate interleukin (IL) and metalloprotease (MMP) expression is influenced by the disease duration.

Methods

Fourteen RA patients, 7 with early RA (< 1 year of evolution) never treated with corticosteroids or disease-modifying antirheumatic drugs, and 7 patients with non-acute RA (> 2 years of evolution) treated with disease-modifying antirheumatic drugs, were studied by ELISA and quantitative and semiquantitative RT-PCR. A group of 14 healthy subjects matched for sex and age was included.

Results

No statistically significant difference in the protein or transcript levels for the cytokines of interest was found between the ENT-RA and NAT-RA groups. The cytokine mRNA expression by freshly isolated PBMC and SFMC in both groups was as follows: IL-1β > TNFα > IL-10 > IL-6, with no mRNA IL-4 expression. In contrast, cytokine serum levels in ENT-RA and NAT-RA patients were detected in inverse order as follows: IL-6 > IL-10, while IL-1β, TNFα and IL-4 were undetectable. MMP-3 mRNA expression by the PBMC of NAT-RA patients was statistically different to that in ENT-RA patients. Similar levels of mRNA expression of MMP-1, MMP-3 and MMP-13 by the PBMC and SFMC in both RA groups were observed.

Conclusions

A close equilibrium between MMP and pro/anti-inflammatory cytokine production is observed in ENT-RA and NAT-RA patients. This balance is apparently not influenced by the length of the disease. Highly sensitive methods such as quantitative RT-PCR and ELISA, and even studying freshly isolated MC, showed sustained cytokine secretion at the local level (synovial fluid/SFMC) and scarce translation at the peripheral level (serum/PBMC). Expression of MMP mRNA needs to be further evaluated in order to know whether their peripheral expression reflects their local activity in RA patients.

Key words

Rheumatoid arthritis, cytokines, synovial fluid, mononuclear cells, quantitative RT-PCR.

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Introduction

Rheumatoid arthritis (RA) is a disabling chronic disease characterized by autoimmune phenomena associated with multiple inflammatory mediators leading to structural damage to the joint (1). It has been postulated that sustained production by synoviocytes and monocyte-macrophages of such mediators, namely interleukin-1β (IL-1β), tumor necrosis factor α (TNF α) and interleukin-6 (IL-6), may be pivotal in triggering articular damage (2).

Disruption of the extracellular matrix and subcondral bone erosions are consequences of the local activation of matrix metalloproteases (3, 4). Metalloproteinase-1 (MMP-1), metalloproteinase-3 (MMP-3) and metalloproteinase-13 (MMP-13) activation play a key role in cartilage destruction (5-7). It is generally accepted that activation of these MMPs occurs via the IL-1β and TNFα up-regulation of genes coding for the metalloproteases in the joint microenvironment. The latter has been demonstrated in several experimental arthritis models (8, 9).

Natural antagonists of these pro-inflammatory cytokines are partially represented by interleukin-4 (IL-4) and interleukin-10 (IL-10) (10, 11). Most studies on clinical improvement in long-standing RA patients have focused on the administration of the monoclonal antibody anti-TNFα (12-14). The vast majority of studies concerning cytokine patterns in RA have concentrated on the production of these mediators *in vitro* by activated monocytes or synoviocytes obtained from RA patients.

Our aim was to study the constitutive expression of IL-1β, TNFα, IL-6, IL-4, IL-10 and MMPs 1, 3 and 13 on freshly isolated mononuclear cells (FIMC) in early, never treated (ENT-RA) and in non-acute, treated RA (NAT-RA) patients at the peripheral and local levels, using a quantitative retro-transcriptase-polymerase chain reaction (RT-PCR), a semi-quantitative RT-PCR and an enzyme-linked immunosorbent assay (ELISA). In doing so we hoped to elucidate whether excessive or inadequate cytokine and MMP expression by FIMC is influenced by the duration of the disease.

Materials and methods

Patients

Fourteen patients attending the Rheumatology Service of the hospital were recruited. Seven of these patients met the criteria for RA as defined by the American College of Rheumatology (1987) (15), with a duration of symptoms < 1 year and inflammatory disease activity, including knee arthritis, for which a synovial fluid test showed a translucent or opaque color, a white blood cell count (WBC) 2000 - 50,000/mm^3 and a neutrophil count of 50-90%. They had never been treated with corticosteroids or disease-modifying antirheumatic drugs (DMARDs). This group comprised the ENT-RA patients.

A second group consisted of 7 patients diagnosed as having RA with a duration of symptoms > 2 years. They were under treatment with DMARDs and presented non-acute inflammatory disease activity, showing knee arthritis with a synovial fluid test exhibiting a transparent straw or yellow color, a WBC count < 2000/mm^3 and a neutrophil count less than 50% (16). They formed the NAT-RA group.

Patients were excluded from this study if they showed evidence of any infectious process or developed another rheumatic disease while under observation. We also studied 14 healthy subjects as a control group.

Rheumatoid factor (RF) and C-reactive protein (CRP) were determined in each patient. A complete study of the synovial fluid, including bacteriological culture, Gramm stain and the bacille acid-fast alcohol resistant (BAAR) stain, was carried out as well. X-ray films of the hands and feet were evaluated at study entry (data not shown).

Isolation of mononuclear cells and RNA extraction

For the isolation of freshly peripheral blood mononuclear cells (PBMC) and synovial fluid mononuclear cells (SF MC), 5 mL of venous peripheral blood (PB) and 5 mL of synovial fluid (SF) were collected into tubes containing heparin. Mononuclear cells (MC) were immediately isolated on a Lymphoprep™ (Nycomed, Oslo, Norway) density gradient. MC were washed twice with phos-

phate buffer solution pH 7.2, at 1500 Xg for 10 minutes.

Isolation of the total RNA was carried out according to the method described by Chomczyski and Sacchi (17). Briefly, MC were homogenized in the presence of TRIZOL™, chloroform was added and the aqueous phase was obtained. The RNA was precipitated with isopropanol at 4°C overnight. The quantity and intactness of the RNA was routinely tested by determining its absorbance at 260/280 nm and by ethidium bromide fluorescence of RNA electrophoresed in 1% formaldehyde-containing agarose gels.

Analysis of cytokine gene expression by the quantitative RT-PCR method

One µg of total RNA obtained from PBMC or SFMC, was reversed transcribed in 0.05M Tris-HCl pH 8.3, 40 mM KCl, 7 mM $MgCl_2$ buffer containing 0.05 µg/µL random hexamers, 1 mM dNTPs mix, 0.05 U/µL RNAse inhibitor and 200 U/µL murine leukemia virus reverse transcriptase M-MLV. Samples were incubated at 4°C for 5 min after being kept at 72°C for 10 min. Samples were then incubated at 37.5°C for 60 min. Reverse transcriptase was further inactivated by heating the sample tubes at 95°C for 10 min. cDNAs obtained were stored at -20°C until use.

Analysis of messenger RNA (mRNA) has been widely used to study minute variations in local cytokine levels. In order to compare the cytokine mRNA levels in different patients, the obtaining of identical cDNA concentrations in each sample was necessary. This was accomplished by the method of Volk *et al.* (18, 19). Briefly, we used two multi-specific control fragments (CF) inserted into two different Bluescript plasmids: pHCQ1 and pHCQ 2, of 471 and 568 bp respectively, which contain the cDNA for different cytokines, including glyceraldehyde 3-phosphate dehydrogenase (GAPDH). After digestion with the appropriate restriction enzymes (Fig. 1, panel A), known amounts of the CF of interest were added in decreasing concentrations to different reaction tubes (12, 1.2 and 0.12 pg/µL), while maintaining the patient's cDNA constant at 1 µL.

Amplification of a housekeeping gene (GAPDH) was performed. The proportion of PCR products amplified from the GAPDH control fragment and the patient's GAPDH cDNA were estimated after separation on 1.5% agarose gel. The bands were stained with ethidium bromide, photographed with 665 Polaroid film and the negatives were scanned with a BECKMAN DU series spectrophotometer (Fig. 1, panel B). All samples were adjusted to contain identical cDNA concentrations at a fixed CF concentration (1.2 pg/µL) (Fig. 1, panel C).

Once the cDNA samples were adjusted, we needed to dilute the CF to 0.06 pg/µL to further quantify IL-1β, TNFα, IL-6, IL-4 and IL-10 mRNA expression, since these molecules correspond to genes with a low expression level. Depending on the amplification efficiency, samples were subjected to a varying number of PCR cycles: 28 (GAPDH), 35 (IL-1β, IL-6 and TNFα) and 40 (IL-10, IL-4).

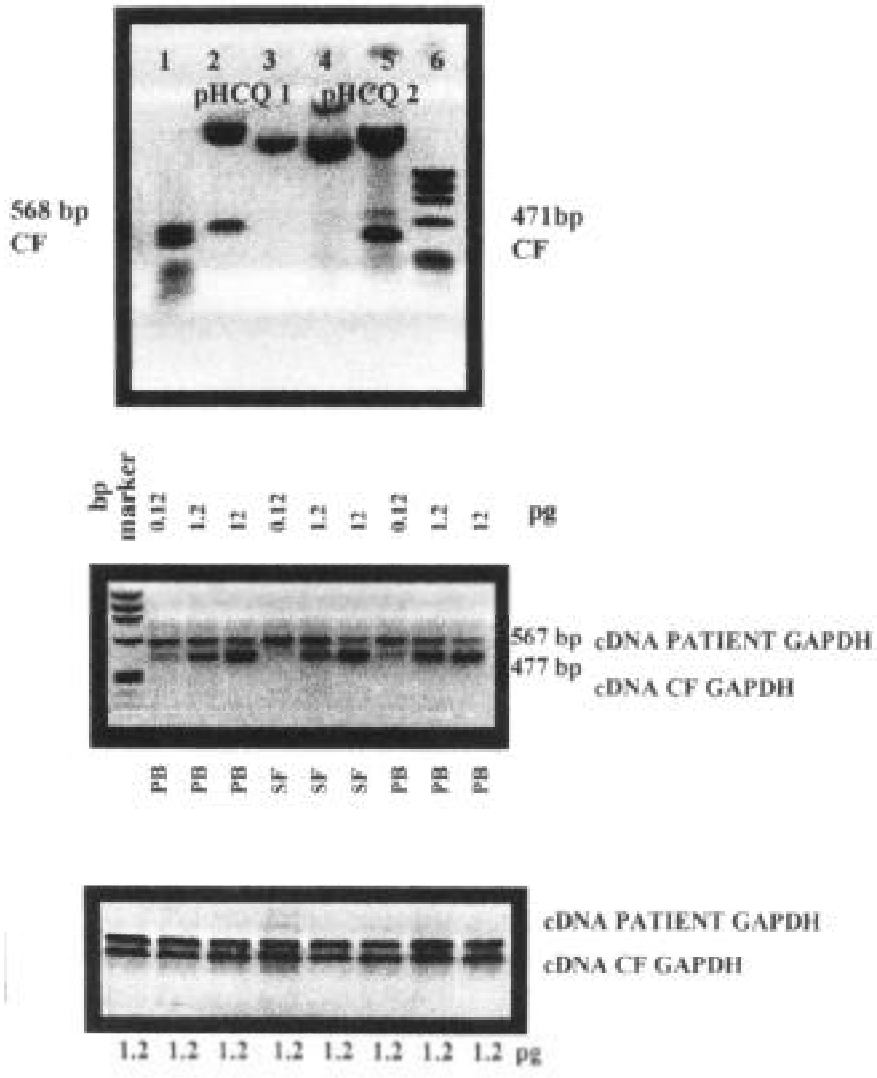

Fig. 1. Overall strategy to quantitatively measure cytokine mRNA expression.

Panel A. Shows the undigested plasmids pHCQ1 and pHCQ2 (lines 3 and 4). Plasmids digested with their multispecific CF: lines 2 and 5 and their respectives lengths: 568 and 471 base pairs, stained with ethidium bromide on 0.8% agarose gel. Molecular weight markers: line 1: phage Φ X 174 Hae III; line 6: pBR 322 Hae III.

Panel B. Adjustment of patient´s cDNA samples with a constitutively expressed gene (GAPDH), using CF at a concentration of 1200 pg/mL, diluted 10^{-2} to 10^{-6}. Known amounts of CF were added in decreasing dilutions in different reaction tubes (12 pg/mL, 1.2 pg/mL, 0.12 pg/mL), to a constant amount of 1 mL of patient cDNA. Equilibration of the patient cDNA sample occured when it was compared to 1.2 pg/mL of CF, meaning that the cDNA sample was already adjusted.

Panel C. Shows a representative example of several cDNA samples obtained from different patients.

Control PCRs without template DNA were performed in all experiments to exclude contamination. Furthermore, total RNA samples without conversion to cDNA were taken from patients and healthy subjects, and reacted with specific primers for IL-1β, TNFα, IL-10, IL-4 and IL-6. No amplified products were detectable, confirming that our RNA samples were not contaminated with genomic DNA. The binding efficiency of the primers for the control fragments versus intact cDNA were identical on the grounds of previous experiments and our own results (data not shown). All of the procedures and material were endotoxin-free.The primers used were:

IL-1β Sense
5'- TGCCCTCTTCCTGG GAGGG-3'
Antisense
5'- GGCTGGGGATTGCCTGAA-3'

TNFα Sense
5'-CTCTGGCCCAGGCAGTCAGA-3'
Antisense
5'- GGCGTTTGGGAAGGTTGGAT-3'

IL-6 Sense
5' -TAGCCGCCCCACACAGACAG-3'
Antisense
5'-GGCTGGCATTTGTGGTTGGG-3'

IL-10 Sense
5'-CTGAGAACCAAGACCCAGACA TCAAGG-3'
Antisense
5'-CAAATAAGGTTTCTCAAGGGGC TGG-3'

IL-4 Sense
5'-GCTTCCCCCTCTGTTCTTCC-3'
Antisense
5'-TCTGGTTGGCTTCCTTCACA-3'

Quantitative measurement of cytokine mRNA expression

To quantify the mRNA expression of these cytokines, samples prepared by electrophoresis were photographed with 665 Polaroid film during exposure to UV light and the negatives of the film containing the dark bands were scanned using a BECKMAN DU series spectrophotometer. After each scan was completed, the band area representing the amplified PCR product of each cytokine was automatically calculated and normalized against the band area represented by the CF at a concentration of 0.06 pg/μL. The results were then expressed as picograms of the cytokine of interest, and the number of molecules was calculated using the following equation:

Moles x 6.023 x 10^{23} / MW = number of molecules of cytokine mRNA
(MW: the molecular weight in gm)

Serum and SF levels of IL-1β, TNFα, IL-6, IL-4 and IL-10 were quantified using commercially available Quantikine kits following the manufacturer's instructions (R & D Systems, Inc. Minneapolis, MN). The sensitivity for IL-1β and TNFα was 15.6 to 1000 pg/mL; the sensitivity for IL-6 was 3.12 - 300 pg/mL; and the sensitivity for IL-10 and IL-4 was 7.8 to 500 pg/mL.

Semiquantitative RT-PCR for mRNA MMP expression by FIMC

Once the cDNA samples from RA and control subjects were adjusted as described above, we determined the mRNA expression of MMP-1 (collagenase-1), MMP-3 (stromelysin-1) and MMP-13 (collagenase-3). The samples were subjected to 35 PCR cycles for amplification using the following primers:

MMP-1 Sense
5' - ATTTCTCCGCTTTTCAACTT-3'
Antisense
5'-ATGCACAGCTTTCCTCCACT-3'

MMP-3 Sense
5' -ATGAAGAGTCTTCCAATCCT-3'
Antisese
5' -GTCCTTTCTCCTAACAAACT-3'

MMP-13 Sense
5' -TGCTGCATTCTCCTTCAGGA-3'
Antisense
5'- ATGCATCCAGGGGTCCTGG-3'

The mRNA expression of each MMP was expressed as relative absorbance units.

The results were analyzed using the Student's t-test.

Results

The duration of symptoms in the ENT-RA group was 9.1 months (range 3 to 12 months), while for the NAT-RA group it was 6.9 years (range 2.5 to 10 years). Clinical and laboratory data are shown in Table I. Our results showed no significant differences on either the protein or the transcript level in the expression of IL-1β, TNFα, IL-6, IL-4 and IL-10 between the ENT-RA and NAT-RA groups by quantitative RT-PCR and ELISA. However, a trend in cytokine mRNA expression by FI-PBMC and FI-SFMC in both groups was found as follows: IL-1β > TNFα > IL-10 > IL-6, with no mRNA IL-4 expression (Fig. 2, panels A-D).

Worth noting is the fact that the cytokine serum levels in ENT-RA and NAT-RA patients were detected in decreasing order as follows: IL-6 > IL-10, while IL-1β, TNFα and IL-4 were undetectable (Fig. 3, panel A and C).

A finding in both groups of RA patients was that cytokine SF levels were up to ten-fold higher than the serum levels, in the following order: IL-6 > IL-1β > TNFα > IL-10, with IL-4 being undetectable (Fig. 3, panel B and D).

Control subjects matched for age and sex showed significant differences in IL-1β and TNFα mRNA expression with respect to the ENT-RA and NAT-RA patients (Table II).

Semiquantitative mRNA expression of MMP-1, MMP-3 and MMP-13

A statistically significant difference between ENT-RA and NAT-RA patients in the PBMC mRNA expression of MMP-3 was found, being higher in the NAT-RA group (Fig. 4, panel C). Based on the relative absorbance units recorded, MMP-1 mRNA expression was prevalent in the ENT-RA group, while MMP-3 mRNA expression was greater in the NAT-RA patients (Fig. 4, panels C and D). Notably, we found that the expression of MMP-1, MMP-3 and MMP-13 by the PBMC and SFMC in both the ENT-RA and NAT-RA groups were almost identical (Fig. 4, panels A-D). mRNA expression of MMPs in the control subjects is also shown on Table II.

Discussion

Previous research has shown that it is now possible to alter the outcome of a variety of inflammatory diseases, either by specifically blocking the effects of a particular pro-inflammatory cytokine, or by intensifying the effects of a given anti-inflammatory cytokine. The current in-

Table I. Patient characteristics and clinical and laboratory data for the early, never-treated rheumatoid arthritis (ENT-RA) and the non-acute treated RA (NAT-RA) groups.

A. ENT-RA group

Patient	Sex	Age	# Tender joints	Duration of symptoms	RF (IU) (normal value < 30)	CRP (normal value < 0.8mg/dl)	X-ray (Radiological stage)
1	F	25	5	3 months	518	8.4	II
2	M	25	4	12 months	394	7.1	II
3	F	20	5	4 months	92	5.3	II
4	F	20	8	12 months	268	6.7	II
5	F	29	4	12 months	360	5.3	I
6	F	33		9 months	101	6.1	I
7	M	53	9	12 months	223	3.8I	

B. NAT-RA group.

Patient	Sex	Age	# Tender joints	Duration of symptoms	Treatment	RF (IU)	CRP	X-ray
1	F	27	3	10 years	Cloroquine, Indometacin	517	1	III
2	F	23	2	4 years	Cloroquine, Indometacin	92	1.4	III
3	F	23	1	4 years	Metotrexate, Ibuprofen	394	1.0	II
4	M	50	3	2.5 years	Metotrexate, Naproxen	72.3	0.9	II
5	F	36	1	4 years	Metotrexate, Ibuprofen	101	1	III
6	F	32	1	10 years	Metotrexate	394	1	IV
7	M	28	3	10 years	Metotrexate	171	0.8	III

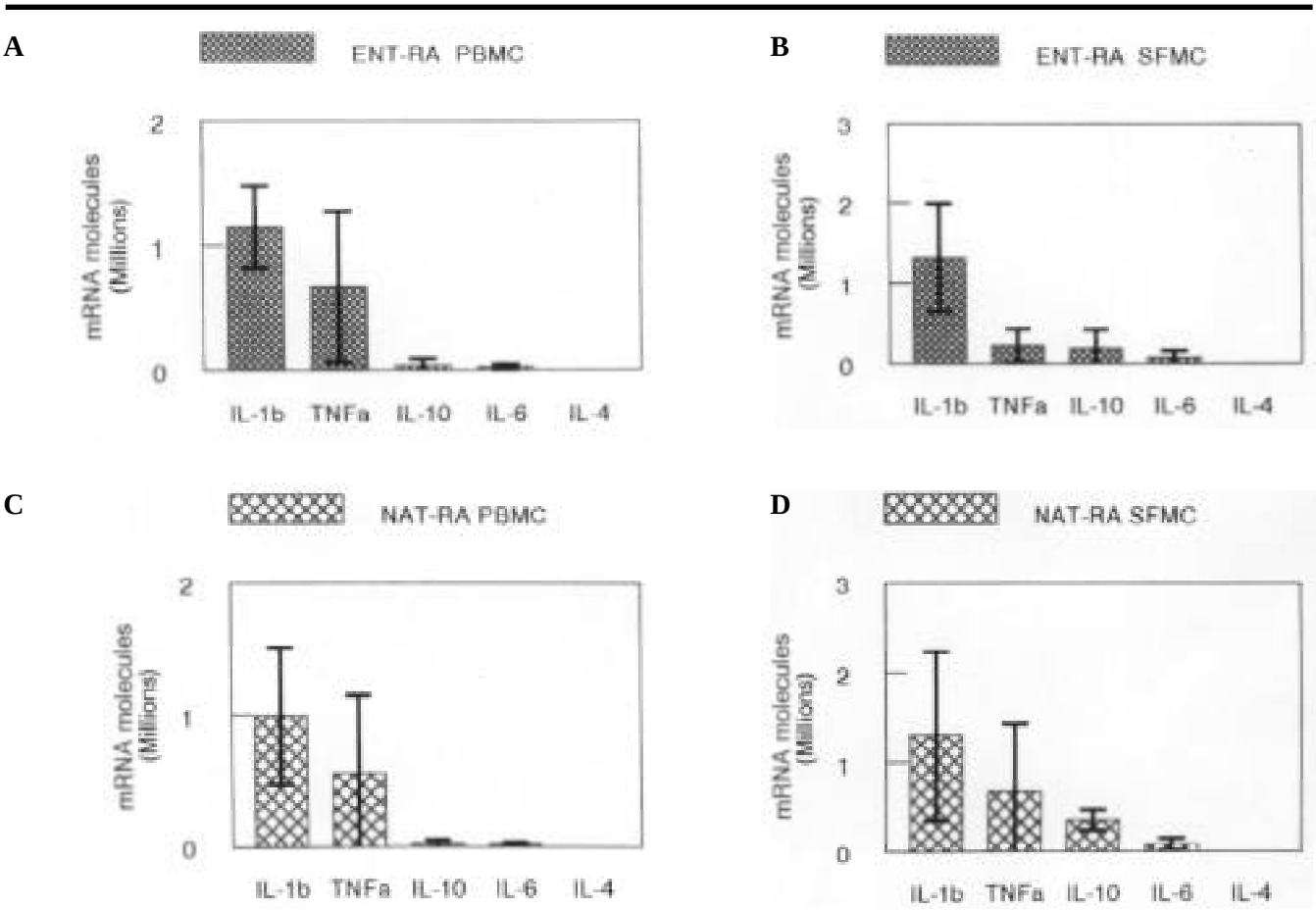

Fig. 2. Cytokine mRNA expression measured by quantitative RT-PCR in PBMC and SFMC obtained from ENT-RA and NAT-RA patients. **Panels A, B, C** and **D**. No significant differences on the transcript level for the cytokines of interest between the ENT-RA and NAT-RA groups were found. However, a trend in cytokine mRNA expression by FI-PBMC and FI-SFMC in both groups was found as follows: IL-1β > TNFα > IL-10 > IL-6, without mRNA IL-4 expression.

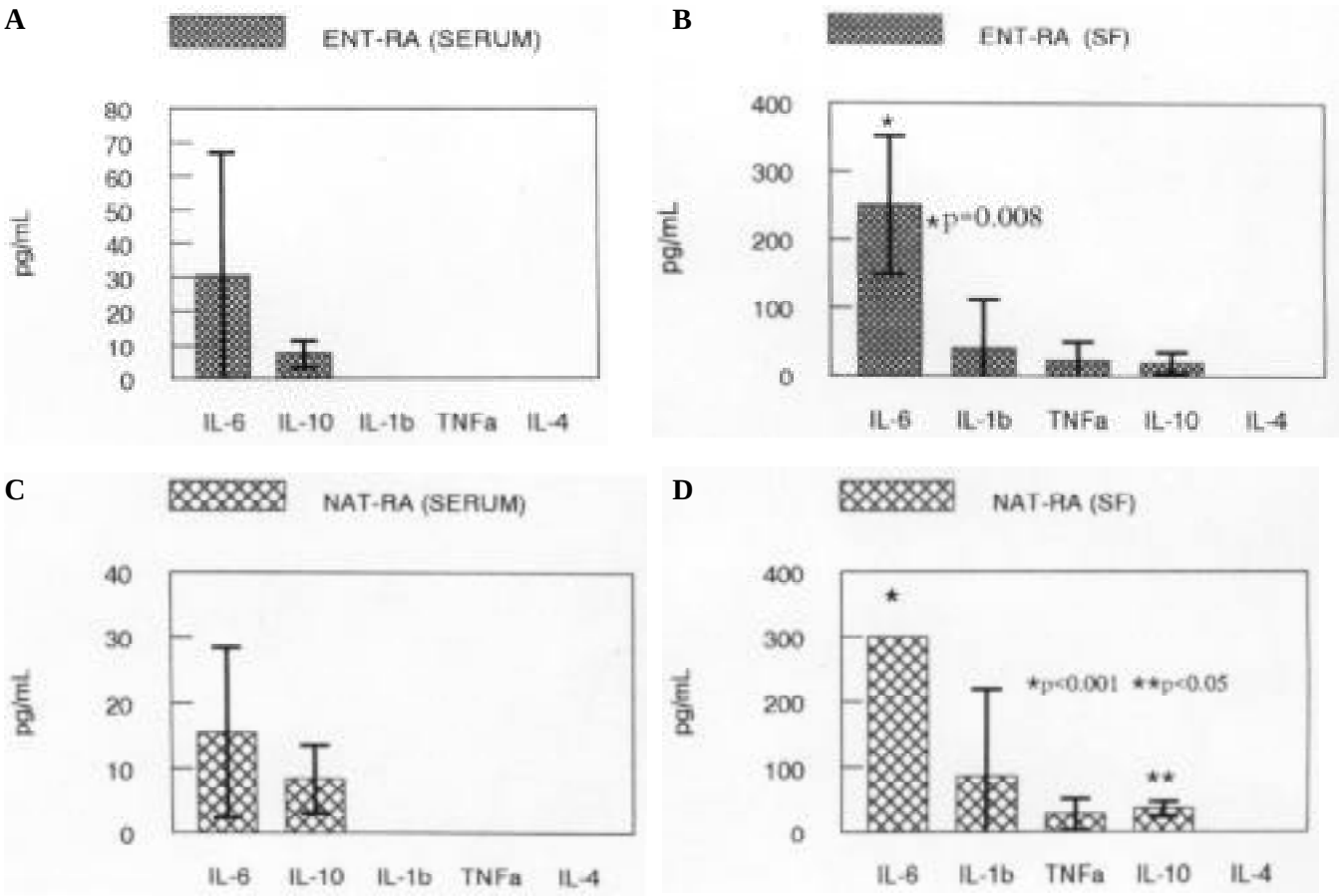

Fig. 3. Serum and SF cytokine expression measured by ELISA from ENT-RA and NAT-RA patients.**Panels A** and **C**. Cytokine serum levels in ENT-RA and NAT-RA patients were detected as follows: IL-6 > IL-10, while IL-1β, TNFα and IL-4 were undetectable. **Panels B** and **D**. Cytokine SF levels were detected which were up to ten-fold higher than the serum levels in the following order: IL-6 > IL-1β > TNFα > IL-10. IL-4 levels were non-detectable in both groups of RA patients.

Table II. Cytokine and MMP expression in healthy subjects compared with the early, never-treated rheumatoid arthritis (ENT-RA) and non-acuted treated RA (NAT-RA) groups.

Cytokine measured (median ± SD)	Healthy subjects (n = 14) Serum (pg/dL)	Healthy subjects (n = 14) PBMC (mRNA molecules)	ENT-RA pts. (n = 7) Serum	ENT-RA pts. (n = 7) PBMC	NAT-RA pts. (n = 7) Serum	NAT-RA pts. (n = 7) PBMC
IL-1β	Not detected	14477 ± 7949*	Not detected	1154529±330864*	Not detected	988614 ± 523777*
TNFα	Not detected	8042 ± 4863*	Not detected	670586 ± 607884*	Not detected	553757 ± 596731*
IL-6	Not detected	Not detected	30.4 ± 36.88	25480 ± 19315	15.4 ± 12.98	10504 ± 15212
IL-10	Not detected	Not detected	7.24 ± 4.16	33814 ± 58534	8.35 ± 5.24	14800 ± 28993
IL-4	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected
MMP-1	-	0.180 ± 0.070	-	.215 ± 0.035	-	0.130 ± 0.048
MMP-3	-	0.095 ± 0.100**	-	0.195 — 0.059	-	0.210 ± 0.121**
MMP-13	-	0.140 ± 0.030	-	0.131 ± 0.023	-	0.142 ± 0.074

* p< 0.01; ** p< 0. 05

ability to predict the actions of individual inflammatory mediators comes in part from the fact that many of their properties have been characterized using *in vitro* culture systems (20).

Our data showed sustained cytokine secretion at the local level (synovial fluid/SFMC) and scarce translation at the peripheral level (serum/PBMC), irrespective of the patient group. We may explain this finding by the notion that asymptomatic synovitis precedes clinically manifested arthritis in both early and established RA, as has already been suggested (21). Notably, there was a trend in cytokine mRNA expression (IL-1β > TNFα > IL-10 > IL-6 without mRNA IL-4 expression) by the PBMC and SFMC in the ENT-RA and NAT-RA patients, while the cytokine serum levels in these patients were detected in inverse order as

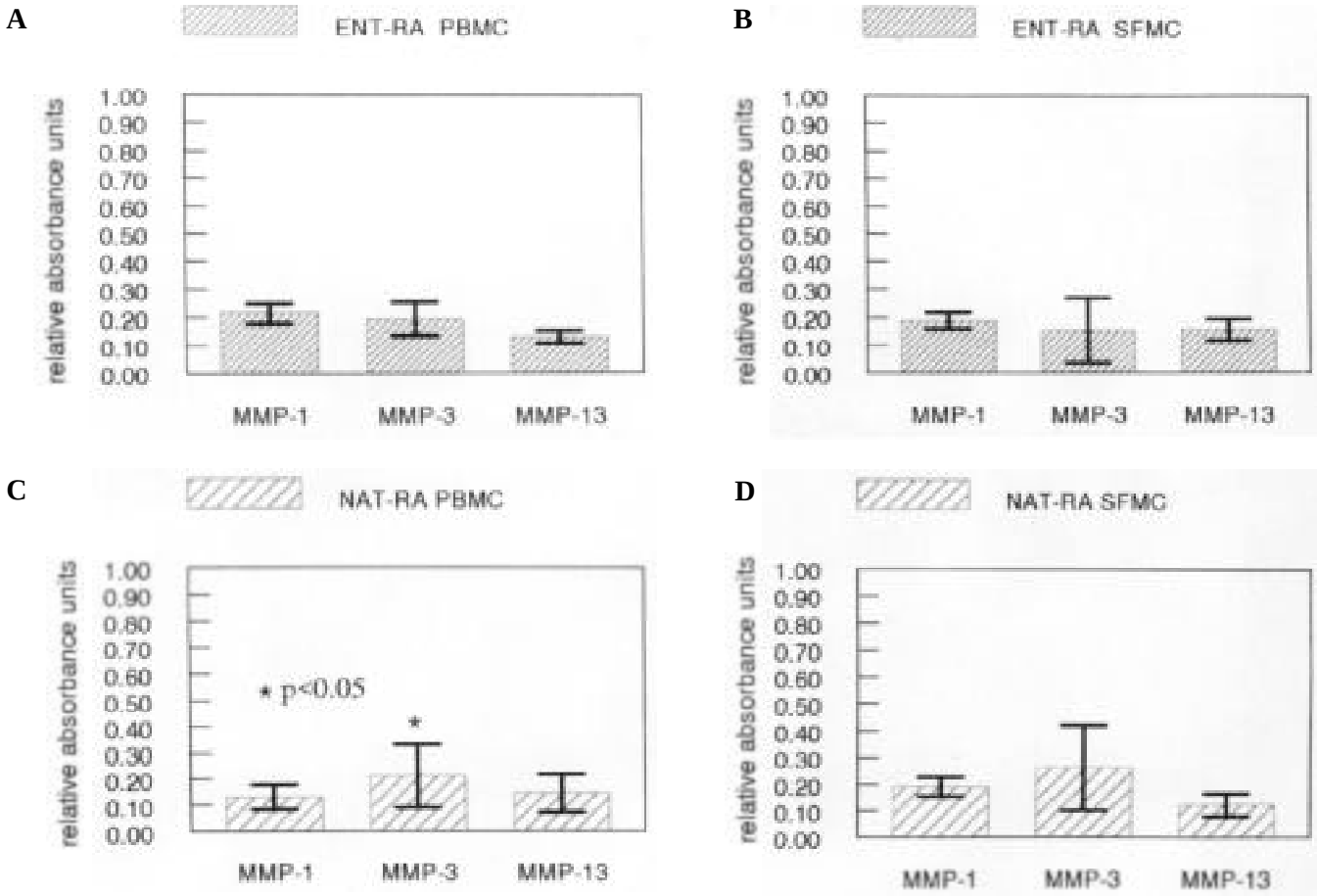

Fig. 4. Semiquantitative mRNA expression of MMP-1, MMP-3 and MMP-13 by PBMC and SFMC in ENT-RA and NAT-RA patients. **Panels A, B, C** and **D**. mRNA expression of MMP-1, MMP-3 and MMP-13 by PBMC and SFMC in both the ENT-RA and NAT-RA groups was found to be very similar.**Panel C**. A statistically significant difference between ENT-RA and NAT-RA patients in the PBMC mRNA expression of MMP-3 was found, being higher in the NAT-RA group.

follows: IL-6 > IL-10, while IL-1β, TNF-α, and IL-4 were undetectable. This is supported by the proinflammatory cytokine dynamic expression reported in animal models of collagen-induced arthritis, in which TNFα and IL-1β were expressed preferentially on the first days of damage, followed by the sustained secretion of IL-6 (22). The low to undetectable levels of peripheral cytokines seen in the ENT-RA and NAT-RA patients, in contrast to the detection of their transcripts, might be explained in several ways:

A) The inflammatory reaction in RA is predominantly triggered at the local level, and it has been demonstrated that the major source of cytokine production is the rheumatoid synoviocyte, so that the noted discrepancy between cytokine protein levels and their transcripts is consistent with the notion that IL-1β, TNFα, and IL-6 are produced mostly by synovium cells rather than in the PBMC or SFMC in RA (23).

B) The autoimmune process may not require the presence of large amounts of cytokines, at least in amounts detectable systemically, in order to develop.

C) Detection of the protein is hampered by the usually low production of cytokines, given their short half-life *in vivo* (24).

D) The binding of circulating cytokine to its cognate receptor, resulting in the formation of a respective cytokine-receptor complex as well as post-transcriptional events, may result in low or absent circulating levels of cytokine proteins (25). The RA process is influenced by the down-regulation of pro-inflammatory cytokines, mainly TNFα, IL-1β, IL-6 and by antagonist molecules known as IL-1 receptor antagonist (IL-1ra) and soluble receptors. IL-1ra is a member of the IL-1 family that binds to IL-1 receptors, but does not induce any intracellular response.

Two structural variants of IL-1ra have been described: a 17kDa form that is secreted from monocytes, macrophages, neutrophils, and a 19 kDa form that remains in the cytoplasm of keratinocytes, monocytes and fibroblasts. IL-1ra has been used in RA gene therapy in humans and in the SCID mouse model where it showed chondroprotective benefits. Moreover, clinical trials using recombinant human IL-1ra have reported improvement in clinical parameters and radiographic evidence of joint damage (26, 27).

On the other hand, the importance of the soluble receptor is shown by its function in inflammatory down-modulation, as reflected by the p75 and p55 TNF receptors. The biological role of the solubilized TNF receptor relates to its removal from the cell surface so that it can no longer serve as a signaling molecule. In this way, formation of the soluble receptor limits the response of the cell

to its ligand. Release of the p75 TNF receptor may limit the cell's TNF sensitivity. Low levels of soluble TNF receptor appear to enhance TNF signaling, perhaps by stabilizing the ligand, whereas higher concentrations inhibit TNF activity. The possible dissociation between mRNA levels and their cognate proteins found in our study could also be explained by a possible increased amount of these antagonist molecules, which also limits the biological activities of the cytokines being studied here (28).

E) The presence of cytokine autoantibodies may have interfered with the assay methods, or the cytokines may have been degraded by proteases or other substances present in the biological samples. In addition, it has been reported that the presence of the mRNA for a cytokine does not necessarily correlate with the production and secretion of biologically active cytokine protein (29).

A finding in both groups of RA patients was that the cytokine SF levels detected were up to ten-fold higher with respect to the serum levels, in the following order: IL-6 > IL-1β > TNFα > IL-10, with IL-4 being non-detectable. Comparison of ENT-RA serum / synovial fluid cytokine levels and NAT-RA serum / synovial fluid cytokine levels showed a significant difference for IL-6, which was detected at levels as much as ten times higher in the synovial fluid than in serum. The predominant secretion of IL-6 in FI-SFMC observed in our patients accords with other studies of the synovial production of cytokines *ex vivo* (22).

An increased secretion of IL-10 in the synovial fluid of chronically damaged joints in NAT-RA patients was noticeable, but the biological significance of this finding is not clear. Perhaps it may be related to B lymphocyte hyperactivity and autoantibody production, particularly rheumatoid factor, as described by Llorente *et al*. (30). IL-10 is recognized as a cytokine able to regulate the levels of surface and soluble receptors of TNF-α, and to limit the effects of TNFα on lymphocytes, fibroblasts and endothelial cells (31). This observation probably reflects the well-known anti-inflammatory action of this cytokine.

On the other hand, important MMPs in RA are MMP-1, MMP-3, MMP-13, MMP-9 and MMP-8. These MMPs are important not only because of their role in the destruction of the extracellular matrix, but also because thay are important regulators of angiogenesis in RA synovium. The regulation of MMP activity is complex and tightly controlled. The specific tissue inhibitors of MMPs [known as the tissue inhibitors of matrix metalloproteinases (TIMPs)] provide fine control by binding in a 1:1 complex with MMPs and inhibiting their action. Recently, it has been reported that RA synovium secretes decreased levels of TIMP-1 (32).

High concentrations of IL-1β, TNFα, and IL-6 derived primarily from synovial macrophages are secreted into the joint millieu. These cytokines at the same time promote MMP secretion by chondrocytes, which in turn will favor the degradation of the extracellular matrix components of the joint finally expressed by bone erosion. Regarding MMP mRNA expression, we found a statistically significant difference in PBMC mRNA MMP-3 expression between the ENT-RA and NAT-RA groups, this expression being higher in the NAT-RA patients probably due to their older age (33). Notably, we found mRNA expression of MMP-1, MMP-3 and MMP-13 by the PBMC and SFMC at almost identical levels, a finding that may be read as a probable peripheral indicator of local RA damage, and merits further evaluation.

Finally, in the light of our results it is clear that a tight equilibrium between MMP and pro/anti-inflammatory cytokine production is demonstrated in ENT-RA and NAT-RA patients, a balance apparently not influenced by the duration of the disease. Highly sensitive methods such as quantitative RT-PCR and ELISA, or even FIMC, have demonstrated sustained cytokine secretion at the local level (synovial fluid / SFMC) and scarce translation at the peripheral level (serum /PBMC). mRNA MMP expression needs to be further evaluated in order to determine whether their peripheral expression reflects their local activity in RA patients.

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