Clin. exp. Immunol. (1988) **73**, 449–455

Interleukin-1 and tumour necrosis factor mRNA expression in rheumatoid arthritis: prolonged production of IL-1α

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(Accepted for publication 24 March 1988)

SUMMARY

In rheumatoid arthritis there is a chronic immune and inflammatory reaction which can lead to the destruction of the diseased joint. Cytokine gene expression was studied in synovial cells using cDNA probes specific for human interleukin 1 (IL-1), -α and IL-1β, tumour necrosis factor (TNF), -α and TNFβ (lymphotoxin); protein molecules which induce cartilage degradation and bone resorption. In all cases studied, IL-1 mRNA was present in freshly isolated synovial cells from fluid or membrane. Compared to levels of IL-1 mRNA found in optimally activated normal blood mononuclear cells, the levels of IL-1α mRNA were high in seven of the nine patients studied, whereas IL-1β mRNA, the dominant form in blood, was relatively lower. TNFα and TNFβ mRNA were also detected. Rheumatoid synovial cells, cultured without any stimulus, continued to express high levels of IL-1α mRNA for up to 5 days, compared to the 24 h response of activated blood cells; IL-1β mRNA in culture was also prolonged. Cultures of rheumatoid joint cells produced IL-1 bioactivity, with roughly equal amounts of IL-1α and β, as assessed using neutralizing antibodies. TNF bioactivity was also detected which may be of importance as TNF induces the production of IL-1. The finding of these mediators produced in large amounts in active rheumatoid synovial cells suggests that mutually stimulatory cell interactions, mediated by these molecules, may be important in the chronic inflammation and tissue destruction in rheumatoid arthritis.

Keywords interleukin 1 tumour necrosis factor mRNA rheumatoid arthritis

INTRODUCTION

Rheumatoid arthritis (RA) is the commonest of a group of chronic inflammatory diseases with autoimmune manifestations. This important group of diseases includes Hashimoto's and Graves' thyroiditis, juvenile onset (type I) diabetes, systemic lupus erythematosus and Sjögren's syndrome (Feldmann, Doniach & Bottazzo, 1985). Current therapy for these diseases is unsatisfactory, involving palliation of symptoms (e.g. joint pains) or replacement of disease-induced deficiency (e.g. insulin in diabetes). However, none of these treatments influence the disease process (Bollet, 1985). Thus further insights into the pathogenesis of these diseases could be valuable, possibly leading to new therapeutic approaches. One of the key features of these diseases, in contrast to the majority of animal models of autoimmune diseases, is their prolonged duration. The mechanisms underlying the chronicity of these diseases are not understood, but are of major importance in their pathogenesis.

There has been much speculation about the role of immune mediators in the pathogenesis of RA, especially interleukin-1 (IL-1). This was based on the presence of IL-1 bioactivity in rheumatoid synovial fluid (SF) (Fontana *et al.*, 1982), on its pro-inflammatory actions on human rheumatoid synovial cells, its stimulation of the release of collagenase and prostaglandin E_2 and its induction of both cartilage degradation (Saklatavala, Sarsfield & Townsend, 1985) and bone resorption (Gowen *et al.*, 1983). The probable importance of IL-1 in RA has been reinforced by the capacity of intra-articular administration of IL-1 in rabbits to reproduce many of the findings of antigen-induced arthritis (Pettipher, Higgs & Henderson, 1986) and by the detection of IL-1 receptors on porcine chondrocytes and synovial fibroblasts (Bird & Saklatavala, 1986).

Tumour necrosis factor (TNF) has also been shown to stimulate the production of prostaglandin E_2 and collagenase by synovial fibroblasts (Dayer, Beutler & Cerami, 1985) and accelerate the degradation of proteoglycans (Saklatavala, 1986).

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Additionally, both TNFα and TNFβ (also known as lymphotoxin) activate osteoclasts to resorb bone *in vitro* (Thomas, Mundy & Chambers, 1987). These activities suggest that TNF could, like IL-1 be involved in the inflammation and destruction occurring in rheumatoid joints. Furthermore, TNFα and β are potent inducers of IL-1 (Dinarello *et al.*, 1986), and so could have an influence on joint destruction both directly and via the production of IL-1. This led us to investigate whether these mediators are produced locally during active RA.

Analysis of the mediator content of synovial fluids has yielded paradoxical results (Buchan *et al.*, 1987), probably due to the complex nature of the immunological events within the lesion. We have therefore made use of a combination of mRNA analysis and specific bioassays to assess the production of IL-1α and β and TNFα and β by mononuclear cells isolated from active RA joints. Here we report that both mRNA and protein for these mediators is produced in the absence of any extrinsic stimulation of the rheumatoid arthritis joint cells. The presence of IL-1α and to a lesser extent IL-1β mRNA was found to be prolonged in culture, suggesting that at least some of the mechanisms involved in the maintenance of chronicity of the joint disease are retained in culture.

MATERIALS AND METHODS

Patients

Mononuclear cells from the 17 synovial fluids (SFs) and two synovial membranes (SMs) used in this study were derived from patients with RA in whom the disease had lasted for at least 2 years and who satisfied American Rheumatism Association criteria for classical disease (Ropes *et al.*, 1959). Fluids were obtained by arthrocentesis of knee joints from individuals with an acute flare on a background of chronic disease; the membranes were specimens obtained during prosthetic replacement. IL-1 mRNA data was compiled from two SM and seven SF samples, TNF mRNA data from five of the above fluid samples and the bioactivity data from supernatants from 24 h cultures of the remaining 10 SFs as supernatants from the other samples were not available.

Cell preparation and culture

Peripheral blood mononuclear (PBM) and SF mononuclear cells (MNC) were separated from heparinized samples on Ficoll–Hypaque (Boyum, 1967). Synovial membranes were digested for 2 h at 37°C with 1 mg/ml collagenase Type IV (204 u/mg, Cooper Biomedical) and 25 mg/ml of DNAse I (Sigma). A single cell suspension was prepared by straining the digest through sterile gauze, followed by three washes in RPMI 1640 containing 10% fetal calf serum. SM and SF cells were then cultured at 1×10^6 cells/ml in 2 ml RPMI 1640 containing 10% fetal calf serum, in 24-well plates (Falcon 3047) for periods ranging from 0–5 days. Healthy PBM were cultured as above for periods of 0–48 h with 0·1% phytohaemagglutinin (PHA) (Sigma) and 5 ng/ml phorbol dibutyrate (PDB) (Sigma). In a number of control experiments we have found that this stimulus is optimal for the simultaneous induction of a variety of cytokines (IL-1, IL-2, TNF etc.).

cDNA probes

IL-1α mRNA was identified using a 460 bp EcoRI-BamHI fragment from *P*Δ3 IL-1α corresponding to the coding region for amino acids 1–132 of the human IL-1α precursor (Gubler *et al.*, 1986). IL-1β mRNA was detected with a 530 bp BamHI-NdeI fragment from *P*Δ11 IL-1β representing the region coding for amino acids 1–139 of the human IL-1β precursor (Auron *et al*, 1984). The control probe was a 708 bp PstI-DraI fragment from p7B6, identified from a cDNA library prepared from a patient with chronic lymphocytic leukaemia (Kaczmarek, Calabretta & Baserga, 1985). An 800 bp, EcoRI fragment from λ42–4 was used to detect TNFα mRNA (Pennica *et al.*, 1984) and a 950 bp, EcoRI fragment derived from λLT11 to detect TNFβ (Gray *et al.*, 1984). All fragments were radiolabelled by random oligo-priming (Feinberg & Vogelstein, 1984) to a specific activity of $6–10 \times 10^8$ ct/min per μg DNA.

mRNA analysis

Total cytoplasmic RNA was extracted from cells using a modified version of the method described (White & Bancroft, 1982). Briefly, cells were spun in the plates at 1,000 *g* for 10 min at room temperature, resuspended in 10 mM Tris, 1 mM EDTA pH 7·5, lysed by the addition of Nonidet P 40 (B.D.H.) and spun at 15,000 *g* to pellet the nuclei. The supernatant was extracted once with phenol, once with chloroform and the RNA precipitated by ethanol and sodium acetate at −20°C. RNA was denatured by heating at 60°C for 15 min in 2·25 M NaCl, 0·225 M Na citrate, 7·5% formaldehyde and blotted onto nitrocellulose in doubling dilutions using a Minifold II slot blot apparatus (Schleicher & Schuell). Filters were prehybridized for 6–16 h at 42°C in a solution containing 50% formamide, 750 mM NaCl. 75 mM sodium citrate, 50 mM phosphate, 500 $\mu g ml^{-1}$ salmon sperm DNA, 0·04% Ficoll, 0·04% polyvinylpyrrolidone, 0·04% bovine serum albumin fraction V. Hybridization was carried out in prehybridization solution to which probe had been added for a further 16–20 h at 42°C. Blots were washed twice at room temperature for 5 min in 300 mM NaCl, 30 mM Na citrate, 0·1% sodium dodecyl sulphate followed by two washes at 50°C for 20 min in 30 mM NaCl, 3 mM sodium citrate, 0·1% sodium dodecyl sulphate and exposed to Fuji X-ray film at −70°C with intensifying screens for 1–12 days. The integral of the OD_{550} across the bands exposed in the linear response range on the autoradiographs was obtained by scanning with a Joyce Loebl Chromoscan 3 densitometer. Values were adjusted using the control probe 7B6, a species of mRNA whose levels are constant throughout the cell cycle (Kaczmarek, Calabretta & Baserga, 1985), to compensate for variations in cell number caused by cell proliferation during the assay, and a positive control of known quantity to compensate for variations in hybridization. As the cDNA probes used for IL-1α and β; and TNFα and β; were of similar length and labelled to similar specific activity, the intensity of the bands on the autoradiographs were used as a semi-quantitative comparison of the amounts of mRNA present.

Bound radioactive DNA was removed from filters by washing in 5 mM Tris/HCl (pH 8), 0·2 mM EDTA, 0·05% sodium pyrophosphate and 0·002% Ficoll, 0·002% polyvinylpyrrolidone, 0·002% bovine serum albumin for 1–2 h at 65°C. This was confirmed by autoradiography.

Assay for IL-1

IL-1 bioactivity was measured using the thymocyte costimulator assay (Paetkau *et al.* 1976). Briefly, thymocytes from male CBA mice were incubated with 50 μl of supernatant in the

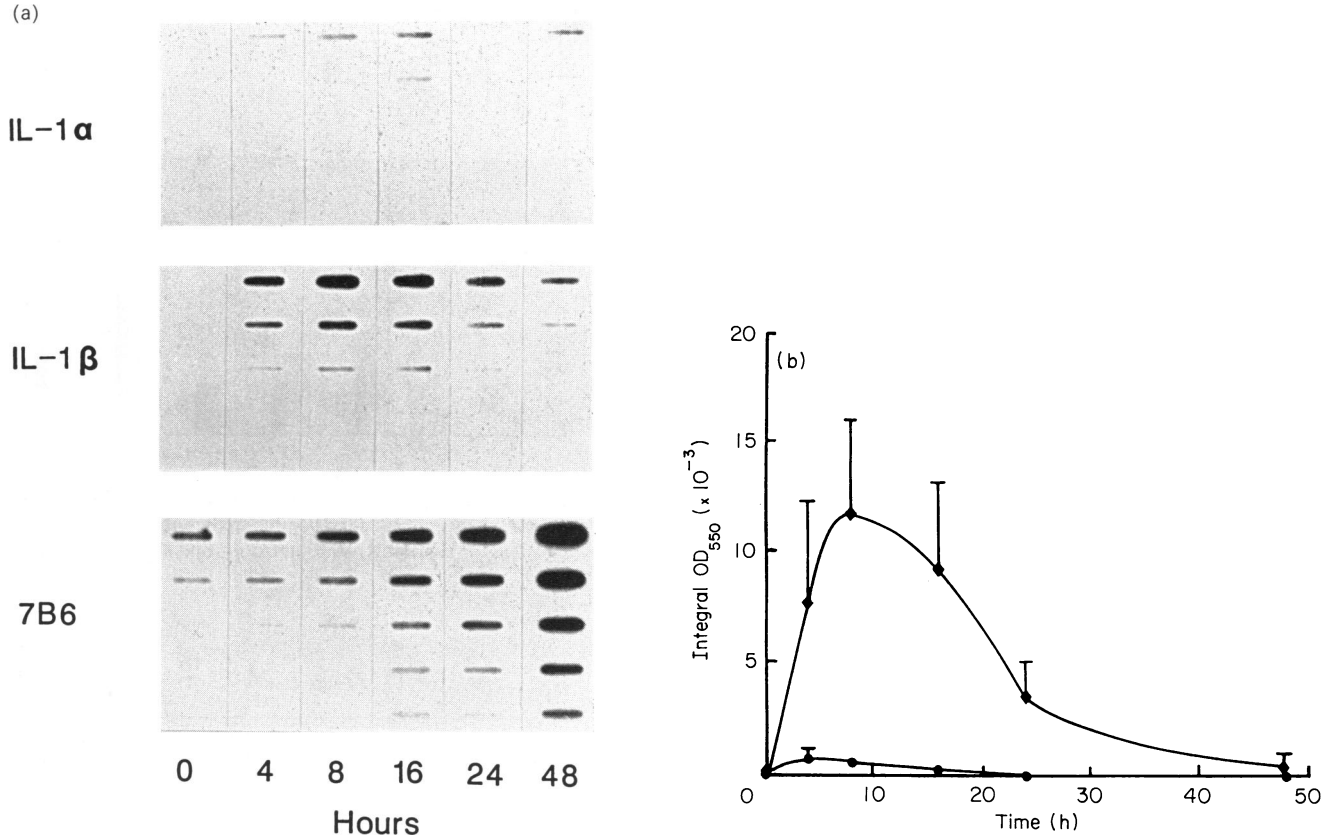

Fig. 1. Kinetics of IL-1α (●) and β (♦) production in PBM from healthy donors. (a) 2×10^6 cells per time point were stimulated with PHA/PDB, the RNA extracted, blotted onto nitrocellulose and probed with IL-1α, IL-1β and 7B6. (b) Autoradiographs from five subjects were scanned using a Joyce Loebl Chromoscan 3 densitometer. The integrals were adjusted to compensate for the increase in mRNA due to cell proliferation. Results are expressed as the integral OD_{550} ($\times 10^{-3}$) ± s.e.m.

presence of a suboptimal dose of phytohaemagglutinin (PHA) (2·5 μg/ml). Cells were cultured in 96-well flat-bottomed plates (1×10^6 thymocytes/well) in a total volume of 200 μl. The specificity of the bioassay was confirmed by preincubation of the supernatants with excess neutralizing polyclonal antisera specific for either IL-1α or IL-1β for 2 h prior to addition to the thymocyte bioassay. ^{3}H thymidine incorporation during the last 16 h of a 72-h incubation was measured. The results are expressed as the mean ct/min ± s.e.m.

Bioassay for tumour necrosis factor

The presence of TNF was measured on WEHI 164 clone 13 as described (Espevik & Nissen-Meyer, 1986). The amount of cytotoxin present was determined from the difference in titre obtained after treating the supernatants with a mixture of anti TNFα and TNFβ monoclonal antibodies.

RESULTS

IL-1 gene expression by normal PBM

Analysis of normal PBMs for expression of IL-1 by Northern blotting showed that the IL-1β cDNA probe bound to a single mRNA species of approximately 1·8 kb, while the IL-1α probe was specific for a mRNA of about 2·1 kb (data not shown). This is consistent with other reports (March *et al.*, 1985). Low levels of IL-1β and IL-1α mRNA were detected prior to stimulation, possibly due to some activation of the cells during separation. To establish the kinetics of IL-1 mRNA production, PBM from five healthy subjects were stimulated with PHA and PDB and the mRNA assayed by slot blotting (Fig. 1). Stimulation resulted in transient increases in the levels of both mRNAs, IL-1β mRNA being more abundant than IL-1α mRNA. Peak values for IL-1α mRNA were seen at an average of 4 h post-stimulation (Fig. 1b), while IL-1β mRNA peaked at an average of 8 h post-stimulation. The expression of IL-1α mRNA is more transient than that of beta, with the level of IL-1α mRNA reaching a nadir by 24 h and IL-1β by 48 h. No significant difference in IL-1α or β mRNA expression could be found between PBM derived from healthy individuals or rheumatoid patients (data not shown).

IL-1 gene expression by RA joint cells

SF and SM specimens from nine patients with acute RA were investigated for their content of IL-1α and β mRNA. A comparison of IL-1α and β mRNA of nine RA samples is shown in Fig. 2, which demonstrates that in all RA samples, IL-1 is detected. The ratio of IL-1α to β in the RA samples varied widely, but averaged 1:1 in the nine samples. In contrast, the ratio of IL-1α to β in activated PBM was 1:16 ($n = 5$). Similar results have been found with normal monocytes (March *et al.*, 1985). In only two patients did the α:β ratio approach the normal for PBM (SM4, SF10).

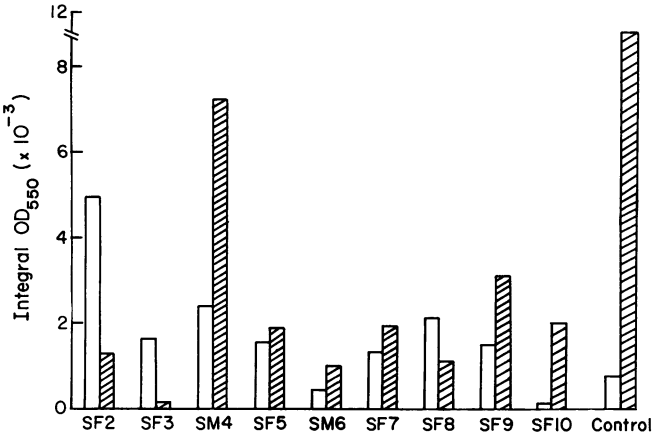

Fig. 2. Comparison of the levels of IL-1α (□) and IL-1β (▨) mRNA in freshly isolated SF and SM MNC from patients with RA. RNA was extracted, blotted onto nitrocellulose and probed with IL-1α and IL-1β. Mean maximum levels of mRNA in stimulated PBM from healthy donors (Fig. 1b) provided as control. Integral values were calculated as described.

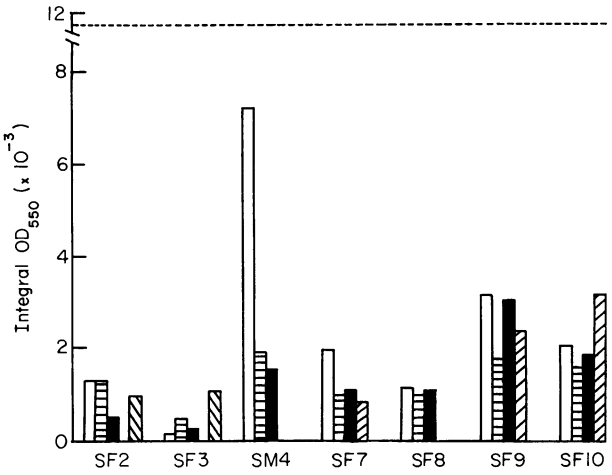

Fig. 4. The blots prepared for Fig. 3 were stripped and reprobed with an IL-1β specific probe. The control value represents the maximum stimulated value for IL-1β mRNA found in normal PBMs. Symbols as Fig. 3.

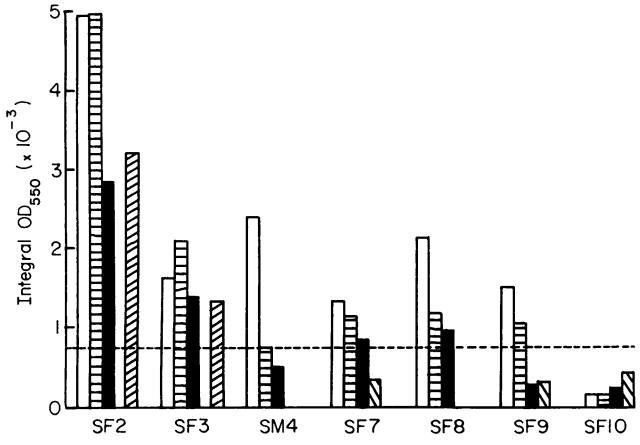

Fig. 3. Slot blot analysis of IL-1α production by RA synovial fluid MNCs cultured in the absence of extrinsic antigen. SF and SM MNC were cultured for 0–5 days, the RNA extracted, blotted onto nitrocellulose and probed with IL-1α. Integral values were calculated as described. The maximum levels of IL-1α mRNA found in PHA/PDB stimulated PBM is shown by the broken line. Unstimulated PBMs expressed negligible amounts of mRNA. SF = synovial fluid, SM = sinovial membrane. (□) day 0; (▤) day 1; (■) day 2; (▧) day 3; (▨) day 5.

Table 1

Sample	LT	TNF	IL-1α	IL-1β
Unstimulated PBMs	0	0	234	145
Peak stimulated PBMs (PHA/PBD)	908	6,831	753	38,188
SF2	0	11,527	4,949	1,284
SF7	7,311	0	1,132	1,939
SF8	6,528	1,973	2,136	1,117
SF9	1,737	81	1,516	3,124
SF10	1,549	265	155	2,032
Mean	4,281	3,461	1,978	1,899
s.e.m.	1,532	2,722	808	354

Comparison of TNFα and TNFβ, IL-1α and IL-1β mRNA expressed by freshly isolated SF MNC from patients with RA. RNA was isolated from 2×10^6 cells, blotted and probed with TNFα and TNFβ. Integral values were calculated as described. Values for IL-1 are as previously described and cannot be directly compared with TNF values.

Persistence of IL-1 mRNA in unstimulated RA joint cell cultures
The temporal pattern of IL-1α and β mRNA expression in RA synovial cell cultures, in the absence of stimulation was determined. The levels of IL-1α mRNA, initially high compared to peak values in PHA/PDB activated PBM, remained elevated for a prolonged period. IL-1α could still be detected after 48 h in cells from five of the seven patients studied while of the two patients studied for 5 days, both still showed high levels of IL-1α (Fig. 3). This contrasts markedly to the normal activated PBM, where IL-1α mRNA returns to baseline 24 hours after stimulation (Fig. 1). IL-1β mRNA, readily detectable in RA joint cells, but at a relatively low level compared to PHA/PDB activated PBM, also remained elevated for 5 days (Fig. 4).

TNF mRNA in Rheumatoid Joint MNCs
The blots of synovial cells were reprobed to look for TNF α and β mRNA, cytokines which can induce inflammation, joint damage and are inducers of the production of IL-1. Either TNFα or β were detected in all of the samples tested (Table 1). The levels of TNFβ were marginally higher than of TNFα. Compared to the levels of mRNA present in PBM activated by PHA and PDB, the levels of TNFβ in the joints were high, averaging approximately 4-fold higher than in PHA/PDB activated PBM. In contrast, TNFα levels were lower than in activated PBM.

Table 2

Sample	TNF u/ml	IL-1 Bioactivity (c.p.m. × 10·0 ± s.e.m.)
SF 12	446	8,989 ± 1,623
SF 13	90	18,342 ± 917
SF 15	250	7,913 ± 1,159
SF 16	24	25,663 ± 2,965
SF 18	169	12,393 ± 461
SF 22	0	12,732 ± 342
SF 29	0	3,252 ± 250

IL-1 and TNF bioactivity in supernatants from RA MNC cultures. Cells were cultured for 24 h as described. Supernatants were filtered and assayed for IL-1 bioactivity using the thymocyte comitogenic assay, the response of thymocytes to PHA alone was 1,818 c.p.m. ± 158. TNF was assayed using the WEHI assay.

Detection of IL-1 and TNF activity

Since IL-1 and TNF mRNA are not invariably translated into protein (Garrett *et al.*, 1987), the supernatants from 24-h cultures of rheumatoid cells were assayed for IL-1 and TNF activity. IL-1 activity was produced by all of the synovial MNC cultures as determined by the thymocyte co-stimulator assay (Table 2). TNF activity, which was completely inhibited by a mixture of anti TNFα and TNFβ antibody, was detected in five of seven samples. There appears to be no correlation between the levels of TNF and IL-1 protein in the same samples (Table 2). However, five of seven samples contained detectable amounts of both factors simultaneously. By using anti-sera specific for each species of IL-1, two of four patients were shown to have equal amounts of IL-1α and β activity and two to four to have more α than β (Table 3).

DISCUSSION

Despite the fact that RA is a systemic disease, the major pathology is in the joints. We have used a combination of mRNA analysis and bioassays rendered specific by neutralizing antibodies to assess the role of the cytokines, IL-1α and β and TNFα and β in the pathogenesis of RA.

Although IL-1 activity has been reported in synovial fluid of patients with RA (Fontana *et al.*, 1982), it was not known whether it was produced by cells in the affected joint. Our demonstration of the presence of IL-1α and β mRNA in mononuclear cells isolated from SF or SM in nine patients (Fig. 1) shows that IL-1 synthesis takes place within the diseased tissue. Detection of cytokine mRNA does not necessarily indicate release of protein from the cells, as intracytoplasmic and membrane forms have been documented (Kurt-Jones *et al.*, 1985; Garrett *et al.*, 1987). However, our findings at the protein level were consistent with the mRNA data (Table 3) indicating that biologically active protein is released from the cells.

IL-1α mRNA was expressed at high levels compared to PHA/PDB activated PBM (Fig. 2) and persisted *in vitro* in the absence of extrinsic stimulation. (Fig. 3), considerably longer than in activated PBM (Fig. 1).

The reasons for the relatively high and prolonged expression of IL-1α are currently being explored. Cellular composition of the three tissue types studied indicates that there is probably insufficient difference between them to explain the altered IL-1α expression. PBM consist of approximately 80% T and Natural Killer cells, 10% B cells and 10% monocytes while both synovial fluid and membrane have less T cells (approximately 40%), more monocytes and macrophages (30%), fewer B cells and in the membrane fibroblasts and endothelial cells (Brennan, F., unpublished data). Since RA SF and SM both produce IL-1, it is unlikely that the tissue cells (e.g. endothelium and fibroblasts) present in membrane but not fluid are the major producers of the IL-1 detected. It is not known at present which cells within RA joints are the major producers of IL-1, but it is of interest that while monocytes chiefly produce IL-1β, T and some B cells produce mostly IL-1α (Tartakovsky *et al.*, 1986, Acres *et al.*, 1987).

It is conceivable that a decreased rate of mRNA degradation could prolong the presence of IL-1α in the cytoplasm, however preliminary experiments suggest that the half-life of IL-1α mRNA in RA joints does not differ from that of PHA/PDB stimulated PBM (Barrett, K., unpublished data). Thus the most likely interpretation for the prolonged IL-1α mRNA production by synovial cells is continual stimulation of IL-1 producing cells by other cells and their products. Self-sustaining mutual cell interactions between T cells and antigen-presenting cells have also been documented in other autoimmune diseases, such as Graves' disease (Londei, Bottazzo & Feldmann, 1985). Since autoreactive T cells in RA joints have been found, recognizing HLA class II, so-called autologous mixed lymphocyte reaction cells (Schlesier *et al.*, 1984), and more recently clones recognizing collagen type II (Londei *et al.*, manuscript submitted), this type of interaction is likely to be of importance in RA.

In this context, it is noteworthy that TNF has been reported to be an inducer of IL-1 production by monocytes and endothelium (Dinarello *et al.*, 1986) and is itself induced by IL-1 (Philip & Epstein, 1986). TNFα and β mRNA was found in the same RA monuclear cell populations which produce IL-1, and cultures of these cells produced TNFα and β protein (Table 2) but there was no clear correlation between IL-1 and TNF levels. Other possible IL-1 inducers such as IFNγ, which synergizes with TNF to augment IL-1 production, have also been found in RA joints (Buchan *et al.*, 1987), and it is significant that T cells cloned from RA joints have recently been shown to produce IFNγ, TNFα and β (Turner, Londei & Feldmann, 1987). The role of other potential IL-1 inducers such as interleukin 4 or granulocyte macrophage colony stimulating factor in RA joints cannot be excluded, nor can the contribution of immune complexes, complement products, leukotrienes and prostaglandins.

Our findings suggest that there is *in vivo* production of IL-1 and TNF by cells within the diseased joint. As both IL-1 and TNF have been reported to induce bone resorption (Gowan *et al.*, 1983; Thomas, Mundy & Chambers, 1987), cartilage degradation (Saklatavala, Sarsfield & Townsend, 1985; Saklatavala, 1986) and collagenase (Dayer & Demczuk, 1984; Dayer, Beutler & Cerami, 1985) they may contribute to the immunopathology of RA. The possible involvement of cytokines in the destructive phase of RA may explain why non-steroidal anti-inflammatory drugs which inhibit prostaglandin production but are poor inhibitors of collagenase, do not appear to arrest

Table 3

Sample	Untreated	Treatment		
		anti-IL-1α	anti-IL-1β	anti-IL-1α and β
Control (PBMs)	104,539±8,304	91,432±4,018	12,983±936	4,902±183
IL-1α (10 μ)	68,402±5,091	4,510±206	71,903±5,062	6,901±205
IL-1β (10 μ)	73,694±4,306	71,011±5,328	5,319±263	7,852±191
SF 16	58,355+1,981	35,274±1,964	33,267±2,143	4,707±491
SF 30	15,421±832	6,959±740	10,013±817	3,110±401
SF 31	21,763±1,011	9,163±293	15,180±692	4,877±231
SF 32	12,985±1,232	6,894±704	6,698±137	3,334±197

The effect of neutralizing antisera specific for IL-1α and IL-1β on the IL-1 bioactivity of RA synovial cell supernatants and supernatants from stimulated PBM. Cells were cultured as described. The levels of IL-1α and IL-1β produced by PHA/PDB stimulated PBMs are shown for comparison. The specificity of the antisera is shown by its ability to inhibit the appropriate recombinant IL-1 species. The response of thymocytes to PHA alone was 3,009 c.p.m. ±151.

collagen degradation in the RA patient (Dayer & Demczuk, 1984). So far there is no clear evidence for any difference in the actions of IL-1α and β (Rupp *et al.*, 1986), which is compatible with binding of both molecules to the same receptor with similar affinities (Bird & Saklatavala, 1986). Thus the functional consequences of the abnormally high and prolonged expression of IL-1α in RA joints are not yet known.

The high and prolonged expression of molecules such as IL-1α, and to a lesser extent IL-1β, in cultures of unstimulated joint cells suggest that these cultures mimic in part the events that occur *in vivo* during a chronic disease process. We have previously shown that IL-2 mRNA is highly expressed and prolonged *in vitro* (Buchan *et al.*, 1987). Using this culture system it may be possible to determine which interacting cells are necessary for prolonged IL-1α production, for example by cell-depletion experiments, and which inducers are involved by using neutralizing antibodies. Furthermore, characterizing those cytokines expressed at the inflammatory site may provide clues about possible therapeutic strategies and disease specificity.

ACKNOWLEDGMENTS

We thank Dr S. Allard for assistance in providing clinical samples, Dr P. Lomedico for IL-1α and IL-1β cDNA, Dr H. M. Shepard for TNFα and β cDNA, Prof. U. Torelli for the 7B6 cDNA, Drs S. Gillis and C. Henney for anti IL-1 antisera, Dr G. Adolf for antibodies to TNFα and TNFβ, Jon Nissen-Meyer for the WEHI clone 13 and Dr Contreras and her colleagues at the National Blood Transfusion Centre, Edgware for buffy coat residues. This work was supported by the Arthritis and Rheumatism Council, ICI plc, the Medical Research Council and the Nuffield Foundation (Oliver Bird Grant).

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