Interindividual and intra-articular variation of proinflammatory cytokines in patients with rheumatoid arthritis: potential implications for treatment

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Abstract

***Objectives*—Assessment of the numbers and spatial distribution of cells producing interleukin 1α (IL1α), interleukin 1β (IL1β), tumour necrosis factor α (TNFα), and interleukin 6 (IL6) in the synovial membranes of patients with rheumatoid arthritis (RA).**

***Methods*—Synovial tissue specimens from 40 patients with RA and eight patients with non-rheumatic disease were obtained by arthroscopy guided biopsy techniques or during joint surgery. A modified immunohistochemical method detecting cytokine producing rather than cytokine binding cells was applied to determine cytokine synthesis in fixed cryopreserved sections. Computerised image analysis methods provided comparative quantitative assessments.**

***Results*—A wide variation between subjects was recorded for both quantities and profiles of expressed cytokines, despite similar macroscopic and histopathological features of inflammation. IL1α and IL1β were the most abundant monokines identified, though produced at different sites. IL1α was predominantly seen in vascular endothelial cells, whereas IL1β staining was mainly shown in macrophages and fibroblasts. Concordant results for the detection of TNFα at protein and mRNA levels were obtained with an unexpectedly low number of TNFα producing cells compared with IL1 expressing cells in many patients with RA. Specimens acquired arthroscopically from areas with maximum signs of macroscopic inflammation showed an increased number of TNFα producing cells in pannus tissue compared with that occurring in synovial villi of a given joint. This clustered distribution was not found for cells expressing any of the other studied cytokines.**

***Conclusion*—The recorded heterogeneous profile of proinflammatory cytokine synthesis in the synovial membrane among patients with RA may provide a clue for an understanding of the wide variation in responsiveness to different modes of antirheumatic treatment between patients.**

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Cytokines, locally produced in the synovial membrane of patients with rheumatoid arthritis (RA), play an important part in regulating the chronic joint inflammation which may lead to serious tissue damage, such as cartilage degradation, bone resorption, and fibrosis. An excessive extracellular presence of the proinflammatory cytokines interleukin 1 (IL1), tumour necrosis factor α (TNFα), and interleukin 6 (IL6), in particular, has been identified and intra-articular as well as systemic levels of these cytokines may to some extent reflect disease activity.[1–5] The paracrine or autocrine mode of action of most cytokines complicates the interpretation of studies of body fluids for reliable assessment of cytokine production. Furthermore, all assays designed to quantify extracellular levels of cytokines will measure the net outcome of secretion, absorption to numerous cellular and soluble cytokine receptors, and degradation by proteases present at inflammatory sites. Techniques based on immunohistochemistry have thus also been widely used to study the expression of cytokines within the synovial tissue in attempts to determine true local production.[6–16] However, most previous studies based on immunohistochemical staining of intra-articular cytokine production in RA have been performed on acetone fixed tissue, a procedure which does not readily allow subsequent discrimination of cytokine producing cells and cytokine binding cells. Antigens present on cell surface membranes and extracellularly in the tissue specimens are generally accurately shown after acetone fixation, while the intracellular morphology is poorly preserved.

A primary reason for performing the descriptive work outlined in this report was to improve quantitative immunohistochemical methods to measure numbers of cytokine producing cells in rheumatoid synovitis. The generation of such technology could provide useful tools to monitor sequential changes in relation to therapeutic intervention. A modified immunohistochemical method, based on commercially available cytokine-specific monoclonal antibodies, has been applied to identify cytokine producing cells rather than cytokine binding cells.[17–19] The use of carefully selected neutralising detecting antibodies against the studied cytokines prevented recognition of surface stained target cells that had bound secreted cytokines by cytokine receptors. A computer aided image analysis method,

also developed by us,[20 21] has been used to quantify the production of IL1α, IL1β, TNFα, and IL6 in rheumatoid synovial membranes. Biopsy specimens taken at different stages of RA have been sampled to enable a comparison of results scored by this technique with those of previously published findings in early and late RA. Synovial tissue acquired during orthopaedic surgery or biopsy specimens obtained at arthroscopy from different parts of a given joint have been used to identify sites of maximum cytokine synthesis.

Patients and methods

PATIENT GROUP

Forty patients with RA, as defined by the American College of Rheumatology criteria,[22] were recruited from outpatient clinics from the rheumatology and orthopaedic services of the Karolinska Hospital, Stockholm, University Hospital, Uppsala and the University Hospital, Malmö. Biopsy specimens were obtained by arthroscopy guided techniques in 20 patients with RA and during orthopaedic surgery in the other 20 patients. All 20 patients studied by arthroscopy had active arthritis (joint pain, swelling, and decreased range of motion) at the time of tissue sampling; 15 of the arthroscopy patients had disease duration <18 months and were described as having early RA. The 20 patients treated with arthroplastic or arthrodesis surgery had end stage destructive arthritis after advanced disease duration. Eight patients without inflammatory joint disease who underwent arthrodesis surgery because of hallux valgus displacement served as controls. Table 1 presents characteristics of the studied patients. All patients gave their informed consent and approval was granted by the ethical committees at the University of Malmö, University of Uppsala, and at the Karolinska Institute, Stockholm.

Table 1 Patient characteristics

Patient number	Sex	Age	Site*	Duration of RA	Treatment*
A Patients with RA providing synovial biopsy specimens at arthroscopy					
RA-1	F	55	Elbow	8 mo	NSAID
RA-2	M	30	Knee	9 mo	NSAID
RA-3	M	25	Knee	4 mo	NSAID
RA-4	F	76	Knee	8 mo	NSAID, AUR
RA-5	F	56	Knee	9 mo	NSAID
RA-6	F	44	Knee	5 mo	NSAID
RA-7	F	46	Knee	8 mo	NSAID
RA-8	M	22	Knee	1 mo	NSAID
RA-9	M	55	Knee	4 mo	NSAID
RA-10	M	80	Knee	1 mo	NSAID, SSA
RA-11	F	54	Knee	2 mo	NSAID
RA-12	F	42	Knee	1 y	AUR
RA-13	F	47	Knee	5 mo	NSAID
RA-14	F	47	Knee	1.5 y	NSAID, SSA
RA-15	F	52	Knee	1.5 y	Pred
RA-16	F	48	Knee	27 y	NSAID, Pred
RA-17	F	34	Knee	8 y	MTX
RA-18	F	45	Knee	6 y	MTX
RA-19	F	27	Knee	6 y	MTX
RA-20	M	48	Knee	29 y	SSA
B Patients with RA providing synovial specimens at surgery					
RA-21	M	66	MTP1	46 y	NSAID, CsA
RA-22	F	69	MTP1	10 y	MTX
RA-23	F	43	MTP1	8 y	None
RA-24	F	71	MTP1	28 y	NSAID, Pred, MTX
RA-25	F	58	MTP1	37 y	NSAID
RA-26	F	51	MTP1	13 y	NSAID, Pred, MTX
RA-27	F	62	MTP1	12 y	NSAID, AMA
RA-28	F	79	MTP1	17 y	NSAID, Pred
RA-29	F	69	MTP1	37 y	NSAID,
RA-30	F	42	MTP1	12 y	CsA, Pred
RA-31	F	72	Knee	32 y	NSAID, Pred
RA-32	F	37	MTP1	15 y	NSAID
RA-33	F	45	Hip	11 y	NSAID
RA-34	F	84	Hip	22 y	NSAID, Pred
RA-35	F	54	Hip	26 y	NSAID
RA-36	F	66	Hip	17 y	None
RA-37	F	86	Knee	30 y	None
RA-38	F	57	Knee	23 y	NSAID
RA-39	F	57	Knee	23 y	NSAID
RA-40	F	49	Hip	23 y	NSAID
C Controls with hallux valgus					
HV-41	M	77	MTP1		None
HV-42	F	55	MTP1		None
HV-43	M	82	MTP1		None
HV-44	F	54	MTP1		None
HV-45	F	49	MTP1		None
HV-46	F	22	MTP1		None
HV-47	F	76	MTP1		None
HV-48	F	53	MTP1		None

NSAID = non-steroidal anti-inflammatory drug; AUR = auranofin; SSA = sulfasalazine; Pred = prednisone 5 mg; MTX = methotrexate; CsA = cyclosporin A; AMA = antimalarial drug; MTP1 - first metatarsophalangeal joint.

TISSUE PREPARATION AND IMMUNOHISTOCHEMICAL STAINING

All biopsy specimens obtained at arthroscopy from 19 knee joints and one elbow joint were sampled from synovial tissue areas that showed maximum macroscopic signs of inflammation, scored as previously described.[23] Whenever possible, specimens were taken both from synovitis occurring in close proximity to the articular cartilage and from synovial villous formations distant from the cartilage. Pannus tissue and articular cartilage specimens were also acquired at surgical resection of synovial tissue at arthroplastic correction of hip or knee joints or at arthrodesis immobilisation of first metatarsophalangeal joints. One biopsy specimen from each patient was analysed, and when both villous and pannus were taken two specimens were analysed. Specimens obtained from arthroscopic and from orthopaedic surgery were snap frozen in liquid isopentane and embedded in OCT compound (Tissue-Tek, Mites Elkhart, IN). All tissues were kept at −70°C until sectioned. Cryostat sections (6–8 μm, cryostat setting 7 μm) were mounted on gelatin coated glass slides (Novakemi, Stockholm, Sweden) and left to air dry for up to 30 minutes. Sections to be stained were initially fixed for 20 minutes with 2% (v/v) formaldehyde (Sigma Chemicals, St Louis, MO) dissolved in phosphate buffered saline pH 7.4 at 4°C and were then left to dry before storage at −70°C.

We have previously shown that formaldehyde fixed sections, stained in the presence of saponin as a detergent to permeabilise cell membranes, preserve the morphology of intracellularly accumulated cytokines in a unique way.[17–20] The use of neutralising cytokine-specific monoclonal antibodies (mAbs) as detecting reagents is of key importance for the assay. Neutralising antibodies recognise epitopes of cytokine molecules that will be unavailable for recognition when bound to cytokine-specific receptors of target cells. Our immunostaining will thus avoid a recognition of cytokine binding cells, but will readily

demonstrate intracellular and cell surface expression of cytokines in producer cells. The staining procedures have been published previously.[19]

Phenotypic characterisation of cells present in the synovial tissue specimens was performed on separate frozen non-formaldehyde treated slides that were fixed in acetone immediately after removal from storage at −70°C in accordance with a previous report.[24]

ANTIBODIES

The following cytokine-specific mAbs were used for immunostaining: a mixture of three anti-IL1α mAbs (all mouse IgG1) (1277–89–7, 1277–82–29, 1279–143–4 from Immunokontakt, Bioggo, Switzerland) detecting different IL1α epitopes were combined to analyse IL1α; two anti-IL1β mAbs (mouse IgG1) were combined (2-D-8 and 1437–96–15 from Immunokontakt, Bioggo, Switzerland); TNFα was detected by pooling two anti-TNFα mAbs (both mouse IgG1, mAb 1 and mAb 11 from PharMingen, San Diego, CA); IL6 staining was performed using one anti-IL6 mAb (rat IgG2a, MQ2–6A3 from PharMingen, San Diego, CA), anti-CD3 mAb (mouse IgG1 from Becton-Dickinson, Mountain View, CA), and anti-CD68 (mouse IgG1, Dakopatts, Glostrup, Denmark).

CONFIRMATION OF CYTOKINE IMMUNOREACTIVITY

Recombinant cytokines were used to block cytokine staining in order to test the specificity. The appropriate cytokine was added in excess at a concentration of 20–50 μg/ml to its corresponding cytokine-specific antibody (2–5 μg/ml) at 4°C overnight. Staining with the complex was performed as previously described and compared with results obtained by combining anticytokine mAbs preincubated with other cytokines as a control. The following cytokines were used in these specificity tests: recombinant IL1α, IL1β, IL6 (Genzyme Corp, Boston, MA), and rTNFα (Bayer Inc, Hannover, Germany).

MICROSCOPIC ANALYSIS AND COMPUTER AIDED IMAGE ANALYSIS

Cytokine stainings and cellular phenotypic characterisation were examined with a Polyvar II microscope (Reichert-Jung, Vienna, Austria). Tissue sections acquired by arthroscopy could be microscopically evaluated using computerised image analysis methods as previously described.[25] A reading of an entire tissue section typically required 50–200 microscope fields using a magnification power of ×250. Tissue sections that were sampled during orthopaedic surgery contained pannus tissue, cartilage, and bone. However, the design of our computer program did not manage quantitative measurements in sections including bone or cartilage tissue, and we thus quantified the results of these specimens by conventional microscopy. The following scores were used: + = 0–10 positive cells; ++ = 11–100 positive cells, and +++ = >100 positive cells in the entire section specimen.

IN SITU HYBRIDISATION

The in situ hybridisation experiments to demonstrate cells expressing TNFα mRNA were performed according to a previous report by us.[26] A mixture of four different oligonucleotide probes was pooled to increase the sensitivity of the detection method. The oligonucleotide sequences were obtained from GenBanks, and TNFα probes of 48 base pairs were designed using Mac Vector software (IBI). Oligonucleotide probes for TNFα were labelled with deoxyadenosine-5'-α-thiotriphosphate ([^{35}S]ATP; New England Nuclear, USA) and terminal deoxynucleotidyl transferase (Amersham, Little Chalfont, UK). Cells expressing at least seven clustered grains were coded positive.

Positive and negative control slides were also generated using fixed human mononuclear peripheral blood cells that had been cultured for one to three hours with 1 μg/ml of lipopolysaccharide (LPS from *E coli* serotype 0128:B12, Sigma Chemical Co, St Louis, MO) or without any exogenous stimulus. Mononuclear cells from five different healthy blood donors were cultured and subsequently stained in parallel for the presence of intracellular TNFα protein and TNFα mRNA. Staining experiments with the mononuclear cells were performed at the same time as the experiments with rheumatoid synovial tissue specimens.

STATISTICAL ANALYSIS

The data were analysed with the Statview SE statistical software. Comparisons of the expression of individual cytokines in paired pannus and villous tissue of a given joint were made using Wilcoxon sign rank test for matched pairs. Comparisons of the production of individual cytokines in arthroscopically obtained biopsy specimens from patients with early RA (disease duration up to 18 months) compared with late disease (duration 6–29 years) were made using the Mann-Whitney two sample test.

Results

CYTOKINE PRODUCTION IN PATIENTS WITH EARLY RA

Immunohistochemical cytokine staining was performed in biopsy specimens acquired by arthroscopy in 15 patients with early RA disease. These specimens were sampled in all patients from synovial villous formations distant from the intra-articular cartilage, and all biopsy tissue expressed maximum macroscopic inflammatory signs (table 2). Arthroscopic inspection showed synovitis proliferating directly on the surface of the intra-articular cartilage in nine out of the 15 studied patients, and biopsy specimens were in addition sampled from these areas (table 2). A distinct intracellular staining signal in individual cells expressing positive immunoreactivity with the different cytokine-specific antibodies was evident in the studied samples (fig 1). Furthermore, an extracellular immunoreactivity of IL1α, IL1β, and TNFα, but not IL6, could often be distinctly seen around producer cells expressing strong intracellular immunostaining (fig 1C). This extracellular staining presumably represented

Table 2 Quantification of cytokine production in biopsy specimens obtained arthroscopically from synovial villi of patients with rheumatoid arthritis with a disease duration of up to 18 months

Patient number	Biopsy site	IL1α*	IL1β*	IL6*	TNFα*	Control	CD3	CD68
RA-1	Villous	4.4	<0.2	6.1	<0.2	0.3	19.4	ND
RA-2	Villous	46.4	20.8	7.8	17.4	0.7	20.7	15.4
RA-3	Villous	31.8	<0.2	0.2	0.3	0.6	3.1	6.8
RA-4	Villous	4.8	8.9	<0.2	<0.2	0.5	6.9	17.0
RA-5	Villous	5.6	11.8	<0.2	<0.2	0.7	8.8	12.7
RA-6	Villous	<0.2	<0.2	<0.2	<0.2	0.9	3.2	11.1
RA-7	Villous	6.2	<0.2	<0.2	<0.2	0.2	12.7	8.5
RA-7	CPJ*	3.6	58.6	<0.2	11.2	0.6	2.7	4.8
RA-8	Villous	1.3	31.0	<0.2	7.2	0.2	6.6	11.3
RA-8	CPJ	2.7	21.0	2.0	10.6	1.5	5.9	13.4
RA-9	Villous	5.3	10.5	1.1	1.8	0.2	5.3	22.6
RA-9	CPJ	3.2	8.0	0.2	0.9	<0.2	1.5	22.5
RA-10	Villous	11.2	20.5	0.6	0.2	0.2	16.8	22.2
RA-10	CPJ	8.8	10.7	0.2	0.2	0.2	6.8	16.1
RA-11	Villous	4.5	<0.2	<0.2	<0.2	<0.2	0.5	6.9
RA-11	CPJ	25.8	3.1	4.6	8.3	0.6	1.4	13.0
RA-12	Villous	15.5	0.5	<0.2	0.7	<0.2	3.5	18.1
RA-12	CPJ	33.1	2.6	4.6	5.7	1.3	1.7	22.1
RA-13	Villous	18.9	1.2	<0.2	0.7	<0.2	2.0	29.8
RA-13	CPJ	12.7	1.4	<0.2	0.7	<0.2	3.3	27.5
RA-14	Villous	0.7	12.2	0.7	0.4	0.2	ND	ND
RA-14	CPJ	1.4	21.2	6.3	11.7	1.8	4.1	12.4
RA-15	Villous	15.8	1.8	<0.2	8.5	<0.2	5.3	31.9
RA-15	CPJ	42.2	7.8	<0.2	11.4	<0.2	5.0	29.3

*IL1α = interleukin 1α; IL1β = interleukin 1β; IL6 = interleukin 6; TNFα = tumour necrosis factor α; CPJ = cartilage-pannus junction.
Cytokine data represent the area occupied by the staining for each cytokine expressed as percentage of the total section area. TNFα was significantly more expressed in pannus tissue than in villous formation of a given joint as judged by the Wilcoxon sign rank test for matched pairs ($p=0.02$). No such corresponding differences could be shown for IL1α, IL1β, or IL6 ($p>0.05$). The size of each tissue section ranged from 2 to 15 mm^2.

secreted cytokines bound to the matrix tissue, as the immunoreactivity could be totally abolished by preincubation of the cytokine detecting mAbs with the relevant corresponding cytokine before staining.

A pronounced variation in quantitative and qualitative cytokine patterns in the synovial specimens was found between individual patients with RA, which was the most striking result of these studies (table 2). IL1α and IL1β were the most abundantly and consistently expressed of the four studied cytokines. IL1α was detected in 14 of the 15 studied joints and the area stained ranged from 0.7 to 46.4% of the total studied tissue. IL1α was predominantly seen in vascular endothelial cells and only to a minor extent in large irregularly shaped cells of macrophage morphology (fig 1B). IL1β synthesis could be shown in 12 out of 15 patients (range in tissue expression 1.4–58.6%). This cytokine mainly appeared in macrophages but also in fibroblast-like cells present at deeper parts of the synovial membrane (fig 1A). In contrast with IL1α, IL1β was never detected in vascular endothelial cells. Macrophages that stained positive for IL1β were often surrounded by extracellular immunoreactivity, while there was a paucity of such extracellular staining around the IL1β expressing cells of fibroblast appearance.

TNFα synthesis could only be shown in the synovial tissue from nine of the 15 studied patients and the detected areas with immunoreactivity (0.3–17.4%) were considerably smaller than those for IL1. The TNFα staining included an intracellular staining in scattered

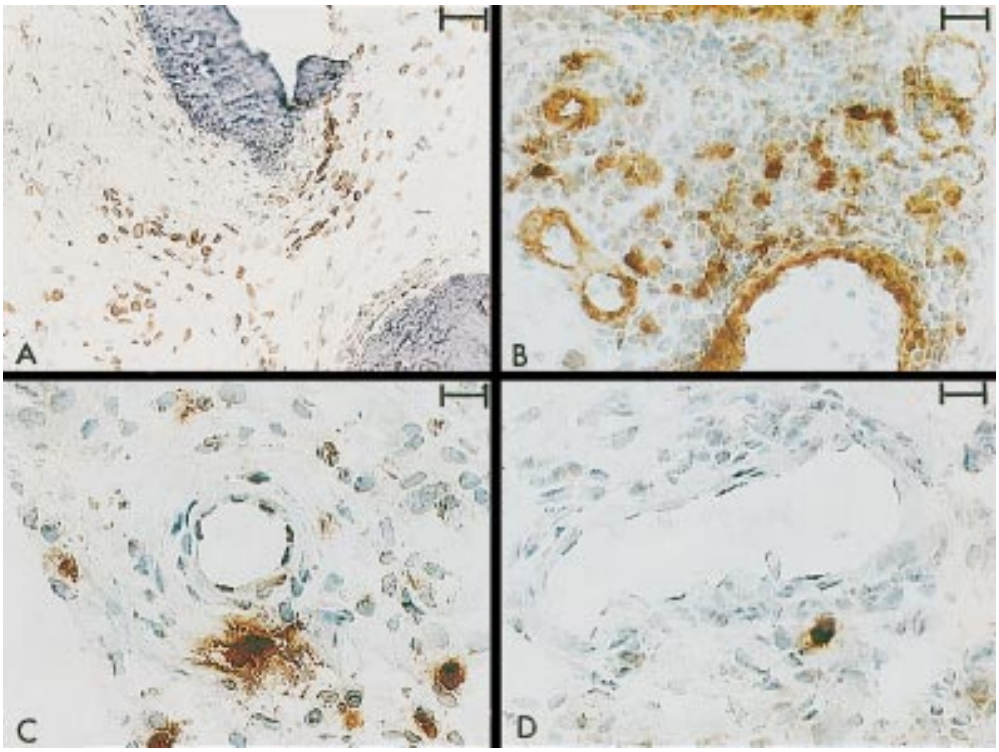

Figure 1 Videoprint photographs illustrating brown (diaminobenzidine) immunoperoxidase staining for cytokine producing cells in cryopreserved synovial membrane biopsy specimens obtained from patients with rheumatoid arthritis (RA). The cells were counterstained with haematoxylin. (A) Interleukin 1β producing cells in pannus tissue penetrating bone (dark blue staining) (original magnification ×200, bar represents 40 μm). (B) Interleukin 1α production occurring in both vascular endothelial cells and individual macrophages (original magnification ×320, bar represents 32 μm). (C) Tumour necrosis factor α (TNFα) producing cells in the sublining layer with additional extracellular TNFα staining encompassing producer cells (original magnification ×400, bar represents 20 μm). (D) Interleukin 6 production was seen in scattered cells with minimum additional extracellular staining (original magnification ×400, bar represents 20 μm).

Table 3 Comparison of cytokine production in biopsy specimens obtained arthroscopically from synovitis in pannus tissue versus distal villous formation of the same joint. Five patients with rheumatoid arthritis with a disease duration of 6–29 years were studied

Patient number	*Biopsy site*	*IL1α**	*IL1β**	*IL6**	*TNFα**	*Control*	*CD3*	*CD68*
RA-16	Villous	<0.2	<0.2	<0.2	<0.2	<0.2	ND	ND
RA-16	CPJ*	<0.2	<0.2	<0.2	<0.2	<0.2	ND	ND
RA-17	Villous	4.1	1.6	<0.2	<0.2	<0.2	13.0	10.7
RA-17	CPJ	6.7	0.8	<0.2	<0.2	<0.2	6.6	6.7
RA-18	Villous	6.4	0.8	<0.2	<0.2	1.1	2.0	1.4
RA-18	CPJ	0.7	1.2	<0.2	<0.2	<0.2	0.7	0.9
RA-19	Villous	3.4	<0.2	<0.2	<0.2	<0.2	8.2	15.4
RA-19	CPJ	0.1	<0.2	0.3	<0.2	<0.2	5.3	12.3
RA-20	Villous	2.1	13.6	<0.2	7.6	<0.2	12.2	28.6
RA-20	CPJ	1.3	9.2	<0.2	0.9	<0.2	1.2	16.3

*IL1α = interleukin 1α; IL1β = interleukin 1β; IL6 = interleukin 6; TNFα = tumour necrosis factor α; CPJ = cartilage-pannus junction.
Cytokine data represent the area occupied by the staining for each cytokine expressed as percentage of the total section area. The results do not indicate any statistically verified differences concerning the expression of the studied cytokines in pannus tissue versus villous formation in individual joints. The size of each tissue section ranged from 2 to 12 mm^2.

macrophages as well as an extracellular immunoreactivity encompassing these cells (fig 1C). TNFα production was increased locally in the pannus tissue specimens compared with paired villous formations in six of the nine studied joints, which represents a statistically verified difference (p=0.02 with Wilcoxon rank test). No such consistent spatial differences could be identified for IL1α, IL1β, or IL6 syntheses. IL6 staining gave a strong signal and was mainly evident intracellularly in macrophage-like cells (fig 1D) and could be shown in seven of the 15 patients with a range of 0.6–7.8% of the total area.

The frequency of infiltrating T cells and CD68 positive cells was recorded (table 2).There was no statistical correlation between these numbers and the incidence of cells producing the studied cytokines.

CYTOKINE FORMATION IN PATIENTS WITH LATE RA

Similar studies to those described in the previous paragraph were also conducted by arthroscopy guided biopsy sampling from pannus and villous tissue in active knee joint synovitis in five patients with RA with a much longer disease duration. The production of each of the studied four cytokines was significantly reduced (p<0.05 with Wilcoxon rank test, no significant differences with sign rank test) in this group of patients with late RA (table 3) compared with the patients with early RA (table 2). No consistent differences for the local production of any of the studied cytokines in the pannus versus the villous tissue could be shown in these samples from patients with late RA.

CYTOKINE FORMATION AT LATE STAGES OF RA AND IN CONTROL PATIENTS

Synovial tissue was sampled from destructive end stage disease in 20 patients with RA undergoing arthroplastic hip or knee joint replacement surgery or arthrodesis surgery of first metatarsophalangeal joints. The tissue specimens thus enabled a study of variations between subjects and profiles of cytokine expression during late RA disease. These studies also made it possible to compare results achieved with the presently employed methodology with those of previous immunhistochemical reports, generally based on staining of synovial tissue obtained during orthopaedic surgery. The synovial tissue was obtained during surgery in patients with late RA and included large acellular areas of cartilage and bone, which prevented scoring of the results by the computer assisted image analysis system owing to the design of the software program. Quantitative data from this part of the study were instead based on an enumeration of individual, immunostained cells or blood vessels using conventional microscopy (table 4). This approach provided readily interpretable data, but did not allow a direct quantitative comparison with results obtained in the first part of this study based on computerised image analysis.

IL1β was most abundantly expressed of the four studied proinflammatory monokines and could be detected in the synovitis of 17/19 patients with RA (table 4). Cells appearing with macrophage as well as fibroblast morphology were positively stained for IL1β (fig 1A) and the frequency of these cells represented

Table 4 Cytokine production in biopsy specimens from synovial tissue obtained at orthopaedic surgery from patients with rheumatoid arthritis (RA21-40) with late disease (a mean duration of 22 years) or from control patients (HV41-48) without rheumatic disease treated for hallux valgus displacement

Patient number	*IL1α**	*IL1β**	*IL6**	*TNFα**	*Control*	*Patient number*	*IL1α*	*IL1β*	*IL6*	*TNFα*	*Control*
RA-21	+	+++	0	0	0	RA-35	0	+++	0	0	0
RA-22	+	+++	0	+	0	RA-36	0	0	+	++	0
RA-23	+	+++	0	0	0	RA-37	0	0	++	++	0
RA-24	+	++	+	0	0	RA-38	+	+++	++	+	0
RA-25	0	++	0	0	0	RA-39	0	++	0	0	0
RA-26	+	++	0	0	0	RA-40	+	++	0	++	0
RA-27	++	+++	++	0	0	HV-41	+	+	+	+	0
RA-28	++	+++	+	+	0	HV-42	0	0	0	0	0
RA-29	++	+++	+	+	0	HV-43	0	0	0	0	0
RA-30	++	+++	+	+	0	HV-44	0	0	0	0	0
RA-31	++	+++	0	0	0	HV-45	0	0	0	0	0
RA-32	+	+++	++	0	0	HV-46	0	0	0	0	0
RA-33	ND*	ND	0	++	0	HV-47	0	0	0	0	0
RA-34	0	++	0	+	0	HV-48	0	+	0	0	0

*IL1α = interleukin 1α; IL1β = interleukin 1β; IL6 = interleukin 6; TNFα = tumour necrosis factor α; ND = not done.
Consecutive tissue sections from synovial tissue samples from orthopaedic surgery were immunohistochemically stained for cytokines and scored. IL1β, IL6, and TNFα expressing cells were quantified: + = 1–10 positive cells per section; ++ = 11–100 positive cells per section; +++ represents >100 positive cells per section. IL1α, a cytokine mainly expressed in the synovial tissue in vascular endothelial cells, was scored in a different way: + = expressed in vascular endothelial cells in large vessels; ++ = in small and large blood vessels; +++ = in small and large blood vessels plus macrophages. The size of each tissue section ranged from 20 to 90 mm^2.

several per cent of the nucleated cells in many specimens. A low number of IL1β producing cells was also found in the synovial membrane of 2/8 control patients. The control cohort included patients surgically treated for hallux valgus displacement of non-rheumatoid origin. The control patients (Nos HV-41 and HV-48) with positive synovial IL1β staining had both developed a mechanically induced moderate synovitis in their treated metatarsophalangeal joints, in contrast with the other six studied control patients. IL1α, mainly expressed in vascular endothelial cells of large blood vessels, could be identified in the synovitis of 13/19 patients with RA and in one control patient. The vascularisation of the rheumatoid synovial tissue obtained at end stage disease was less pronounced than in biopsy specimens selected from active synovitis in early RA. This difference in the organisation of the synovial tissue in early compared with advanced stages is a likely explanation for the identified reduced IL1α expression during late disease.

COMPARATIVE STUDIES OF TNFα PRODUCTION USING IMMUNOSTAINING AND IN SITU HYBRIDISATION

IL6 and TNFα producing cells could only be detected in the synovial specimens from 9/20 and 10/20 patients with RA, respectively. In addition, the frequency of these cells was very low, well below 1% of the total cells in the synovial specimens. The unexpected paucity of cells expressing TNFα protein was further examined using in situ hybridisation on consecutive tissue section specimens to quantify the number of synovial cells expressing TNFα transcripts. Synovial tissue from nine patients with RA was studied in three separate experiments for the presence of cells expressing TNFα mRNA, and the results confirmed those obtained with immunohistochemical TNFα protein staining (data not included). The frequency of cells expressing TNFα mRNA in the synovial tissue specimens was well below 1% of the total synovial cell population in all experiments. The sensitivity of the TNFα in situ hybridisation method and the immunostaining of TNFα protein was concomitantly examined using harvested, fixed human blood mononuclear cells that had been cocultured with *E coli* derived LPS for one to three hours. Experiments performed with cells from five different normal blood donors indicated an excellent agreement of the results scored with the two methods in the number of cells that expressed TNFα mRNA (a mean of 54% and a range of 42–57% of the monocyte population) and TNFα protein (a mean of 46% and a range of 34–55% of the monocyte population).

Discussion

We have studied the formation of four well known proinflammatory cytokines in the synovial tissues of patients with early and late stage RA using a modified immunohistochemical method developed at our laboratory.[17 19 27 28] The most important and, in part, new findings we believe to have come out of these studies are identification of (*a*) a pronounced interindividual diversity of patterns of cytokine production in synovial biopsy specimens displaying similar histopathological features; (*b*) a quantitative dominance of synovial cells producing IL1; and (*c*) an unexpected paucity of cells synthesising TNFα and IL6.

A striking heterogeneity in the qualitative and the quantitative pattern of the identified synovial production of these studied cytokines was seen in both early and late stages of RA (tables 2–4). This information is not readily disclosed in many published reports, which present means and standard errors of pooled patient data. However, the results concord with those obtained when synovial membrane cytokine transcription was semiquantified using polymerase chain reaction (PCR) and liquid phase hybridisation[29] or with quantitative reverse transcription PCR,[30] as well as with one previous study by us on a small group of patients with early RA.[19]

The design of this study does not allow any firm conclusions to be drawn about the isolated influence of disease duration on the recorded quantitative aspects of synovial cytokine synthesis. The reason for this is that all the 15 patients with early RA had active continuing arthritis and were selectively biopsied by arthroscopy at sites expressing maximum macroscopic inflammation, whereas the 20 patients with late RA all had end stage disease and the biopsy specimens obtained at orthopaedic surgery did not necessarily express signs of maximum inflammation. Data deduced from table 3, based on arthroscopically obtained samples from five patients with active arthritis during late RA and compared with a corresponding group of 15 patients with early RA (table 2), may suggest a reduced cytokine expression as a consequence of disease duration. However, the small number of patients with late RA enrolled in this cohort and the fact that these patients received a more potent pharmacological treatment than the group with early arthritis (table 1) hampers any straightforward conclusions about a causal relation between disease duration and quantitative cytokine formation. Nevertheless, an obvious diversity in the qualitative cytokine patterns, despite comparable synovial immunohistology within each of these three studied groups of patients, remains a common denominator. This was an unexpected finding, particularly in the group of 15 patients with early RA (table 2), bearing in mind that their biopsy specimens had been sampled from areas with uniform signs of maximum macroscopic inflammation. A better understanding of the observed variation of cytokine expression among patients with RA may, possibly, provide a tool to predict individual responsiveness to different modes of treatment.

The quantitative dominance of cells producing IL1 compared with cells making TNFα or IL6 in many, but not all, studied RA synovial biopsy specimens, was the second major finding of this study (tables 2–4). Although it is a well established fact that IL1 and TNFα are considered master cytokines in the pathogenetic process of RA synovitis, we were surprised by the abundant expression of IL1α and

IL1β compared with that of TNFα. This increase was seen both in the number of patients with RA identified as expressing each cytokine (IL1α 31/39 patients, IL1β 32/39, and TNFα 21/40 patients), and even more in the strongly enhanced frequency of cells producing IL1 within a given synovial biopsy specimen.

The production of IL1α will be grossly underestimated by any assay relying on assessments of its extracellular presence as IL1α is a poorly secreted protein.[31] The literature on IL1α synthesis in RA synovitis is thus confusing, and reliable reports are preferably based on intracellular detection.[7 15 32] In agreement with studies based on immunohistochemistry by Deleuran and coworkers,[14] we observed IL1α to be extensively expressed in vascular endothelial cells in both small and large vessels (fig 1B). This localisation might possibly enable IL1α to signal and functionally affect immunocompetent cells passing from blood vessels into the rheumatoid synovitis. However, a strong IL1α expression in blood vessels does not represent an RA-specific finding as we have found the same vascular staining pattern for IL1α in other chronic inflammatory disorders.[33] In addition to the dominant localisation of IL1α in vascular endothelial cells in RA synovitis, we also noted an IL1α expression in a minor fraction of synovial cells with macrophage morphology in certain specimens (fig 1B). These findings differ from previous studies which reported much higher frequencies of synovial cells simultaneously expressing IL1α.[15 30] These discrepancies might be explained by the fact that all previous immunohistochemical studies have been conducted using acetone fixed material, which will not enable discrimination between cytokine binding and cytokine producing cells.

IL1β, together with IL1α, was the quantitatively dominating identified cytokine detected in cells with macrophage and fibroblast morphology, but never in vascular endothelial cells. It was a consistently observed phenomenon that the extracellular immunoreactivity of IL1β was much more intense and extended around IL1β producing macrophages than around IL1β positive fibroblasts. Whether these findings reflect a decreased capacity for IL1β release in the fibroblasts compared with the macrophages needs to be addressed in separate studies. IL1β was strongly expressed at the cartilage-pannus junction in many specimens as well as in villous synovitis occurring at a great distance from the intra-articular cartilage. These findings of a strongly increased IL1β formation, in general, in the rheumatoid synovitis are in good agreement with most,[34–36] but not all,[13] previous immunohistochemical studies. It has recently been shown that functional IL1 blockade by subcutaneously administered IL1 receptor antagonist (IL1RA) offers a new efficient mode of treating RA, including a retardation of the rate of development of joint erosions.[37] Large doses given daily are needed to obtain these successful results. We believe that the extensive production of IL1α and IL1β in the rheumatoid synovitis, identified in this and several other studies, in part explains why successful IL1RA treatment demands both frequent and high dosage.

Our studies showed an unexpected paucity of TNFα formation in the RA synovial membrane (tables 2–4) compared with several previous studies using an immunohistochemical technique,[13 38] though they confirmed results of two recent studies using mRNA detection methods[30 38] as well as our previous pilot study.[19] Data from the second part of this study based on surgically obtained synovial samples at end stage RA and evaluated by conventional microscopy can be readily compared with previously published results by other groups owing to comparable study designs. We only recorded cells expressing TNFα in 10/20 studied patients with RA and the frequency of these cells was low, well below 1% of the total cell population (table 4). These results thus diverge from some previous studies reporting TNFα immunoreactivity in 30–60% of all synovial cells.[10 15 38] These studies were all performed on acetone fixed tissue sections, which led to detection of both cytokine producing and cytokine binding cells, as already discussed. However, more recent studies reflecting TNFα production at an mRNA level in RA synovitis based on PCR or RT-PCR methods showed a continuing synthesis at the time of biopsy in about half of the patients.[30 39] In the same studies IL1β mRNA transcripts were detected in the great majority of the same patients. These results are firmly comparable with the results we have recorded at a protein level using our modified immunohistochemical technique.

As we considered the issue of relative expression of TNFα and IL1β in the RA joint to be a question of considerable interest, not least with regard to the mechanisms of action of current cytokine blockade treatments, we performed additional methodological controls in our TNFα detection procedure. This was achieved by comparing the specificity and sensitivity of the immunohistochemical procedure for TNFα protein detection with in situ hybridisation for TNFα mRNA on smeared human LPS stimulated peripheral blood cells and in parallel on consecutive sections from synovial tissue biopsy specimens. A striking similarity was noted in both cases in the numbers of cells expressing TNFα at protein and mRNA levels. The numbers of TNFα positive cells in the LPS stimulated cultures correlated well with earlier published data on expected frequencies of TNFα producing cells after LPS stimulation of blood mononuclear cell cultures.[40]

TNFα production could only be identified in approximately two out of three patients with early RA in this study, though the biopsy specimens were selectively sampled from synovial areas with macroscopic signs of uniform, maximum inflammation (table 2). This part of the study was evaluated by computerised image analysis measuring the combined area occupied by TNFα producing cells and encompassing secreted TNFα bound to the matrix. These data cannot unfortunately be quantitatively directly compared with results obtained by conventional microscopy. However, this approach allowed a

more accurate comparison of cytokine synthesis in different compartments of a given joint than would have been possible with conventional microscopy. An arthroscopically guided biopsy technique enabled sampling of specimens with similar features of intense inflammation from pannus tissue and from villous synovitis occurring at a distance from the intra-articular cartilage in the same joint. Staining of tissue sections from such paired samples showed that TNFα producing cells were clustered in the pannus tissue compared with villous synovitis in patients with early RA (table 2). No such consistent differences between pannus and villi could be identified for IL1 or IL6 production. A strong expression of TNFα, IL6, and IL1α, but not of IL1β, in the pannus region has previously been reported.[13]

Multiple clinical trials using TNFα blockade have achieved excellent results in many but not all patients with RA studied, confirming that TNFα is a cytokine of major importance in RA. It has previously been shown that TNFα is a key regulator of subsequent production of other proinflammatory cytokines and mediators.[41 42] The new finding of this study is that the number of these regulatory cells producing TNFα is considerably lower than reported earlier. The fact that control of RA can be achieved by providing TNFα blockade systematically with prolonged intervals would be easier to explain if the true frequency of TNFα producing cells was as low as reported here, rather than comprising a major part of the total synovial cells at any given moment. Furthermore, we could not identify production of TNFα in all the patients with RA studied, suggesting that although TNFα is a major regulator in many patients with RA, there may be exceptions to this rule. The current results of the heterogeneity of TNFα expression in various patients with RA may thus be of relevance for the variability, including lack of response, to TNF blockade as a treatment in RA.

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1 Eastgate JA, Symons JA, Wood NC, Grinlinton FM, di Giovine FS, Duff GW. Correlation of plasma interleukin-1 levels with disease activity in rheumatoid arthritis and osteoarthritis. Lancet 1988;ii:706–9.
2 Arvidsson NG, Gudbjörnsson B, Elfman L, Ryden AC, Tötterman T, Hällgren R. Circadian rhythm of serum interleukin-6 in rheumatoid arthritis. Ann Rheum Dis 1994;53:521–4.
3 Westacott CL, Whicher JT, Barnes IC, Thompson D, Swan AJ, Diepppe PA. Synovial fluid concentrations of five different cytokines in rheumatic diseases. Ann Rheum Dis 1990;49:676–81.
4 Tetta C, Camussi G, Modena V, Di Vittorio C, Baglioni C. Tumour necrosis factor in serum and synovial fluid of patients with active and severe rheumatoid arthritis. Ann Rheum Dis 1990;49:665–7.
5 McNiff PA, Stewart C, Sullivan J, Showell HJ, Gabel CA. Synovial fluid from rheumatoid arthritis patients contains sufficient levels of IL-1 beta and IL-6 to promote production of serum amyloid A by Hep3 B cells. Cytokine 1995;7: 209–19.
6 Husby G, Williams R. Immunohistochemical studies of interleukin-2 and interferon-γ in rheumatoid arthritis. Arthritis Rheum 1985;28:174–81.
7 Buchan G, Barrett K, Turner M, Chantry D, Maini RN, Feldmann M. Interleukin-1 and tumor necrosis factor mRNA expression in rheumatoid arthritis: prolonged production of IL-1 alpha. Clin Exp Immunol 1988;73:449–53.
8 Xu WD, Firestein GS, Taetle R, Kaushansky K, Zvaifler NJ. Cytokines in chronic inflammatory arthritis. II. Granulocyte-macrophage colony stimulating factor in rheumatoid synovial effusions. J Clin Invest 1989;83:876–82.
9 Brennan FM, Zachariae COC, Chantry D, Larsen CG, Turner M, Maini RN, *et al.* Detection of interleukin-8 biologic activity in synovial fluids from patients with rheumatoid arthritis and production of interleukin-8 mRNA by isolated synovial cells. European Journal of Rheumatology 1990;20:2141–4.
10 Chu CQ, Field M, Feldman M, Maini RN. Localisation of tumor necrosis factor alpha in synovial tissue and the cartilage-pannus junction in patients with rheumatoid arthritis. Arthritis Rheum 1991;34:1125–32.
11 Alvaro-Gracia JM, Zvaifler NJ, Brown CB, Kaushansky K, Firestein G. Cytokines in chronic inflammatory arthritis. VI. Analysis of the synovial cells involved in granulocyte-macrophage colony stimulating factor production and gene expression in rheumatoid arthritis and its regulation by IL-1 and tumor necrosis factor-α. J Immunol 1991;146: 3365–71.
12 Field M, Chu C-Q, Feldman M, Maini RN. Interleukin-6 localisation in the synovial membrane in rheumatoid arthritis. Rheumatol Int 1991;11:45–50.
13 Chu C-Q, Field M, Allard S, Abney E, Feldman M, Maini RN. Detection of cytokines at the cartilage/pannus junction in patients with rheumatoid arthritis: implication for the role of cytokines in cartilage destruction and repair. Br J Rheumatol 1992;31:653–61.
14 Deleuran BW, Chu C-Q, Brennan FM, Mitchell T, Feldman M, Maini RN. Localisation of tumor necrosis factor receptors in the synovial tissue and cartilage-pannus junction in patients with rheumatoid arthritis. Arthritis Rheum 1992; 35:1170–8.
15 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.
16 Woods J, Haines K, Shah M, Rayan G, Koch A. Low-level production of interleukin-13 in synovial fluid and tissue from patients with arthritis. Clin Immunol Immunopathol 1997;85:210–20.
17 Sander B, Andersson J, Andersson U. Assessment of cytokines by immunofluorescence and the paraformaldehyde/saponin procedure. Immunol Rev 1991;119: 65–93.
18 Dolhain RJEM, Andersson U, ter Haar NT, Brinkman BMN, Verwej CL, Daha MR, *et al.* Detection of intracellular IFNγ by light microscopy using an immunoperoxidase technique: correlation with the corresponding mRNA and protein product. J Leukoc Biol 1993;54:545–51.
19 Ulfgren A-K, Lindblad S, Klareskog L, Andersson J, Andersson U. Detection of cytokine producing cells in synovial membrane from patients with rheumatoid arthritis. Ann Rheum Dis 1995;54:654–61.
20 Björk L, Fehringer T, Andersson U, Andersson J . Computerized assessment of multiple human cytokines at the single cell level using image analysis. J Leukoc Biol 1996;59:297–95.
21 Litton M, Dohlsten M, Hansson J, Rosendahl A, Ohlsson L, Kalland T, *et al.* Tumor therapy with an antibody targeted superantigen generates a dichotomy between local and systemic immune response. Am J Pathol 1996;150:1607–18.
22 Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, *et al.* The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315–24.
23 Lindblad S, Hedfors E. Intraarticular variation in synovitis. Local macroscopic and microscopic signs of inflammatory activity are significantly correlated. Arthritis Rheum 1985; 28:977–86.
24 Klareskog L, Forsum U, Malmnäs-Tjernlund U, Kabelitz D, Wigren A. Relationships between HLA-DR-expressing cells and T lymphocytes of different subsets in rheumatoid synovial tissue. Scand J Immunol 1981;14:183–92.
25 Cunnane G, Björk L, Ulfgren A-K, Lindblad S, Fitzgerald O, Breshnihan B, *et al.* Quantitative analysis of synovial membrane inflammation: a comparison between automated and conventional microscopic measurements. Ann Rheum Dis 1999;58:493–9.
26 Khademi M, Wallström E, Andersson M, Piehl F, Di Marco R, Olsson T. Reduction of both pro- and anti-inflammatory cytokines after 6 months of interferon beta-1a treatment of multiple sclerosis. J Neuroimmunol 2000;103:202–10.
27 Andersson J, Abrahms J, Björk L, Funa K, Litton M, Ågren K, *et al.* Concomitant in vivo production of 19 different cytokines in human tonsils. Immunology 1994;83: 16–24.
28 Andersson U, Litton MJ, Fehniger TE, Ulfgren AK, Andersson J. Detection and quantification of cytokine-producing cells by immunostaining, In: Paterson BK, ed. *Techniques in quantification and localization of gene expression.* Birkhauser, 2000:55–80.
29 Klimiuk P, Goronzy J, Björnsson J, Beckenbaugh R, Weynad C. Tissue cytokine patterns distinguish variants of rheumatoid synovitis. Am J Pathol 1997;151:1311–19.

30 Wagner S, Fritz P, Einsele H, Sell S, Saal JG. Evaluation of synovial cytokine patterns in rheumatoid arthritis and osteoarthritis by quantitative reverse transcription polymerase chain reaction. Rheumatol Int 1997;16:191–6.
31 Dinarello CH. Interleukin-1. Cytokine Growth Factor Rev 1997;8:253–65.
32 Deleuran BW, Chu C-Q, Field M, Brennan FM, Katsikis P, Maini RN. Localisation of interleukin 1 alpha, type 1 interleukin receptor and interleukin-1 antagonist in the synovial membrane and cartilage/pannus junction in rheumatoid arthritis. Br J Rheumatol 1992;31:801–9.
33 Lundberg I, Ulfgren A-K, Nyberg P, Andersson U, Klareskog L. Cytokine production in muscle tissue in idiopathic inflammatory myopathies. Arthritis Rheum 1997;40:865–74.
34 Kirkham B. Interleukin-1, immune activation pathways, and different mechanisms in osteoarthritis and rheumatoid arthritis. Ann Rheum Dis 1991;50:395–400.
35 Yanni G, Whelan A, Feighery C, Quinlan W, Symons J, Duff G, *et al*. Contrasting levels of in vitro cytokine production by rheumatoid synovial tissues demonstrating different patterns of mononuclear cell infiltration. Clin Exp Immunol 1993;93:387–95.
36 Cauli A, Yanni G, Panayi GS. Interleukin-1, interleukin-1 receptor antagonist and macrophage populations in rheumatoid arthritis synovial membrane. Br J Rheumatol 1997;36:935–40.
37 Breshnihan B, Cunnane G, Youssef P, Yanni G, Fitzgerald O, Mulherin D. Microscopic measurement of synovial membrane inflammation in rheumatoid arthritis: proposals for the evaluation of tissue samples by quantitative analysis. Br J Rheumatol 1998;37:636–42.
38 Brennan FM, Field M, Chu C-Q, Feldman M, Maini RN. Cytokine expression in rheumatoid arthritis. Br J Rheumatol 1991;30:76–80.
39 Canete JD, Llena J, Collado A, Sanmatri R, Gaya A, Gratacos J, *et al*. Comparative cytokine gene expression in synovial tissue of early rheumatoid arthritis and seronegative spondyloarthritis. Br J Rheumatol 1997;36:38–42.
40 Henter J-I, Söder O, Andersson U. Identification of individual tumour necrosis factor/cachectin-producing cells after lipopolysaccharide induction. Eur J Immunol 1988;18:983–8.
41 Brennan F, Chantry D, Jacksson A, Maini RN, Feldman M. Inhibitory effect of TNF-α antibodies on synovial cell interleukin-1 production in rheumatoid arthritis. Lancet 1989;ii:244–7.
42 Feldmann M, Brennan F, Paleolog E, Taylor P, Maini RN. Anti-tumor necrosis factor alpha therapy of rheumatoid arthritis. Mechanism of action. Eur Cytokine Netw 1997;8:297–300.