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# *In situ* hybridization of IL-6 in rheumatoid arthritis

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## SUMMARY

IL-6, an important mediator of the acute phase response, has been implicated in the pathogenesis of rheumatoid arthritis (RA). Many cell types including macrophages, T cells, B cells, endothelial cells and fibroblasts can produce this cytokine and production is largely regulated at the level of gene transcription or mRNA stabilization. In this paper we have first measured the levels of IL-6 activity in synovial fluid (SF) and serum from patients with RA and then localized IL-6-producing cells in the synovium by *in situ* hybridization combined with immunophenotyping. Patients with RA had raised levels of IL-6 in both SF and serum compared with patients with osteoarthritis and age-matched healthy controls. In individual RA patients tested serially after admission to hospital, serum IL-6 was initially raised and, unexpectedly, increased with clinical improvement. *In situ* hybridization of IL-6 mRNA showed positive cells both in the lymphocyte-rich aggregates and adjacent to small blood vessels. With immunophenotyping it was found that cells containing IL-6 mRNA were often in contact with $CD14^+$ tissue macrophages and double immunophenotyping revealed that immunoreactive IL-6 was often associated with synovial T cells.

**Keywords** IL-6 rheumatoid arthritis *in situ* hybridization gene expression

## INTRODUCTION

IL-6 is a pro-inflammatory cytokine previously known as B cell stimulating factor 2 (BSF-2) [1], 26-kD protein [2] and interferon-beta 2 [3]. Human IL-6 is produced from a single gene [4] that encodes a primary product of 212 amino acids. The hydrophobic sequence at the N-terminal is cleaved before secretion to produce a 184-amino acid peptide that is variably glycosylated with a molecular weight varying from 23 kD to 32 kD (reviewed by [5]). IL-6 gene expression can be induced in a number of cell types including monocytes [6,7], fibroblasts [8,9], endothelial cells [10,11], T cells and B cells [12] and by a variety of stimuli such as bacterial endotoxin, plant lectins, viruses, double-stranded RNA, IL-1, tumour necrosis factor (TNF) and platelet derived growth factor [5].

Many of the biological actions of IL-6 appear relevant to inflammatory diseases such as rheumatoid arthritis (RA) where chronic immune activation and recurrent acute phase responses are characteristic. One of the functions of IL-6 appears to be the induction of differentiation of activated B cells into antibody-secreting cells [1,13,14]. In RA this could contribute to the frequent presence of plasma cells in the joint synovium and the production by this tissue of immunoglobulins including pathogenic anti-IgG autoantibodies (rheumatoid factors) [15,16]. Recombinant IL-6 is a potent inducer of hepatocyte acute phase proteins [17] and can act as an endogenous pyrogen [18]. IL-6 could potentiate chronic inflammation by augmenting T cell activation [19,20] and by inducing IL-2 receptor expression [21].

Several tumour cells such as cardiac myxoma, cervical and bladder cell carcinomas, have been found to produce large amounts of IL-6 [13]. Patients with such tumours were frequently found to have circulating autoantibodies and a clinical syndrome resembling RA which resolved with surgical removal of the tumour. It has therefore been suggested that IL-6 production may contribute to the pathogenesis of RA, at least during acute inflammatory exacerbation of this disease.

In the present study we have measured synovial and serum levels of IL-6 bioactivity and have identified IL-6 mRNA in synovial tissue both by Northern blot analysis and *in situ* hybridization in patients with active RA. It appears that the gene for IL-6 is not constitutively expressed [12] and only activated cells contain detectable mRNA for this cytokine. Detection by *in situ* hybridization of snap-frozen specimens therefore localizes cells activated *in vivo* for IL-6 gene transcription.

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## PATIENTS AND METHODS

*Patients*

Synovial samples from eight patients with classical or definite RA [22] were obtained at the time of joint replacement surgery and frozen immediately after excision in liquid nitrogen to

preserve the *in vivo* mRNA expression. Tissue was stored at −80°C until sectioned in a cryostat. Additionally, synovial fluid from the inflamed knee joints (therapeutic aspirations) and simultaneous peripheral blood samples were obtained from 26 out-patients (age 52·8 ± 5·2 years, mean ± s.e.m.) with classical or definite RA or from 12 patients with osteoarthritis (OA) and joint effusions (mean age 64·2 ± 4·8 years). All patients were receiving non-steroidal anti-inflammatory drugs only. Control populations consisted of age- and sex-matched healthy volunteers (mean age 48·2 ± 3·5 years, $n = 16$).

*Synovial fluid and serum samples*

Synovial fluids (SF) were taken into EDTA tubes and blood into glass tubes for serum collection. Samples were centrifuged at 400 ***g*** for 10 min then again at 10 000 ***g*** for 5 min, aliquoted and stored at −30°C until used. Before bio-assay, SF samples were treated with hyaluronidase (bovine testes, type VIII 100 U/ml, 45 min, 37°C; Sigma, Poole, Dorset), followed by centrifugation at 10 000 ***g***. SF and serum samples then underwent complement inactivation by incubation at 56°C for 30 min.

Longitudinal studies of serum IL-6 were performed on three patients admitted to hospital with active RA. Clinical assessment of disease activity was performed by the same physician at 3-day intervals. The following standardized measures were used [23]: Ritchie articular index; duration of morning stiffness (EMS); patients' analogue pain score; and physician's global assessment. Laboratory monitoring of disease activity was performed by haemoglobin concentration, total leucocyte count, platelet count and erythrocyte sedimentation rate (ESR). Onset of clinical improvement was defined as the day when Ritchie articular index was below 10; ESR < 20 mm/h; and EMS < 30 min [23].

*Bioassay for IL-6 activity*

IL-6 bioactivity was measured as hybridoma growth factor using the B9 cell line as previously described [24]. B9 cells ($4 \times 10^3$/well) were cultured with four dilutions of each sample (SF 1%, 0·1%, 0·01% and 0·001% and serum 10%, 5%, 2·5% and 1·25%) for 72 h at 37°C in a humidified 5% $CO_2$/95% air atmosphere. B9 proliferation was assessed by the MTT colourimetric assay described by Mosmann [25]; IL-6 activity was compared with that obtained from a standard curve of human recombinant IL-6 (hrIL-6) (National Institute for Biological Standards and Control (NIBSC)).

*Neutralization of IL-6 activity*

To check the specificity of the bioassay results appropriately diluted serum and SF samples (25 μl) were incubated with a 1/2000 dilution (25 μl) of either goat pre-immune serum or immune sera raised against human recombinant IL-6 protein (gift from Dr S. Poole, NIBSC). After incubation for 1 h at room temperature the samples were tested for IL-6 activity.

*Preparation of IL-6 probe*

Human IL-6 cDNA pGEM was kindly provided by Dr E. Kawashima (Glaxo Institute of Molecular Biology, Geneva, Switzerland). A 505bp *Hin*dIII/*Hin*cII fragment encoding amino acids 44–212 of the IL-6 precursor protein was labelled using random oligomers and [$^{32}$P]-alpha dCTP (New England Nuclear, Cambridge, MA), to a specific activity of $2 \times 10^8$ ct/min per μg DNA. *Pst*I restricted pGEM lacking the IL-6 insert was radiolabelled to the same specific activity and used as a routine negative control for *in situ* hybridization experiments. A Sephadex G50 column (Sigma) was used to separate unincorporated label from DNA probe.

*Northern analysis*

Total RNA was isolated from approximately 2 g of synovial tissue using a guandinium isothiocyanate/cesium chloride method. Polyadenylated RNA was separated by two cycles of binding to oligo-(DT) cellulose (GIBCO/BRL, Paisley, UK). The mRNA (5 μg) was electrophoresed in a 1·3% agarose-formaldehyde gel, transferred onto Hybond N (Amersham, Amersham, UK) and crosslinked by exposure to UV light. The membrane was pre-hybridized, hybridized and washed as previously described [26]. The size of the RNA was determined by size markers (0·24–9·5 kb RNA ladder, BRL) stained with methylene blue on the nylon membrane.

*Tissue section preparation and immunophenotyping*

Immunophenotyping of rheumatoid synovial sections was performed as described elsewhere [26a]. Briefly, 8 μm sections, cut on the day of the experiment, were thaw-mounted onto baked microscope slides coated with 0·5% gelatin, 0·25% CrK $(SO_4)_2$ and 0·02% diethyl pyrocarbonate (DEP). Slides for *in situ* hybridization alone were immediately immersed for 15 s in cold Carnoy's fixative (1 acetic acid/6 ethanol/3 chloroform) while those also to be immunophenotyped were fixed for 10 min in cold acetone. These slides were then air dried.

Mouse monoclonal antibodies with well-defined specificities denoted Leu-M3, recognizing 80–92% of synovial mononuclear phagocytes and Leu-4 recognizing the pan T cell surface antigen CD3 were obtained from Becton Dickinson (Oxnard, CA), all other immunophenotyping reagents being supplied by Vector Laboratories (Burlingame, CA). Controls for immunophenotyping were included in every experiment. A mouse monoclonal antibody of the same sub-class as the other first antibodies (IgG1 κ) directed against bromodeoxyuridine (Dako, High Wycombe, UK), an antigen not found in normal human tissue, was used as a first antibody control. The biotinylated second antibody was replaced with TS buffer (0·05 M Tris-HCl/0·15 M NaCl, pH 7·6).

In situ *hybridization*

*In situ* hybridization was performed as previously described in detail [27].

*Combined immunolocalization/immunophenotyping*

Immunolocalization of IL-6 protein using a mouse anti-human IL-6 monoclonal antibody (a generous gift from Dr Jo Van Damme, Leuven, Belgium) at 2% (v/v) dilution and the ABC-AP method was performed as described elsewhere [26a]. After rinsing in TS buffer, the sections were immunophenotyped with anti-CD3 (Leu-4, Becton Dickinson) using avidin-biotin-peroxidase complex (ABC, Vector, Burlingame, CA) (Wood *et al.* submitted for publication).

## RESULTS

*IL-6 activity in serum and synovial fluid*

The detection limit of the B9 assay to hrIL-6 was 0·5 pg/ml and hybridoma growth factor (HGF) activity was detected in all samples tested. Twenty-six patients with RA had a mean serum IL-6 of 161 ± 25 pg/ml (mean ± s.e.m.) (Fig. 1). This was significantly higher than in a healthy age-matched control group (22·5 ± 3·0 pg/ml; $P < 0·001$). In paired synovial effusion samples from these patients the IL-6 levels were much higher (24 ± 11 ng/ml; $P < 0·001$). In a group of 12 OA patients neither the serum nor the synovial fluid levels were significantly different from the serum concentration in the age- and sex-matched control group.

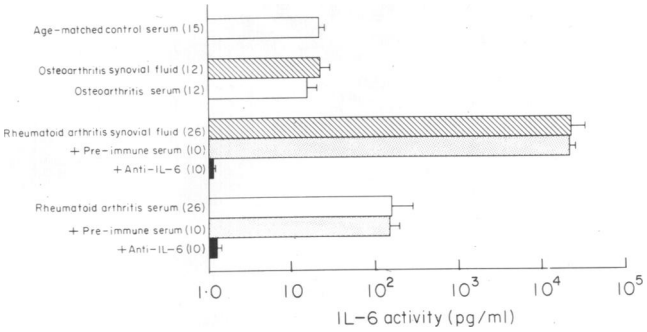


**Fig. 1.** Serum and synovial fluid IL-6 levels in patients and controls. Paired sera (□) and synovial fluid ▨ obtained from patients with RA, osteoarthritis and controls were assessed for IL-6 activity by B9 bioassay. Specificity was confirmed by addition of pre-immune sera (▨) or anti-IL-6 sera (■). Bars represent mean ± s.e.m.

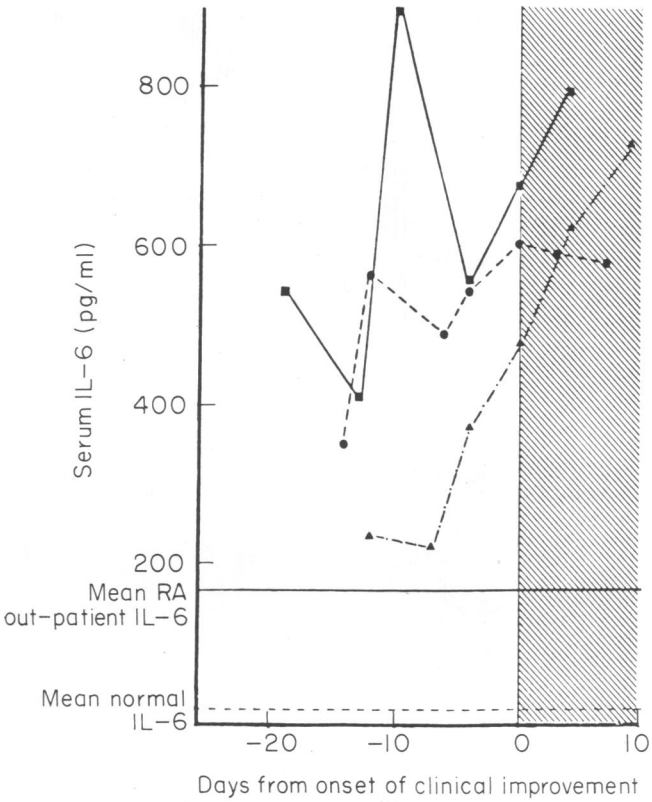


**Fig. 2.** Serial IL-6 serum levels in three patients with active rheumatoid arthritis. The first IL-6 measurement for each patient was on the day of admission to hospital. Onset of clinical improvement is defined in Patients and Methods section and is indicated by the shaded region

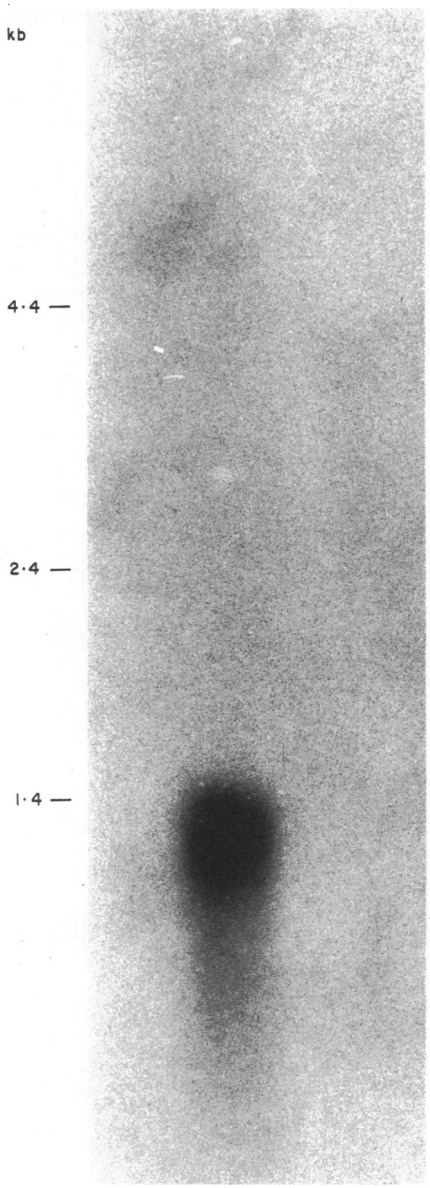


**Fig. 3.** Northern blot analysis of IL-6 mRNA from rheumatoid arthritis synovial tissue.

To confirm that this HGF activity was due to IL-6 we tested the ability of pre-immune and polyclonal anti-IL-6 sera to inhibit the proliferation of the B9 cells. Samples were chosen with high titres of IL-6 activity (up to 300 ng/ml). The IL-6 activity in the synovial fluid and serum samples was completely neutralized by incubation with the anti-IL-6 serum but was not significantly altered by incubation with pre-immune serum.

*Serum IL-6 activity and clinical improvement*

To determine whether the raised serum levels of IL-6 noted in patients with RA were related to variations in disease activity, sequential studies were performed on three individuals. Figure 2 shows the serum IL-6 levels in three patients admitted to hospital with clinically active RA. Initially levels were signifi-

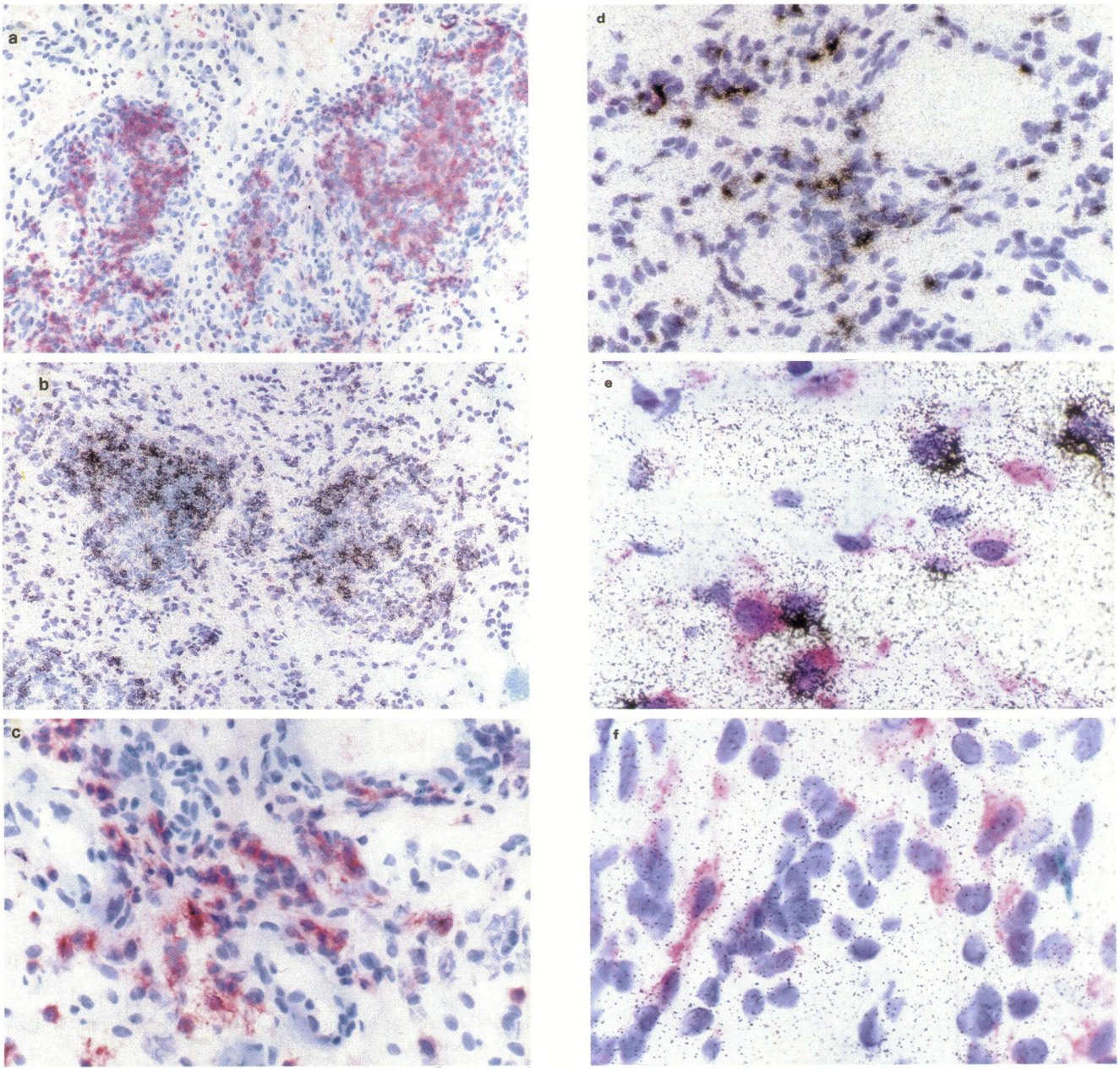


**Fig. 4.**

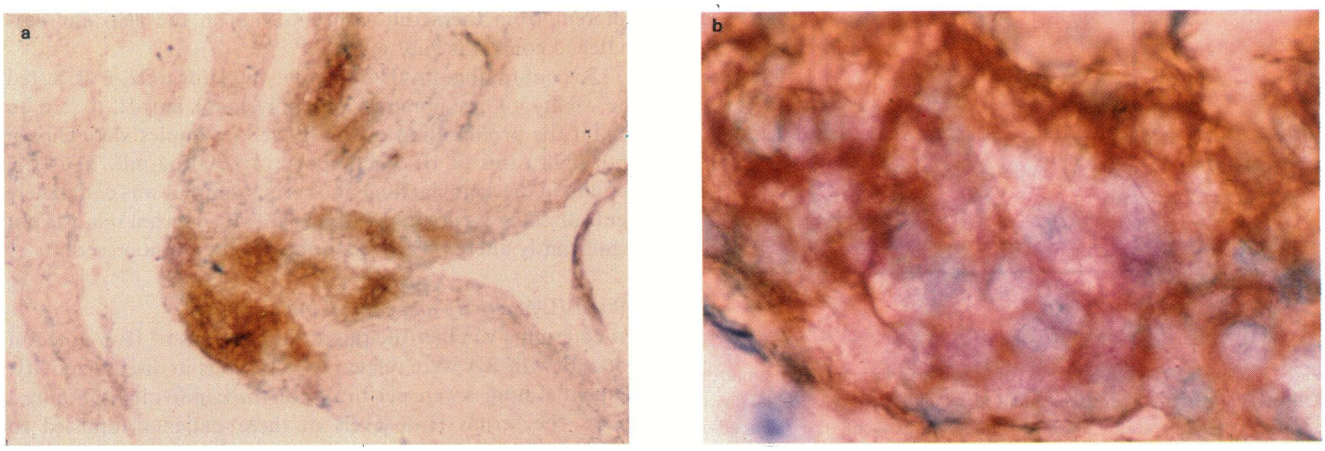


**Fig. 5.**

cantly higher than both healthy controls and RA out-patients. As the patients' disease activity decreased, however, the level of serum IL-6 rose. When the patients entered clinical remission, as defined by clinical and laboratory measures of inflammation [23] serum levels of IL-6 continued to rise until the patients left hospital.

*Northern analysis*

A single species of IL-6 mRNA was detected migrating at 1·3 kb as predicted for the mature transcript [1] (Fig. 3).

*Immunophenotyping*/in situ *hybridization of RA synovium*

After Northern blots had determined that cells within the rheumatoid synovium were expressing the IL-6 gene *in vivo*, *in situ* hybridization and immunophenotyping alone, and in combination, were used to locate and identify cells containing IL-6 mRNA. Although the pattern of the mononuclear cell infiltrate of rheumatoid synovium is largely non-specific, four types of areas can be identified. These are the proliferating lining cell layer, focal lymphocyte-rich aggregates [28,29], a transitional area composed of macrophages, lymphocytes and plasma cells [30] which occurs at the periphery of lymphocyte aggregates and also extends from the lining cells to the fourth region, the interstitial area. This area is relatively less cellular but is rich in collagen fibres and contains fibroblasts and elongated macrophage-like cells [31]. Several groups have reported that the majority of cells with the lymphocyte-rich aggregates in rheumatoid synovium are T cells [28,29]. Figure 4a shows RA synovium immunophenotyped with anti-Leu-4 antibody which recognizes the pan-T cell surface antigen, CD3. Strong staining of the aggregate areas is seen.

Figure 4b is a section from the same area as that in Fig. 4a, where *in situ* hybridization has been used to detect IL-6 mRNA. Many cells within these lymphocyte-rich aggregates are expressing the IL-6 gene. T cells are also found in the transitional area of the synovium as illustrated in Fig. 4c. Many of these T cells are close to small blood vessels. A section from the same area as that in Fig. 4c was tested by *in situ* hybridization (Fig. 4d) which identified a similar perivascular distribution of cells hybridizing with the IL-6 probe.

A combined immunophenotyping/*in situ* hybridization experiment is illustrated in Fig. 4e using anti-Leu-M3 antibody to stain synovial macrophages. Macrophages were frequently found in association with cells containing IL-6 mRNA.

Control experiments were performed routinely using radiolabelled plasmid DNA. Figure 4f shows the consecutive section to that in Fig. 4e that has been immunophenotyped with anti-Leu-M3 antibody and probed with [$^{32}$P]-labelled pGEM. No non-specific hybridization was present in this or other control sections.

*Combined immunolocalization/immunophenotyping*

Combined immunolocalization of IL-6 (staining red) and immunophenotyping using anti-Leu-4 antibody (staining brown) of rheumatoid synovium is shown in Fig. 5a. Combined staining is localized in a cellular aggregate. A higher power view of this RA synovial membrane demonstrates the co-localization of IL-6 peptide and CD3$^+$ T cells (Fig. 5b). For technical reasons we were unable to demonstrate simultaneous *in situ* hybridization of IL-6 mRNA and immunophenotyping CD3$^+$ cells.

## DISCUSSION

Production of IL-6 may be an important pathogenic mechanism in chronic inflammatory diseases such as RA. Using a sensitive bioassay, we measured levels of IL-6 in the synovial fluid and serum of patients with RA and OA and compared them with serum levels in a control population. Serum levels in RA patients were signficantly raised compared with osteoarthritis and aged-matched disease-free controls. Within the RA patients, synovial fluid levels were significantly higher than paired serum levels, compatible with the proliferating synovial tissue being the likely source of the IL-6 detected in the circulation.

Interestingly, in patients with active RA, serum levels of IL-6 rose with clinical improvement. Different results were obtained when the samples were tested for IL-1 beta [32] and soluble IL-2 receptor [23] where levels were high during active disease and fell with clinical remission. The results could be explained by postulating a source of IL-6 production distinct from the inflamed joints. However, synovial fluid levels of IL-6 were always significantly higher than corresponding serum levels. It is possible that cytokines such as IL-1 and TNF induce the production of IL-6 that is released from the joint at a later time and so blood levels of IL-6 reflect synovial inflammatory activity that occurred previously. Alternatively, IL-6 may have anti-inflammatory or restorative functions in RA. IL-6 plays a major role in the production of acute phase proteins during a systemic inflammatory response [17]. The acute phase response consists of the production of a large number of protease inhibitors and potential cytokine inhibitors and these may suppress both systemic and local immune responses. Additionally, it has been reported that IL-6 can inhibit the production of both IL-1 and TNF-$\alpha$ at the level of transcription [33]. Therefore, IL-6 may provide a negative feedback signal to limit the cytokine cascade.

The main source of IL-6 in culture of human leucocytes has been found to be the monocyte/macrophage population [6,7] and we were therefore interested to identify which cells were producing IL-6 within the synovial membrane *in vivo*.

Many cells that hybridized with the IL-6 DNA probe were located within lymphocyte-rich aggregates and adjacent to small blood vessels. Immunophenotyping of these same areas

**Fig. 4.** Immunophenotyping and *in situ* hybridization for CD3$^+$ T cells and IL-6 mRNA in rheumatoid synovium. CD3$^+$ T cells are indicated by red staining, IL-6 mRNA-positive cells by overlying dense silver granules. (a) Localization of CD3$^+$ T cells in perifollicular areas of rheumatoid synovium. (b) Consecutive section to (a) showing localization of IL-6 mRNA expressing cells. (c) Immunolocalization of CD3$^+$ cells in transitional area. (d) Consecutive section to (c) showing IL-6 mRNA expressing cells; (e) high power field of RA synovium showing combined localization of CD14$^+$ cells and IL-6 mRNA; (f) consecutive section to (e) showing no hybridization to a control probe with combined CD14$^+$ immunohistochemistry.

**Fig. 5.** Combined Immunolocalization and immunophenotyping of IL-6 peptide (red staining) and CD3$^+$ cells (brown staining). (a) Low power view of rheumatoid synovium. (b) High power view of lymphocytic aggregate as seen in left field of (a).

revealed abundant T cells suggesting that this cell is activated *in vivo* to produce IL-6. It has been shown that activated purified human T cells express the IL-6 gene in the presence of macrophages [12]. Previously, IL-6 gene expression has been detected in the hyperplastic lining layer of rheumatoid synovium [34]. Within the synovial membrane, macrophage cells identified by Leu-M3 reactivity ($CD14^+$), very rarely contained IL-6 mRNA but were commonly found in contact with, or in the immediate vicinity of cells hybridizing with the IL-6 probe. This raises the possibility that activation of the IL-6 gene may be the result of cell–cell interaction. IL-1 is the most potent endogenous stimulator of IL-6 production so far described [8] and seems a likely candidate for an IL-6-inducer in RA, having been identified in synovial macrophages *in vivo* in separate experiments [26a].

Our finding of T cells expressing the IL-6 gene is in agreement with immunohistochemical analysis of consecutive sections of RA synovium reported by Hirano *et al.* [35], who found IL-6 protein located in the same areas as T and B cells and also noted a perivascular distribution of IL-6 corresponding to that of T cells. In agreement with this data, we found that IL-6 protein and T cells, identified using specific monoclonal antibodies, co-localized in the lymphocyte-rich aggregates in rheumatoid synovium. This immunolocalized IL-6 may, of course, represent peptide bound to receptors on these cells, but synthesized elsewhere.

In addition to perivascular cells expressing the IL-6 gene we also identified cells in the vessel lining that hybidized with the IL-6 probe. Human endothelial cells are a recognized source of IL-6 [10,11]. Some IL-6 produced adjacent to the abundant small blood vessels of the rheumatoid synovial membrane might be expected to pass back into the circulation and exert distant effects. IL-6 is known to be a potent inducer of acute phase protein synthesis [17] and can also act as an endogenous pyrogen [18], both compatible with the clinical features of active RA. IL-6 bioactivity [36] and immunoreactivity [35] have been described previously in the synovial fluid and serum of patients with RA. In one study [36] serum IL-6 bioactivity correlated with a number of acute phase proteins and other studies [37] have found a significant positive correlation in RA serum between IL-6 and levels of C-reactive protein and a negative correlation with albumin. This lends support to the idea that some of the systemic manifestations observed in RA are due to circulating IL-6.

This is the first report of combined immunohistochemical and *in situ* hybridization analysis of IL-6. The results are non-quantitative but show that IL-6 is produced within the RA synovium probably by activated T cells (though not exclusively) and, while blood levels are raised in active disease, changing levels are inversely related to disease activity. The latter raises the possibility that IL-6 may have direct or indirect anti-inflammatory functions.

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