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Local synthesis of both macrophage and T cell cytokines by synovial fluid cells from children with juvenile rheumatoid arthritis

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SUMMARY

The production of tumour necrosis factor-alpha (TNF-α), TNF-β and IL-6 in synovial fluid was studied in 50 samples of synovial fluid from 44 children with juvenile rheumatoid arthritis (JRA) by identifying cytokine production at a single-cell level. Post Ficoll-separated synovial fluid mononuclear cells were permeabilized and then intracellular TNF-α, TNF-β and IL-6 protein production was examined using indirect immunofluorescence and murine anti-cytokine MoAbs. All three cytokines were measured in 37 of the 50 samples. In 25 of the 37 samples there was complete concordance; all three cytokines were present in six and absent in 19 samples. At least one cytokine was present in 27/50 (54%) of synovial fluid samples. Overall, TNF-α was detected in 22/49 (45%) samples, TNF-β in 15/41 (37%) and IL-6 in 16/45 (36%) samples. Five patients had serial arthrocentesis, and in these samples there were two patients who had initially positive cytokine production, which on subsequent measurement was negative; in the other three patients there was no change from the previous cytokine production. We provide evidence that synovial fluid mononuclear cells produce monocyte and T cell cytokines in JRA. These findings suggest a role for both T cell and macrophage products in the pathogenesis of JRA, and the potential for modulation of cytokine production as a target for therapeutic intervention.

Keywords cytokine synovial fluid single cell juvenile rheumatoid arthritis

INTRODUCTION

Cytokines have been implicated as mediators of both inflammation and joint destruction in rheumatoid arthritis (RA) [1–4]. They are released from activated T cells and macrophages present in the joint, and may mediate their damage locally by causing synovial fibroblasts to release enzymes that damage cartilage and bone. While the individual cytokines involved in this inflammation are still being defined, it appears macrophage products such as IL-1, tumour necrosis factor-alpha (TNF-α) and IL-6 are prevalent in the synovial fluid and tissue, while T cell products such as IL-2, IL-4 or TNF-β are rarely detected [1–7]. However, most studies have been performed on patients who have had long-standing RA, and these findings may not accurately reflect what is occurring at disease inception in the joint.

The early literature regarding synovial fluid cytokines was very inconsistent. There seemed to be little correlation between indices of joint inflammation such as the number of cells in the joint and the cytokine concentration. There were different levels of cytokine production from different joints in the same patient, while most anti-rheumatic medications seemed to have no effect on synovial fluid cytokine levels [2,4,8,9]. The synovial fluid is a 'soup' composed of a mixture of enzymes, proteins, cytokine inhibitors and hyaluronic acid all of which have the potential to affect assay systems, which may explain some of these differences. Additionally, cytokines are present at extremely low concentration in synovial fluid, further contributing to the inaccuracy of measurements.

We often perform arthrocentesis and corticosteroid joint injection on children early in the course of their arthritis, often before any systemic treatment has commenced. Thus we are in the unique position of being able to study the cytokine repertoire in untreated patients at disease onset. To overcome many of the previously discussed difficulties, we have adapted a recently described immunofluorescent technique which measures intracellular cytokines [10]. More importantly, detection of intracellular cytokine allows for the identification of individual cells secreting individual cytokines, thereby providing a possible means for studying the different cytokine interactions. Extrapolation of these results could be used to direct future cell-targeted specific therapies.

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We studied three cytokines: TNF-α and IL-6, both macrophage products, and TNF-β, a T lymphocyte product. We found that mononuclear cells from most patients were actively synthesizing at least one cytokine. In 55% of patients at least one macrophage cytokine was produced, while 36% of patients produced the T cell product, TNF-β.

PATIENTS AND METHODS

Patients

We studied 44 consecutive children (29 female, 15 male) who had a knee joint injection, and sufficient synovial fluid could be obtained for the study. In five of these children joint aspirations were performed on more than one occasion, twice in four patients and three times in one patient. Therefore 50 samples of synovial fluid were available for analysis. All the patients fulfilled the American College of Rheumatology criteria for juvenile rheumatoid arthritis (JRA) [11]. The group consisted of 28 patients with (17 female, 11 male) pauciarticular, 13 patients with (10 female, three male) polyarticular, and three patients with (two female, one male) systemic JRA. The mean age at joint injection was 12 years (range 5–18 years). Forty-nine samples from 43 patients had TNF-α measured in the synovial fluid mononuclear cells, 41 samples from 35 patients were tested for TNF-β, and 45 samples from 40 patients had IL-6 measurements.

At the time of joint aspiration intracellular cytokines were simultaneously determined in unstimulated peripheral blood from 12 patients. Additional studies included serum albumin, total protein and IgG levels. Haematology testing consisted of a peripheral blood count with erythrocyte sedimentation rate (ESR), total leucocyte count and a differential count. Where possible a leucocyte count was performed on the synovial fluid which included total leucocyte number, polymorphonuclear cell, lymphocyte and monocyte count. A chart review was performed on all patients, and a record of the type of arthritis and age at diagnosis of the arthritis recorded, along with the joint count at time of joint aspiration, medication at time of joint aspiration, history of previous joint injections, and any changes in medication in the previous 12 months.

The peripheral blood from 10 healthy adult volunteers was used for controls.

Cell preparation and immunofluorescence

Peripheral blood lymphocytes (PBL) and synovial fluid (SF) mononuclear cells were isolated from heparinized peripheral blood or SF by Ficoll–Hypaque density centrifugation. The cells were washed and counted, then 3×10^6 cells per cytokine were fixed in 4% paraformaldehyde and examined by immunofluorescence, according to previously described methods [10]. Briefly, cells were washed in a mixture of phosphate-buffered salt solution and a detergent, saponin, and labelled with an anti-human cytokine-specific (IL-6, TNF-α or β) mouse MoAb. Following incubation at 37°C for 30 min with the antibody, the cells were further washed in PBS-saponin and labelled with an FITC-coupled goat anti-mouse IgG isotype-specific antibody, fixed on microscope slides and examined under a fluorescent microscope (Polyvar, Reichert Optische Werke AG, Vienna, Austria). As previously described, cells were considered positive if there was specific juxtanuclear staining within the cell [12]. Positive cells were counted and expressed as a percentage of total cells. A minimum of 1000 cells were counted. If the percentage of positive cells was 0·5% or greater, then the sample was recorded as having the cytokine present. Despite repeated attempts to use FACS analysis, we were unable to quantify accurately cytokine-producing cells using this technique. This is probably the result of the small number of cytokine molecules with specific staining within each cell and the relatively low intensity of the staining.

Immunofluorescent staining of cell surface antigen was performed only on SF samples. To determine cell surface phenotype, before fixation, 3×10^6 cells per cytokine were incubated for 30 min on ice with predetermined optimal concentrations of Mo2 (CD14) (monocyte), CD2 (pan T cell), CD3 (pan T cell) and/or CD4 (helper T cell) MoAbs, then extensively washed in PBS. The Mo2-RDI was directly conjugated with PE while the CD2, CD3 and CD4 samples were further incubated with PE (R-PE)-conjugated AP F(ab')$_2$ fragment goat anti-mouse IgG Fc fragment-specific antibody on ice for 30 min and then extensively washed. Following this, cytokine production was determined as outlined above.

The TNF-α IgG1 and TNF-β IgG2b MoAbs were used in a concentration of 5 μg/ml, while the IL-6 IgG1 MoAb was used in a concentration of 10 μg/ml. FITC-conjugated goat anti-mouse IgG1 and IgG2b were diluted to a 1:300 concentration.

Anti-TNF-α MoAbs were generously supplied by Genentech (South San Francisco, CA), anti-TNF-β antibodies were generously supplied by Dr G. Adolf (Ernst-Boehringer Institut fur Arzneimittel-Forschung, Vienna, Austria), while IL-6 was a gift from Dr T. Hirano (Division of Molecular Biology, Biomedical Research Centre, Osaka, Japan). Goat anti-mouse IgG1 and IgG2b-labelled FITC were obtained from Caltag (South San Francisco, CA). The Mo2-RDI murine MoAbs were purchased from Coulter Immunology (Hialeah, FL), while Clonab CD2, CD3 and CD4 (T11) MoAbs were supplied by Biotest (Dreieich, Germany) and R-PE was purchased from Jackson Immunoresearch (West Grove, PA).

Statistical analysis

All results were expressed as a mean ± s.d. Variables within the patient groups were compared with the Student's *t*-test, using the Statview program, Abacus Concepts Inc. (Berkeley, CA), on a Macintosh SE computer. The Bonferroni correction was used when multiple variables were tested.

RESULTS

Figure 1 illustrates the presence of cytokine within SF mononuclear cells. The cytokine has a distinct fluorescence pattern, with a juxtanuclear staining pattern which reflects cytokine accumulation within the Golgi organelle [10]. The percentage of positive cells varied from patient to patient, but ranged from 1% to 5% of SF cells.

At least one cytokine was present in 27/50 (54%) SF samples. TNF-α was positive in 22/49 (45%), TNF-β was positive in 15/41 (37%) and IL-6 was positive in 16/45 (36%) samples. There was no statistical difference in the percentage of patients who had detectable intracellular cytokine/s between the pauciarticular (55%) and polyarticular (50%) JRA patients (Table 1). Owing to the small numbers, only 6% of the study group, the systemic patients were not compared.

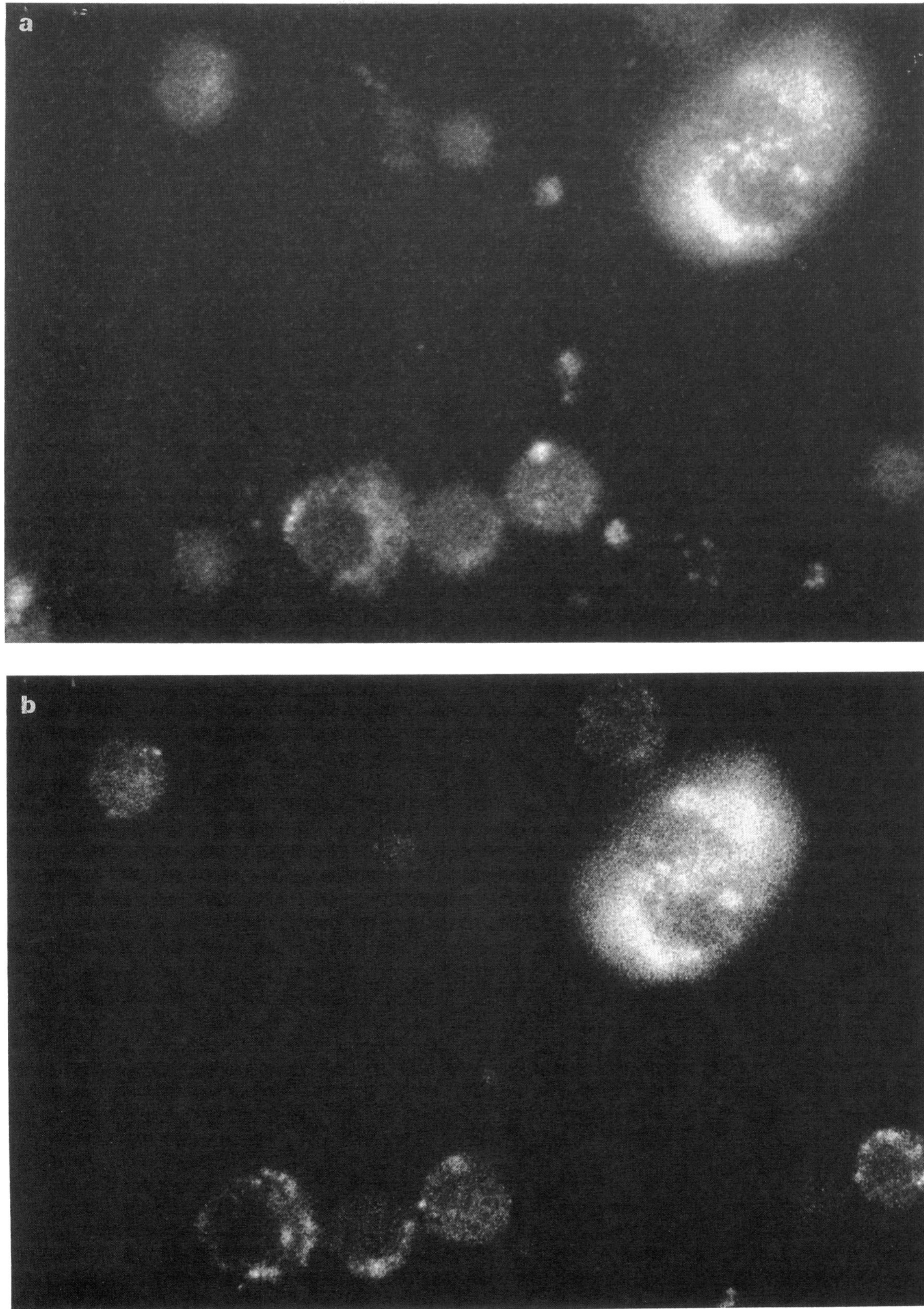

Fig. 1. (a) Double immunofluorescent staining of tumour necrosis factor-beta (TNF-β)-producing T cells. This figure demonstrates TNF-β-producing cells from synovial fluid of a patient with arthritis. The typical immunofluorescent FITC cytokine staining pattern of TNF-β is shown by the bright staining of the first and third cells in the group of three in the upper right portion of the figure. (b) CD2 staining. The same cells as above were stained with anti-CD2 conjugated with PE. The positive cells have peripheral surface staining as demonstrated in all four cells in the upper portion of the figure. When compared with a, it can be seen that the TNF-β-positive cells by FITC also were positive for CD2 by PE staining.

Table 1. Correlation of type of arthritis with cytokine production

Type of arthritis	Cytokine					
	TNF-α		TNF-β		IL-6	
	+	−	+	−	+	−
Pauciarticular	14/32	20/32	9/28	19/28	9/29	21/29
Polyarticular	6/14	6/14	5/11	6/11	4/13	8/13
Systemic	2/3	1/3	1/2	1/2	3/3	0/3
Total	22/49	27/49	15/41	26/41	16/45	29/45

+, Number positive/number of samples; −, number negative/number of samples.

Comparison of the patients with at least one cytokine produced (cytokine-positive) with patients who were negative for all three cytokines (cytokine-negative), revealed that cytokine-positive patients tended to be older at presentation and tended to have higher IgG levels (Table 2). When individual cytokine production was examined, there was no statistical difference in any of the clinical or laboratory parameters for TNF-α-positive and TNF-α-negative patients, although the TNF-α-positive patients tended both to be older at diagnosis of their arthritis, and to have a higher ESR (data not shown). In the IL-6 group there was a statistically different total SF leucocyte count, with the IL-6-positive patients having a mean count of $20{\cdot}6 \times 10^9$ compared with the IL-6-negative patients with a mean count of $14{\cdot}7 \times 10^9$ ($P = 0{\cdot}0071$), and there tended to be a higher joint count, with the IL-6-positive patients having a mean joint count of 13 compared with the IL-6-negative patients with a mean joint count of 6 (Table 3).

The use or type of systemic medication did not influence cytokine production. Medication used included non-steroidal anti-inflammatory drugs (NSAID), methotrexate and corticosteroids (Table 4). All five patients who were not on any systemic medication were cytokine-negative. However, joint injection, if performed within the previous 12 months, appeared to influence the production of cytokine. Only 3/18 patients with a steroid injection in the tested joint within 12 months of the study had detectable cytokine, compared with 17/32 who had not received a previous steroid injection ($P = 0{\cdot}001$) (Table 5).

In 37 (93%) samples there was concordance between TNF-α and TNF-β, with both cytokines being absent or present together; the remaining two samples were positive for TNF-α alone. However, comparing IL-6 and TNF-α, there was a lower rate of concordance, with 33/45 (75%) samples having both IL-6 and TNF-α either present or absent together, while in 12/45 samples, eight were positive for TNF-α and four samples were positive for IL-6 only. Similarly for TNF-β and IL-6, where 28 (76%) samples were concordant but nine samples had only one cytokine present: six were positive for TNF-β alone and three were positive for IL-6 alone.

During the course of the study five patients had repeat joint injections performed on the same joint. In two patients cytokines were not detected at either the initial or the repeat examinations. One patient had three samples taken over a 2-year period. In this patient neither TNF-α nor TNF-β were detected at any of the three arthrocenteses. IL-6 was present only in the initial but not in the subsequent samples. In the fourth patient, TNF-α and TNF-β were detectable on both examinations. The final patient had TNF-α and TNF-β detected at both examinations, while IL-6 was present at the first examination but not at the second examination.

In order to determine the phenotype of the cytokine-secreting cell, we attempted double staining of the SF mononuclear cells for cytokine and cell surface phenotype in 26 patients. Positive cytokine immunofluorescence was detected in only nine of these patients. In four of these nine patients using CD3 and Mo-2 to detect T cells and monocytes respectively, we did not detect any cell which was positive for both cell surface marker and cytokine. This is probably the result of having too few cytokine-positive cells to detect the double-staining cells. However, in five patients using cell surface staining with CD2 and/or Mo-2, simultaneous cell surface and cytokine staining was found. TNF-α/Mo-2 double-staining cells were seen in 4/4

Table 2. Correlation of patients' clinical and laboratory parameters with cytokine production

Patient characteristics	Cytokine production: Present (mean ± s.d.)	Cytokine production: Absent (mean ± s.d.)	P
Age at presentation (years)	8 ± 4·7	4·7 ± 3·9	0·01*
Age at diagnosis (years)	8·1 ± 4·8	4·75 ± 4·1	0·008*
Disease duration (years)	6·3 ± 4·8	4·7 ± 3·8	0·23
SF total leucocyte count ($10^{-9}/l$)	15·9 ± 11·9	13·5 ± 14·0	0·5
SF monocyte count (% of total joint leucocyte count)	45·8 ± 32	35·3 ± 23·7	0·25
ESR (mm/h)	36 ± 36	20·2 ± 15	0·24
Leucocyte count ($10^{-9}/l$)	9·24 ± 2·1	8·24 ± 1·8	0·03
Total serum protein level (g/l)	75·6 ± 6·4	76·8 ± 5·5	0·8
Serum albumin level (g/l)	38 ± 3·9	41 ± 3·5	0·3
Serum IgG level (g/l)	14·1 ± 4·2	11·65 ± 1·1	0·012*
Joint count	11 ± 11·3	5 ± 9·5	0·17

*Not statistically significant after correction for multiple testing.
SF, Synovial fluid; ESR, erythrocyte sedimentation rate.

Table 3. Presence or absence of IL-6 production in comparison with patients' laboratory and clinical parameters

	IL-6		
	Present (mean ± s.d.)	Absent (mean ± s.d.)	*P*
Age at presentation (years)	8±5	5·5±4·7	0·57
Age at diagnosis (years)	7·9±5·4	6·1±5	0·68
Total SF leucocyte count ($10^{-9}/l$)	20·6±8	14·7±11·6	0·007*
SF monocyte count (as % of total leucocyte count)	44±33	40·4±26·9	0·4
ESR (mm/h)	39±40	22±23	0·09
Peripheral blood leucocyte count ($10^{-9}/l$)	9·2±1·5	8·3±1·7	0·2
Serum protein (g/*l*)	75±7·6	77±7·1	0·2
Serum albumin (g/*l*)	7·6±4·5	41±1·4	0·87
Serum IgG (g/*l*)	15·4±4	10·3±2·4	0·2
Joint count	13±12	6·2±9	0·02†

* Statistically significant after Bonferroni correction.
† Not statistically significant when corrected for multiple testing.
SF, Synovial fluid; ESR, erythrocyte sedimentation rate.

Table 4. Comparison of drug administration and production of intracellular cytokine

	Cytokine	
	Produced ($n=27$)	Not produced ($n=23$)
*Drug**		
NSAID ($n=45$)	24	21
Methotrexate ($n=5$)	1	4
Corticosteroids ($n=8$)	4	4
Nil ($n=5$)	0	5

* Patients could be on more than one medication.
NSAID, Non-steroidal anti-inflammatory drug.

patients, IL-6/Mo-2 double-staining in 4/4 patients and TNF-*β*/CD2 in 1/3 (Table 6). Therefore, as expected we confirmed monocytes as the source of IL-6 and TNF-*α*, and T cells as the source of TNF-*β*. We did not detect any TNF-*α*- or IL-6-secreting $CD2^+$ or $CD3^+$ T cells.

In 12 patients, peripheral blood mononuclear cells (PBMC) were collected at the same time as the SF samples. In all 12 patients there was no evidence of spontaneous peripheral blood cytokine production. Absence of spontaneous peripheral blood cytokine production was also found in 10 healthy controls.

DISCUSSION

Cytokines are important proteins in normal cellular function. They operate through a series of intricate networks, which can regulate their secretion in an autocrine and paracrine manner. In addition, naturally occurring inhibitors can modify the effects of secreted cytokines [2]. Subsequent pathologic effects are probably mediated by a change in this delicate balance, as an increase or decrease in secretion of various cytokines or their inhibitors is capable of producing significant abnormalities in target tissues. Studies on joint damage in adult RA patients provide a good example of this theory. It appears that joint inflammation and damage is secondary to an increased production of macrophage products such as IL-1, TNF-*α* and IL-6 and an associated down-regulation of lymphocyte products including TNF-*β* or interferon-gamma (IFN-*γ*) [5,13]. We undertook this study to define the cytokine repertoire in SF in JRA. We found evidence of macrophage products, TNF-*α* and IL-6, and we also demonstrated that these cytokines are produced by SF macrophages. Significantly, we were also able to demonstrate the presence of TNF-*β*-producing T cells in the synovial fluid. This demonstrates that T cells as well as macrophage products may play a role in the synovitis of JRA.

Despite the fact that cytokines are probably important contributors in the continuing damage in the rheumatoid joint, the presence or absence of individual cytokines alone may not be sufficient to produce arthritis. *In vitro*, TNF-*α* induces cartilage resorption, inhibits proteoglycan release, and alone or in association with other cytokines, such as IL-6, induces synoviocyte proliferation [1,2,13]. However, *in vivo*, TNF-*α* may play only a minor role in joint inflammation, as intra-articular injections of TNF-*α* into rabbit knees resulted in only a mild synovitis which was less than that produced by either IL-1 or a combination of IL-1 and TNF-*α* [14,15]. In the chronically inflamed joint, TNF-*α* has been believed to be secreted primarily by synovial lining cells and interstitial macrophages, and TNF-*α* has been detected in at least 50% of the SF samples studied from patients with RA, and at greater levels than in other arthritides [2,16,17]. Although there is uncertainty as to the exact role of TNF-*α* in joint inflammation, these studies found a correlation with active disease, as defined by a high synovial leucocyte count, elevated ESR, and low haemoglobin and serum iron levels and elevated concentrations of TNF-*α*. We provide evidence that in JRA, SF macrophages secrete TNF-*α*. We were unable to correlate any measured parameter of inflammation,

Table 5. Effect of intra-articular steroid (within the past 12 months) on synovial fluid cytokine production

Steroid treatment	Cytokine present in synovial fluid	Number of patients tested	Per cent positive
No prior intra-articular steroid	17	32	53
Previous intra-articular steroid	3	18	17

Table 6. Synovial fluid cell origin of cytokine production

	Cell surface phenotype		
Cytokine	Mo-2 (monocyte)	CD3/CD2 (pan T cell)	CD4 (helper T cell)
IL-6	4/4	0/3	0/1
TNF-α	3/4	0/3	0/1
TNF-β	ND	1/5	0/1

including SF leucocyte count, ESR and IgG levels, and the SF cell production of TNF-α.

TNF-β acts in synergy with TNF-α [1]. These two cytokines share the same receptor and mediate similar responses. Either alone or with TNF-α, TNF-β has been shown to stimulate cartilage breakdown *in vitro* and could therefore play a role in the pathogenesis of arthritis [1]. We detected TNF-β in 36% of JRA patients, but we were again unable to correlate the presence of TNF-β and increase in SF leucocyte count or other parameters of inflammation. However, the presence of TNF-β, a T cell product, implicates T cells in the development or persistence of the synovitis in JRA. This is in contradiction to the adult studies, where T cell products, such as TNF-β and IFN-γ, have been identified only in very small amounts in RA SF [16,17]. The difference in TNF-β production again emphasizes the difference between patients with adult RA and JRA. In fact JRA resembles adult RA in only 5–10% of patients in terms of both clinical and laboratory features and prognosis. Therefore it may not be surprising that disease pathogenesis may also be very different. In our study, only three of our patients had rheumatoid factor-positive arthritis, a number too small for meaningful comparison.

In the joint it has been felt that IL-6 probably acts to augment the effects of IL-1 and TNF-α rather than directly to cause joint damage [2,18]. IL-6 also produces systemic effects such as an increase in acute phase reactants, and is capable of fever induction [18]. We detected IL-6 in 36% of patients, and were able to correlate the presence of IL-6 with a higher SF total leucocyte count. There was no association of IL-6 with laboratory parameters of systemic inflammation such as raised ESR or hypergammaglobulinaemia. Increased serum IL-6 levels have been documented during periods of systemic disease activity in patients with systemic JRA [18]. It appears IL-6 could therefore be used as an indicator of inflammation in JRA, with its presence suggesting increased disease activity or severity [18,19].

Cytokines are secreted by activated T lymphocytes and macrophages or monocytes. As we were unable to detect any cytokine in the simultaneously obtained peripheral blood samples, SF cytokine-producing cells may either be activated in the joint, or activated cells may home to the joint. It has been previously demonstrated in JRA and RA that there is an increased number of activated SF T cells (DR^+) compared with peripheral blood [20–24]. More recently we have shown both an increase in DR^+ SF T cells and an increase in the $CD4^+$ $CD29^+$ cells in the joint [25]. The $CD4^+$ $CD29^+$ cells are not only 'memory cells', which respond to recall antigen, but the CD29 molecule recognizes the common β chain of the VLA family of molecules [26]. This family of molecules may be important as homing receptors and as receptors for extracellular matrix proteins such as collagen [27]. Therefore the TNF-β-secreting T cells could either have been activated in the joint or were activated peripherally but 'homed' to the inflamed joint. Similarly, SF monocytes, which we have shown to secrete TNF-α and IL-6, can be activated as they traverse the synovium, can be activated in the SF, or can be activated peripherally and target the inflamed joint. Whatever the mechanisms, we have shown that both SF T cells and macrophages produce cytokines which are capable of mediating cartilage damage.

Treatment may be an important modifier of the cytokine repertoire in synovial fluid. NSAIDs have been shown *in vitro* to modify some of the effects of IL-1, but the addition of prednisone was required for complete ablation of the effect of IL-1[28]. Most of our patients were on an NSAID. However, five patients were on no treatment at the time of arthrocentesis, and we could not detect any cytokines in these five samples. It may be that some of these children had been given small amounts of an NSAID before the joint aspiration without our knowledge. However, it appears that NSAIDs do not significantly modify cytokine production. In contrast, intra-articular corticosteroid injection was a powerful inhibitor of cytokine production. In the patients who had serial arthrocenteses less than 12 months apart, IL-6 production which initially had been positive became negative. However, TNF-α and TNF-β were persistently detected in the one patient with less than 1 year between joint injections. Significantly fewer patients had cytokines detected if they had been given a steroid joint injection in the tested joint within 1 year of our study. This suggests that at least part of the anti-inflammatory effect of corticosteroid is probably through cytokine modulation.

We found that children whose SF cells produced cytokines tended to be older at onset of their arthritis than the cytokine-negative group. There was no correlation of cytokine production with any measure of disease activity. To our knowledge cytokine production does not appear to be age-dependent. One reason for our finding of the correlation of age and cytokine production may be that generally younger children tend to have a more self-limited, less destructive form of arthritis. This is true

when comparing young ANA-positive pauciarticular patients with the older HLA B27$^+$ pauciarticular patients, and when comparing RF-negative polyarticular patients and the older RF-positive polyarticular JRA patients. As our polyarticular and pauciarticular groups were too small to subdivide by age, this may also explain the lack of correlation of cytokine production and disease subtype. A much larger study would be required to address this issue.

Our data demonstrate that in JRA synovial fluid T cells and macrophages both locally produce cytokines. This SF cell production of proinflammatory cytokines may be the cause of the ongoing local inflammatory process characteristic of JRA. This finding further supports our previous observation implicating a direct role for SF cells in the pathogenesis of joint destruction in JRA [25]. Therefore modulation of activated SF T cell and monocyte cytokine production provides a new and exciting avenue for drug therapy able to decrease cartilage destruction and improve long-term joint function in JRA.

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