Tumour necrosis factor alpha and its soluble receptors in juvenile chronic arthritis

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Abstract

Objective. To identify possible imbalance of tumour necrosis factor alpha (TNFα) and its soluble receptors in the different subgroups of juvenile chronic arthritis (JCA).

Methods. Serum and synovial fluid samples from 45 children were examined, 25 pauciarticular JCA, 13 polyarticular JCA and seven spondyloarthropathy. TNFα, sTNFRI and sTNFRII levels were measured by EASIA and enzyme-linked immunosorbent assay (ELISA). Analysis of the results was carried out using non-parametric tests: Kruskal–Wallis one-way analysis of variance was used to compare the three clinical subgroups; the Mann–Whitney U-test was used to compare group medians.

Results. Thirty-three serum samples were assayed for TNFα. There was no significant difference between the three groups using the Kruskal–Wallis analysis of variance. Analysis of synovial fluid TNF levels showed significantly lower levels in the spondyloarthropathy group compared with the pauciarticular JCA (P = 0.01) and the polyarticular group (P = 0.002). Significantly higher levels of sTNFRI were observed in the synovial fluid of the polyarticular JCA group compared with the pauciarticular JCA group (P = 0.004) and similarly for sTNFRII (P = 0.03). Molar ratios were calculated for TNF vs sTNFRI. The sTNFRI/TNFα ratio was significantly higher in the spondyloarthropathy group compared with the pauciarticular group and the polyarticular group (P = 0.01) and compared with the polyarticular group (P = 0.05).

Conclusion. These results suggest that the increased joint destruction observed in polyarticular disease compared with the other two subtypes may be related to the lower sTNFR/sTNFα ratios observed.

Key words: Juvenile chronic arthritis, Synovial fluid, Cytokines, Tumour necrosis factor alpha, Soluble TNF receptors.

Juvenile chronic arthritis (JCA) refers to a group of idiopathic arthritides of childhood. Although arthritis is the salient feature of all these diseases, there are important clinical differences which suggest that the underlying pathology may be different.

Pauciarticular JCA, involving four or fewer joints [1], usually runs a benign course. While clinically quite marked inflammation can be observed in individual joints, destruction of that joint is an uncommon finding long term [2, 3]. In contrast, polyarticular disease is frequently associated with joint damage and erosive changes [4, 5]. While serological indices of inflammation are more frequently raised in polyarticular disease, there is little to distinguish between the clinical findings of these two subgroups with respect to individual joints. The juvenile spondyloarthropathies are members of a different clinical group. Although the laboratory markers of inflammation can be impressive, the natural history of juvenile spondyloarthropathies is towards new bone formation, fibrosis and ankylosis rather than bone resorption [6–8]. This would suggest that different mechanisms of inflammation and/or repair occur in the subgroups of JCA.

The inflammatory cytokines have long been implicated in the pathogenesis of inflammation and in particular arthritis [9–12]. Tumour necrosis factor alpha (TNFα) exhibits many biological actions both in vitro and in vivo pertinent to arthritis. It can induce an inflammatory response, collagenase production [13, 14], bone and cartilage resorption [15, 16] and cachexia [17]. TNFα is one of the primary stimulants of interleukin-1 (IL-1) in synovial cell cultures of adult rheumatoid arthritis patients [18]. This, along with its ability to induce HLA class I molecule expression on vascular endothelial cells and dermal fibroblasts [19], suggests
that TNFα plays a central role in the cytokine network in rheumatoid arthritis as well as cellular immunity.

The cytokine network is essential to host defence and thus regulatory mechanisms are essential for homeostasis [20, 21]. Several mechanisms are currently known to down-regulate the pro-inflammatory cytokines including anti-inflammatory cytokines [22, 23] and cytokine antagonists, which are present in synovial fluids [24, 25]. TNFα binds to two high affinity cell surface receptors, TNFR1 and TNFRII, which are present on virtually all cell types [26, 27]. The extracellular domains of these receptors exist in a soluble form and have been shown to bind circulating TNFα and thus inhibit its biological activity [28]. Among the factors which may influence cellular responses induced by TNFα are the number of cell-associated receptors and the concentration of soluble TNFRI and TNFRII receptors in the extracellular fluid.

The relative proportion of TNFα to its soluble receptors in biological fluids has proved to be important in a number of clinical conditions [29–32]. In meningococcal septicaemia, a diminished ratio of soluble TNFRI and TNFRII to TNFα was observed in those patients with a fatal outcome compared with survivors [30]. We postulated, therefore, that levels of TNFα and its soluble receptors might be different in the synovial fluid of the various subgroups of JCA thus reflecting their contrasting clinical courses.

**Patients and methods**

**Patients and clinical characteristics**

Fifty children with JCA according to the EULAR criteria [1] entered the study. All required intra-articular steroid injection for synovitis of the knee. None had had a steroid injection to that knee in the preceding 6 months. Clinical details recorded included subtype of JCA, age, sex, disease duration and current medication. The degree of joint inflammation observed clinically was scored on a scale of 1–3 indicating mild, moderate or marked synovitis, by the same observer. Three children had systemic JCA and due to their small numbers were excluded from the study. Two children, one from the polyarticular group and one from the pauciarticular group had synovial fluid TNFRII and RII/TNFα ratios 10 times higher than the maximum ratios observed in either group and were excluded as these results were probably incorrect, and a re-test was not possible due to the small number of samples available. Thus, 45 children completed the study in which 47 knees were aspirated. There were 25 in the pauciarticular group, 13 in the polyarticular group and seven with spondyloarthropathy. Values for erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) were available on 12 pauciarticular, seven polyarticular and four patients with spondyloarthropathy and the clinical details and results are included in Table 1.

Synovial fluid was aspirated using an aseptic technique. Samples were immediately spun and stored at −70°C until assayed.

Blood samples were available on 31 children. CRP was measured by nephelometry and ESR using the Westergren method. Serum samples were spun and rapidly frozen at −70°C until used.

Ethical Committee approval was obtained for this study at Great Ormond Street Hospital National Health Service Trust and patient assent and parent consent given.

**Cytokine determination**

TNFα determination in synovial fluid and serum. Synovial fluid and serum samples were initially assayed for TNFα by EASIA (Medgenix, Fleurus, Belgium) with a limit of sensitivity of 20 pg/ml. In 60% of samples assayed, TNFα levels were below the limit of detection of the assay. These negative samples were subsequently reassayed using a high sensitivity enzyme-linked immunoassay technique. Samples were immediately spun and stored at −70°C until used. The relative proportion of TNFα to its soluble receptors in biological fluids has proved to be important in a number of clinical conditions [29–32]. In meningococcal septicaemia, a diminished ratio of soluble TNFRI and TNFRII to TNFα was observed in those patients with a fatal outcome compared with survivors [30]. We postulated, therefore, that levels of TNFα and its soluble receptors might be different in the synovial fluid of the various subgroups of JCA thus reflecting their contrasting clinical courses.

**Determination of soluble TNF receptors R1 and RII in synovial fluid and serum.** Initial samples were assayed by a method previously reported [25]. Subsequent samples were assayed by ELISA (R & D Systems Europe, Abingdon, Oxon) with a limit of sensitivity of 2 pg/ml. Both assays were chosen as their antibodies detected both free and complexed TNFα (as confirmed by the manufacturers). Intra-assay variation was 11.7% with an interassay variation of less than 10% for the individual assays used. There was a tendency for the high sensitivity kit to give lower values to high TNFα levels than observed using the low sensitivity kit. This was probably due to the fact that the TNFα antibodies used in these kits are directed against different TNFα epitopes, the different standards used for the calibration curves, or to a different sensitivity because of interference with other proteins. Since the values obtained from these two assays were not linearly related, the results obtained from both assays were analysed separately using non-parametric tests (vide infra).

**Statistical analysis**

Since specimens were available in limited amounts only it was not possible to test them by both assays. As synovial fluid values for the TNFα and TNFRI and TNFRII were obtained by different assays, non-parametric analysis by the Kruskal–Wallis one-way analysis of variance was used to compare the three clinical subgroups. Subsequently the Mann–Whitney U-test was used to compare each pair of group medians. For analysis of TNFα values using the low sensitivity kit,
samples below the limit of detection were given a value of 20 pg. For analysis of results obtained using the high sensitivity kit, values were censored at 33 pg, the highest value observed using this assay. The Bonferroni correction was used to adjust the \( P \) values obtained from the Mann–Whitney \( U \)-tests to take into account multiple comparisons.

All serum samples were assayed by the same assay for TNF, sTNFRI and sTNFRII and values were found to be normally distributed if log transformed using Shapiro and Francia’s \( W \)-test. However, the assumption of constant variances were not valid. Therefore, the results were expressed as medians and analysed by the Kruskal–Wallis one-way analysis of variance.

### Results

Thirty-three serum samples were assayed for TNF\( \alpha \) and all samples were assayed by the high sensitivity kit from 17 pauciarticular JCA, 10 polyarticular JCA and six spondyloarthropathy patients (Fig. 1). Although the median value for the polyarticular group appears higher than the median of the other two groups, this was not significant.

In 16 patients sTNFRI and II were measured. All samples were measured by the same assay system [27] as were the synovial fluid samples used for comparison. For all patients the mean serum sTNFRI was 1.25 (range 0.6–2.1 ng/ml). The level of serum sTNFRII was twice that of sTNFRI, mean 3.5 (range 2.3–6.0 ng/ml) \( P = 0.001 \). Insufficient serum samples were available for analysis of sTNFRs within the disease subgroups.

Synovial fluid TNF\( \alpha \) levels were compared in the three subgroups and the results obtained using both the low and high sensitivity assays are shown in Fig. 2a, b. Using the low sensitivity assay, TNF\( \alpha \) levels were higher in the polyarticular group [median 64, 95% confidence interval (CI) <20–136 pg/ml] when compared with the pauciarticular group (median <20, 95% CI <20–36 pg/ml) but not significantly so, \( P = 0.12 \) (Fig. 2a). The lowest levels of TNF\( \alpha \) were observed in those children with spondyloarthropathy (median <20, 95% CI <20–20 pg/ml) which were significantly lower than the polyarticular group, \( P = 0.02 \), but not the pauciarticular group, \( P = 0.16 \).

Using the high sensitivity assay, TNF\( \alpha \) levels were again highest in the polyarticular group (median >33, 95% CI 18 to >33 pg/ml) (Fig. 2b). Intermediate values were observed in the pauciarticular group (median 30.4, 95% CI 13.5 to >33 pg/ml), which were not significantly different than the polyarticular group, \( P = 0.3 \). The lowest levels were observed in the spondyloarthropathy group (median 9, 95% CI 5.9–18.6 pg/ml), which were significantly lower than both the pauci- and polyarticular groups, \( P = 0.01 \) and \( P = 0.002 \), respectively.

Significantly higher levels of sTNFRI were observed in the synovial fluid of the polyarticular group than in the pauciarticular group (median 9.2, 95% CI 7.3–11.4 pg/ml vs median 6.3, 95% CI 5.6–7.5 pg/ml) \( P = 0.004 \) (Fig. 3a). Levels were intermediate in the
spondyloarthropathy group but were not significantly different from the other two groups ($P = 0.4$ and $P = 1.0$, respectively).

As with sTNFRI, significantly higher sTNFRII levels were observed in the polyarticular group when compared with the pauciarticular group (median 12.8, 95% CI 11.5–17.9 pg/ml vs median 9.7, 95% CI 7.4–12.1 pg/ml) $P = 0.03$ (Fig. 3b). Again there was no significant difference between the subset with spondyloarthropathy (median 8.0, 95% CI 2.0–14.5 pg/ml) and the other two groups ($P = 0.11$ and $P = 1.0$, respectively). Analysis of 16 paired serum and synovial fluid samples revealed sTNFRII to be almost eight times higher than serum sTNFRI: mean 9.1 (range 5.0–16.0 ng/ml) vs mean 1.25 (range 0.6–2.1 ng/ml) $P = 0.001$. sTNFRII levels were four times higher in synovial fluid than in serum: mean 13.7 (range 7.8–28.9 ng/ml) vs mean 3.5 (range 2.3–6.0 ng/ml) $P = 0.001$.

Since the biological activity of TNFα appears to be related to the ratio of TNFα to its soluble receptors in vivo, these were calculated for the synovial fluid samples using the results of the high sensitivity assay. Molar ratios were calculated taking the molecular weight of TNFα to be 17 kDa. Since TNFα circulates in a trimolecular complex, apparent molecular weight values for TNFα were taken to be 51. Values obtained using the high sensitivity assay were used. The molecular weights of sTNFRI and sTNFRII were taken to be 55 kDa and 75 kDa respectively, and molar concentrations of TNFα and its receptors calculated accordingly. The results are displayed in Fig. 4a–c. The TNFRI/TNFα ratio was much higher in the spondyloarthropathy group (median 815, 95% CI 581–1447) than in the pauci- and polyarticular groups (median 286, 95% CI 224–468; $P = 0.003$ and median 353, 95% CI 257–447; $P = 0.003$; respectively). There were no significant differences between the polyarticular and pauciarticular groups ($P = 1.0$ and $P = 0.11$, respectively). Analysis of 16 paired serum and synovial fluid samples revealed sTNFRII to be almost eight times higher than serum sTNFRI: mean 9.1 (range 5.0–16.0 ng/ml) vs mean 1.25 (range 0.6–2.1 ng/ml) $P = 0.001$. sTNFRII levels were four times higher in synovial fluid than in serum: mean 13.7 (range 7.8–28.9 ng/ml) vs mean 3.5 (range 2.3–6.0 ng/ml) $P = 0.001$.

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Discussion

A number of clinical findings differentiate JCA from adult rheumatoid arthritis. In all forms of JCA erosive change is much less commonly seen. On the contrary, new bone formation leading to ankylosis is a frequent occurrence particularly in the spondyloarthropathies [7]. In pauciarticular disease, even with recurrent episodes of inflammation within a given joint, neither erosion nor new bone formation is common, particularly in early disease [4]. In adult rheumatoid arthritis the inflammatory cytokines have been implicated in the observed erosive disease. TNF is widely expressed and detected in the tissues and fluid of the synovial joint, and has been shown to induce collagenase production [13] and to resorb both cartilage and bone both in vivo and in vitro [15, 16]. We and others have shown that the inflammatory cytokines are involved in the different subgroups of JCA [12, 33–36]. Lepore et al. observed elevated levels of IL-6 and TNFα in the synovial fluid of children with JCA [33]. Mangge et al. [35] noted a correlation between laboratory markers of inflammation and IL-6, TNFα and the soluble IL-2 receptor in JCA, which were markedly elevated in children with systemic disease. However, the soluble TNFRI receptor was the best indicator of disease activity [35]. Prieur et al. have shown that sTNFRII but not TNFα correlated with the fever spikes in children with systemic JCA [34].

In recent years there has been increasing interest in the role of the soluble TNF receptors in a variety of diseases [30–32]. Girardin et al. [30] noted that in children with severe meningococcal sepsicaemia, levels of sTNFRI and sTNFRII initially increased as TNFα increased. However, at TNFα levels greater than 500 ng/ml no further increase in the soluble receptors was observed and this was associated with a high mortality. Thus, the investigators concluded that an imbalance between TNFα and its naturally occurring inhibitors was implicated in the increased morbidity and mortality. Van Zee et al. [31] observed that soluble TNF receptors increased with inflammation in experimental bacteraemia and circulate at sufficient levels in critically ill patients to block TNFα cytotoxicity in vitro. This would suggest that in experimental bacteraemia, soluble TNF receptors protect against the TNF-mediated effects observed in septic shock.

The clinical situation is somewhat different in JCA when compared with acute septic episodes. First, serum TNFα levels are either low or undetectable and certainly never reach the levels observed in septic shock. However, in our study synovial fluid levels in the 100 pg/ml range were not unusual and unlike acute septic episodes may remain elevated for months or years while synovitis persists.
In contrast to our findings, Madson et al. [36] observed increases in a number of inflammatory cytokines but not TNFα in both the serum and synovial fluid in JCA. The explanation for this discrepancy may be a technical one, in that a number of anti-TNF antibodies used in assays do not detect complexed TNFα, its predominant form in biological fluids [37]. Cope et al. [24] and Roux-Lombard et al. [25] observed increased levels of both TNFα and its soluble receptors in the synovial fluid of patients with rheumatoid arthritis and that sTNFRs were four to five fold higher in synovial fluid than in serum levels. These results concur with our own where synovial fluid sTNFRs were on average six times greater than serum levels. Furthermore, as they observed, synovial fluid sTNFRII levels were on average 1.5 times higher than TNFRI. Interestingly, in our patients with JCA serum sTNFRII was twice as high as sTNFRI. This contrasts with the findings in sepsis where sTNFRII levels are at least three to four times higher than sTNFRI. These variations in the ratios of the sTNFRs in different disease states and biological fluids may be due to the different standards used in the individual assays employed. Alternatively, they may reflect the different mechanisms of shedding of the two receptors, in particular variations in responsiveness of TNFRI and TNFRII to TNFα stimulation [38], or the effect of proteolytic enzymes, particularly elastase or other metalloproteinases, known to be present in inflamed joints [39]. Furthermore, the cell populations within the joint are quite distinct from those observed in the peripheral blood. Thus, the observed sTNFRI and RII levels may reflect different receptor expression known to occur on different cell types [28–40]. Since the vast majority of synovial fluid cells are neutrophils it is surprising that TNFRII, the most abundant polymorphonuclear leukocytes TNFR, is not higher in JCA synovial fluid when compared with serum. However, the relative increase of TNFRII might be explained by endothelial activation, where TNFRI is in greatest abundance.

In this study we observed the highest levels of synovial fluid TNFα in patients with polyarticular disease, with significantly lower levels observed in the pauciarticular group. However, the lowest levels were observed in children with spondyloarthropathy. These findings are not related to the degree of knee joint inflammation observed clinically, since similar proportions of moderate and marked knee joint inflammation were present in the polyarticular and spondyloarthropathy groups.

For all children there was a tendency for the sTNFRs to rise with increasing TNFα levels (data not shown) and this concurs with the findings in other inflammatory conditions [30]. However, in the spondyloarthropathy group, despite having the lowest levels of TNFα, the levels of sTNFRI and RII were similar to the other two groups. Thus, significantly higher ratios of sTNFRs/TNFα were observed in this group. High concentrations of sTNFRs have been shown to abrogate the biological activity of TNFα both in vitro and in vivo [38]. While 300–500 molar excess may be required for almost complete loss of TNFα bioactivity, as little as 30 molar excess of the soluble receptors can have some inhibitory activity [31]. In our study, sTNFRs were present in between 100 and 5000-fold molar excess, thus it is conceivable that there would be considerable differences in the bioactivity of TNFα in synovial fluid with low and high sTNFR levels. Since significantly lower ratios were observed in the polyarticular group (on average 180 molar excess) when compared with the spondyloarthropathy group (molar excess 770), it could be anticipated that the resorptive effects of TNFα on bone and cartilage within the joint in the former group would be expected to be more marked when compared with the latter. This would provide at least part of the explanation for the different clinical courses of these disease subgroups. It could be argued that disease-modifying and anti-inflammatory agents such as methotrexate, sulphasalazine and steroids might in themselves alter cytokine and receptor concentrations. These drugs were taken by approximately 50% of both the spondyloarthropathy and polyarticular groups: 3/7 children with spondyloarthropathies were given sulphasalazine compound, and 6/13 children with polyarticular JCA were treated with methotrexate.

In conclusion we have observed a significant difference in the levels of TNFα and the sTNFRs TNFα ratios in the different subgroups of JCA. Whilst recognizing that TNF is only one of the many cytokines, not to mention growth factors, involved, and this system of agonist/antagonist is only one of many methods of regulation which might influence bone destruction and remodelling in JCA, these findings reflect different pathophysiologicals at play within the different subgroups of JCA, in particular, juvenile spondyloarthropathy.

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References


