Elevated Concentrations of Monocyte Derived Cytokines in Synovial Fluid of Children with Enthesitis Related Arthritis and Polyarticular Types of Juvenile Idiopathic Arthritis

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ABSTRACT. ***Objective.*** Cytokines are the major mediators of joint damage in chronic arthritis. Data on synovial fluid (SF) cytokine concentrations in patients with juvenile idiopathic arthritis (JIA), especially enthesitis related arthritis (ERA), are limited. We measured levels of different monocyte derived cytokines, T cell derived cytokines, and a proinflammatory chemokine in SF specimens from children with ERA or polyarticular (Poly) rheumatoid factor (RF)-negative JIA.

Methods. Macrophage products [tumor necrosis factor-α (TNF-α), interleukin 1ß (IL-1ß), IL-6, IL-12p40)], T lymphocyte products [IL-2, IL-4, interferon-γ (IFN-γ)], and a proinflammatory chemokine (IL-8) were assayed using ELISA in SF specimens from 53 patients with JIA [ERA 34, polyarticular RF-negative 19] and 40 patients with rheumatoid arthritis (RA).

Results. In the ERA group, median SF cytokine levels were higher compared to RA (all values are pg/ml): IL-1ß [< 15.6 (< 15.6–213) vs < 15.6 (< 15.6–41); $p < 0.01$], IL-12p40 [236 (< 15.6–1714) vs 21 (< 15.6–520); $p < 0.0001$], and IL-6 [1139 (< 4.6–2187) vs 835 (< 4.6–875); $p < 0.0001$]. TNF-α and IFN-γ levels were similar to RA. IL-8 levels were significantly less than RA ($p < 0.0001$). The median levels of IL-1ß [39.4 (< 15.6–558) vs < 15.6 (< 15.6–41); $p < 0.0001$] and IL-12p40 [209 (< 15.6–849) vs 21 (< 15.6–520); $p < 0.0001$] were higher in patients with Poly-JIA compared to RA. TNF-α, IL-6, and IL-8 levels in Poly-JIA were comparable to RA. IL-2 and IL-4 were not detectable in any patient with JIA. Cytokine profile comparison between the 2 subsets revealed that the median IL-6 [1139 (< 4.6–2187) vs 790 (17.4–2119); $p < 0.01$] and IFN-γ levels [235 (< 4.6–600) vs < 4.6 (< 4.6–412); $p < 0.0001$] were higher in ERA than in Poly-JIA. In contrast, median IL-8 levels were higher in Poly-JIA [200 (3.8–200)] compared to ERA [74.6 (4–200); $p < 0.001$]. However, there was no difference in levels of TNF-α, IL-1ß, and IL-12p40 between patients with these 2 subtypes of JIA.

Conclusion. SF levels of IL-1ß and IL-12p40 are increased in both Poly-JIA and ERA as compared to RA. IL-6 levels were higher in ERA compared to RA. Levels of TNF-α were comparable to RA in both Poly-JIA and ERA. This suggests that joint inflammation in JIA is mediated predominantly by monocytes. In ERA the levels of IL-6 and IFN-γ are higher than in Poly-JIA. The increase in IFN-γ in children with ERA with undetectable IL-4 suggests a Th1-dominant immune response in this disease subset. (J Rheumatol 2005;32:1349–53)

Key Indexing Terms:
INTERLEUKINS JUVENILE RHEUMATOID ARTHRITIS
PATHOGENESIS CHEMOKINES ENTHESITIS RELATED ARTHRITIS

Juvenile idiopathic arthritis (JIA) is the most common cause of chronic arthritis in childhood. Synovitis in JIA is associated with persistent infiltration of synovium by mononuclear cells, comprising T lymphocytes and macrophages. These cells exert their action through production of various mediators including cytokines[1].

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Ms Saxena is supported by a fellowship from CSIR, India.

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Accepted for publication February 17, 2005.

In patients with systemic onset JIA, serum concentrations of interleukin 1 (IL-1), tumor necrosis factor-α (TNF-α), IL-8, and IL-6 have consistently been shown to be elevated during periods of disease activity compared to healthy controls as well as inactive disease, suggesting that monocyte derived cytokines play an important role in causation of this type of JIA[1-6]. However, studies on alterations in cytokine concentrations in patients with polyarticular (Poly-JIA) and oligoarticular JIA have shown no consistent pattern[1-6]; the variability may be attributed at least partly to variations in the specimens tested and partly to disease heterogeneity.

Using immunohistochemistry, Murray, *et al*[7] found expression of both Th1 and Th2 cytokines in synovial tissue. However, another study found a Th1 bias, with high levels

of interferon-γ (IFN-γ)[8]. In a study using intracellular cytokine staining, synovial fluid (SF) cells were found to produce both T cell and monocyte derived cytokines[9].

Data on cytokine levels in SF from patients with JIA are limited and contradictory, based on studies with small sample sizes[3,4,10-12]. Such findings, however, are important as they provide valuable information on key mediators of inflammation. In addition, the spectrum of JIA is different in people of the Indian subcontinent, with large numbers of children having enthesitis related arthritis (ERA) and polyarticular rheumatoid factor (RF)-negative arthritis[13]. The oligoarticular disease occurring in young girls with antinuclear antibody positivity is distinctly rare[14,15]. Further, there is male preponderance, in contrast to the female preponderance seen in Western countries[13-15].

We therefore investigated the concentrations of different monocyte derived cytokines (TNF-α, IL-1ß, IL-6, and IL-12p40), T cell derived cytokines (IL-2, IL-4, and IFN-γ), and a proinflammatory chemokine (IL-8) in SF specimens obtained from a group of children with ERA or polyarticular RF-negative JIA.

MATERIALS AND METHODS

Fifty-three SF specimens obtained from patients with JIA, diagnosed according to criteria of the International League of Associations for Rheumatology[16], at the time of therapeutic intraarticular corticosteroid injection were studied. All patients had active disease at the time of specimen collection. The parents or the patient gave informed consent for collection of SF.

SF specimens obtained from 40 patients with rheumatoid arthritis (RA) were used as inflammatory disease controls.

Cytokine estimation in SF. SF was centrifuged (2000 rpm, 10 min) to remove cellular debris and was stored at –80°C in aliquots until analysis. The sample was thawed only once to avoid degradation. Levels of various cytokines including TNF-α, IL-1ß, IL-6, IL-12p40, IL-2, IL-4, IFN-γ, and IL-8 in the SF were measured using enzyme immunoassays (Opt EIA human cytokine sets; BD, PharMingen, San Diego, CA, USA); assays were carried following manufacturer's instructions. In brief, capture antibodies against the cytokine of interest were coated overnight on 96-well ELISA plates (Nunc, Roskilde, Denmark). Plates were then blocked using phosphate buffered saline (0.15 M, pH 7.2) with 10% fetal calf serum. After washing, the wells were incubated with test specimens for 2 h. Bound cytokine was then detected using a biotinylated anticytokine antibody and avidin-horseradish peroxidase conjugate. Tetra-methyl-benzidine was used as the substrate, and absorbance was measured at 450 nm using an ELISA reader (Tecan). The minimum detection limits of the assays were as follows: TNF-α, IL-12p40, IL-2, and IL-1ß 15.6 pg/ml; IL-6 and IFN-γ 4.6 pg/ml; IL-4 7.8 pg/ml; and IL-8 3.8 pg/ml.

Statistical analysis. The data are shown as median (range), and cytokine levels are expressed as pg/ml. Mann-Whitney U test and Fisher's exact test were used for intergroup comparisons. Pearson test was used for correlation analysis.

RESULTS

Among our 53 patients, 34 had ERA and 19 had polyarticular RF-negative arthritis (Poly-JIA). The median age of these patients was 15 (3–35) years, the median age at onset of symptoms was 10 (1–16) years, and the median duration of disease was 5 (0.5–26) years (Table 1). In the RA control group (25 women, 15 men), median age was 35 years (20–51). No patient or control was receiving or had received anti-TNF agents.

IL-12p40 levels were higher in patients with ERA [236 (< 15.6–1714)] than in those with RA [21 (< 15.6–520); $p < 0.0001$]. Median IL-1ß levels were higher in patients with ERA [< 15.6 (< 15.6–213)] than in those with RA [< 15.6 (< 15.6–41); $p < 0.01$]. IL-6 levels were higher in ERA compared to RA ($p < 0.0001$). Median level of both TNF-α and IFN-γ was comparable to RA. Median IL-8 levels in ERA [74.6 (4–200)] were lower than in RA [111 (17–1333); $p < 0.001$].

In Poly-JIA the median levels of TNF-α [93 (< 15.6–1000)], IL-6 [790 (17.4–2119)], and IL-8 [200 (3.8–200)] were comparable to those in RA.

IL-12p40 levels were higher in Poly-JIA patients [209 (< 15.6–849)] than in RA [21 (< 15.6–520); $p < 0.0001$]. Median IL-1ß levels were higher in Poly-JIA patients [39.4 (< 15.6–558)] than in RA [< 15.6 (< 15.6–41); $p < 0.0001$]. Median IFN-γ levels in Poly-JIA patients [< 4.6 (< 4.6–412)] were lower than in RA [218 (< 4.6–1462); $p < 0.0001$] (Table 2).

IL-2 was not detectable in SF specimens from any patient with JIA and was detectable in only 2 patients with RA. IL-4 was undetectable in SF specimens from JIA patients, and was detectable in 4 patients with RA.

Positive correlation was found between IL-12p40 and IFN-γ levels ($r = 0.4$; $p < 0.01$) in patients with JIA. No correlation was found between TNF-α, IL-6, and IL-1 levels. There was no correlation between cytokine levels and age of the patient.

Subtype comparison. The median IL-6 [1139 (< 4.6–2187) vs 790 (17.4–2119); $p < 0.01$] and IFN-γ levels [235 (< 4.6–600) vs < 4.6 (< 4.6–412); $p < 0.0001$] were higher in ERA than in Poly-JIA. IFN-γ was detected more often in ERA (22/34) than in polyarticular RF-negative samples (2/19) ($p < 0.001$).

In contrast, median IL-8 levels were higher in Poly-JIA [200 (3.8–200)] compared to ERA [74.6 (4–200); $p < 0.001$]. However, there was no difference in TNF-α, IL-1ß, and IL-12p40 levels between patients with these 2 subtypes of JIA (Figure 1).

DISCUSSION

This is the first study to investigate elevated concentrations of monocyte derived cytokines and IFN-γ in synovial fluids obtained from patients with ERA. The levels were either higher than or similar to levels in patients with RA. In addition, patients with Poly-JIA also had elevated levels of monocyte derived cytokines and IL-8. IL-2 and IL-4 were undetectable in both types of JIA.

Elevated levels of monocyte derived cytokines like IL-6, TNF-α, and IL-1 in both ERA and Poly-JIA suggest that monocytes/macrophages play a major role in synovitis in

Table 1. Clinical details of patients with juvenile idiopathic arthritis.

	Enthesitis Related Arthritis, n = 34	Polyarticular RF-negative, n = 19
Age at presentation, yrs	15 (6–35)	16 (3–22)
Age of onset, yrs	10 (4–16)	8 (1–15)
Disease duration, yrs	5 (1–26)	6 (0.5–15)
Male:female	33:1	16:3
Treatment		
NSAID	19	3
Prednisolone	2	5
DMARD	5	6
None	8	5

NSAID: nonsteroidal antiinflammatory drugs; DMARD: disease modifying antirheumatic drugs.

Table 2. Synovial fluid cytokine concentrations in 3 groups. All values are expressed in pg/ml and as median (range).

	Enthesitis Related Arthritis, n = 34	Polyarticular RF-Negative JIA, n = 19	Rheumatoid Arthritis, n = 40
TNF-α	96.5 (< 15.6–2000)	93 (< 15.6–1000)	42 (< 15.6–1000)
IL-6	1139 (< 4.6–2187)	790 (17.4–2119)*	835 (< 4.6–875)***
IL-1ß	< 15.6 (< 15.6–213)	39.4 (< 15.6–558)	< 15.6 (< 15.6–41)*
IL-12p40	236 (< 15.6–1714)	209 (< 15.6–849)	21 (< 15.6–520)***
IFN-γ	235 (< 4.6–600)	< 4.6 (< 4.6–412)***	218 (< 4.6–1462)
IL-8	74.6 (4–200)	200 (3.8–200)**	111 (17–1333)**

* $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$ compared to ERA group.

patients with JIA. Previous studies have also shown elevated levels of SF IL-6, IL-1, and TNF-α in different subtypes of JIA[3,4,10,11]. Kutukculer, *et al*[3] found elevated levels of IL-6, TNF-α, and IL-1 in pauciarticular and polyarticular juvenile RA samples, whereas De Benedetti, *et al*[4] reported higher IL-6 levels in samples from patients with systemic onset disease as compared to RA.

We found higher IL-6 levels in ERA samples compared to Poly-JIA, but no difference in levels of IL-1ß and TNF-α. Rooney, *et al*[17] had found TNF-α levels to be higher in patients with polyarticular disease compared to pauciarticular juvenile RA and juvenile spondyloarthropathy. Most other studies[3,4,10] did not find any difference in different subtypes of JIA, but these studies either did not include patients with ERA or had a small number of patients with late onset pauciarticular JRA. Grom, *et al*[18], using immunohistochemistry and reverse transcription-polymerase chain reaction on synovial tissues, found similar expression of TNF-α in patients with Poly-JIA and ERA, but this was higher than in patients with pauciarticular disease.

TNF-α and IL-1 are potent inducers of inflammatory responses and act synergistically in inducing joint damage. Indeed, many stimuli induce both IL-1 and TNF-α simultaneously. However, we found no correlation between concentrations of these 2 cytokines in SF samples, suggesting that their secretion may be independent of each other. IL-6 is a potent stimulator of osteoclasts, and may thus induce the destruction of bone and cartilage.

This is the first study to observe elevated levels of the monocyte derived cytokine IL-12p40 in SF of patients with JIA. In 2 previous studies[19,20], only serum levels of IL-12p40 were measured. Higher serum levels of IL-12p40 were found in children with active JIA as compared to children with inactive disease and healthy children[19]. All our patients had active disease and hence we could not study the relationship of this variable with disease activity. IL-12p40 is a strong chemoattractant for macrophages, and also assists in selective expansion of Th1 cells[21]. Indeed, in our study a good correlation was found between levels of IL-12p40 and IFN-γ.

ERA is quite similar to adult spondyloarthropathy, a disease in which a microbial trigger is supposed to play a major role in pathogenesis. Recently it has been shown that microbial products such as peptidoglycan, lipopolysaccharide, and flagellin are recognized by macrophages using Toll-like receptors (TLR). Recognition by TLR activates the nuclear factor-κB pathway and leads to production of proinflammatory cytokines like TNF-α[21]. Monocyte activation through the innate pathway can also activate Th1 cells via production of IL-12p40, thus leading to production of IFN-γ[22]. Indeed, in our study, the majority of children with ERA had detectable levels of Th1 cytokine IFN-γ. In contrast, Th2 cytokine IL-4 could not be detected with our methodology. These observations suggest a possible Th1 bias in patients

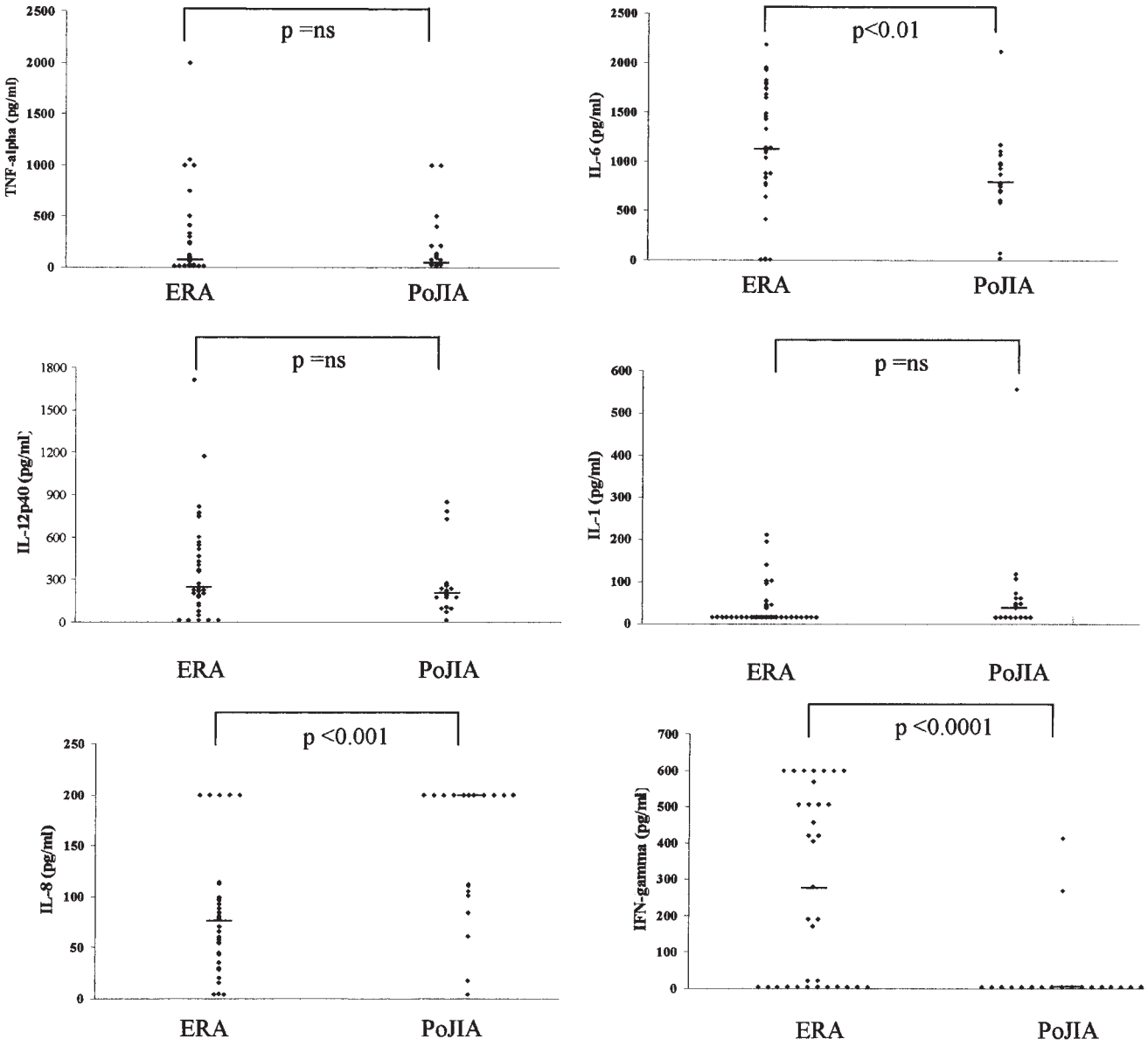

Figure 1. Scatter plots showing cytokine levels in enthesitis related arthritis (ERA) and polyarticular rheumatoid factor-negative JIA (PoJIA). All intergroup comparisons by Mann-Whitney U test.

with ERA. Kutukculer, *et al*[3] also failed to find IL-4 in SF samples in JIA. However, these data require confirmation using more sensitive techniques like intracellular cytokine staining and measurement of mRNA levels. In recent studies using these techniques, both a Th1[8] and a Th2[23] bias were observed, but these studies had very few children with ERA.

Elevation in SF IL-8 concentration in patients with Poly-JIA, as observed in our study, has also been reported by De Benedetti, *et al*[12] in a study of 30 specimens obtained from patients with different subtypes of juvenile RA. They found comparable levels in all 3 subtypes. IL-8, a potent neutrophil chemoattractant, is produced by synovial macrophages and fibroblasts. A correlation between IL-8 levels and SF leukocytosis has been reported[12]. What can be the trigger for production of IL-8 in Poly-JIA? SF immune complexes from children with Poly-JIA are known to induce synoviocytes to produce IL-8 as well as other proinflammatory cytokines[24]. As well, IL-1 and TNF-α can also upregulate IL-8 production.

In recent years, specific inhibitors of various cytokines have been developed for treatment. For instance, TNF-α blockers are routinely used to treat patients with RA and JIA[25]. IL-1 receptor antagonists are being used in patients with RA, and anti-IL-6 receptor antibody in those with systemic onset JIA[26], with promising results. Anti-IL-12[27] and anti-IL-8[28] have shown beneficial effects in animal models of arthritis and are soon likely to undergo human trials.

Our results suggest that monocyte derived cytokines may play an important role in pathogenesis of synovitis in polyarticular JIA and ERA, and that the immune response in ERA is predominantly Th1 mediated.

ACKNOWLEDGMENT

We thank Dr. Rakesh Aggarwal for helpful discussion and critical reading of the manuscript.

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