Abnormal Regulation of Interleukin 6 in Systemic Juvenile Idiopathic Arthritis

PATRIZIA PIGNATTI, MARINA VIVARELLI, CRISTINA MEAZZA, MARIA GRAZIA RIZZOLO, ALBERTO MARTINI, and FABRIZIO DE BENEDETTI

ABSTRACT. ***Objective.*** To evaluate the *in vitro* production of interleukin 6 (IL-6) by peripheral blood mononuclear cells (PBMC) of patients with systemic juvenile idiopathic arthritis (JIA), its regulation by IL-10, and the association of abnormal regulation of IL-6 production with presence of polymorphisms in the regulatory sequence of the IL-6 gene.

Methods. PBMC were cultured in the absence or presence of lipopolysaccharide (LPS). IL-6 and IL-10 levels were measured by ELISA. Polymorphisms in the regulatory sequences of the IL-6 gene were assessed by restriction fragment length polymorphism analysis and sequencing of amplified regions.

Results. Patients' PBMC produced high amounts of IL-6 compared to controls in unstimulated conditions. The IL-10 50% inhibitory dose of LPS stimulated IL-6 production was significantly higher in patients than controls. IL-10 levels produced in the absence or presence of LPS were comparable between patients and controls. The –174 G/C polymorphism in the IL-6 gene does not appear to be correlated with the high unstimulated IL-6 production or with the reduced inhibition by IL-10 observed in patients with JIA. No differences in patients compared to controls and with respect to the published sequence were found in the 3' untranslated region (UTR) of the IL-6 gene.

Conclusion. Most patients with JIA have increased unstimulated production of IL-6 and reduced inhibition of IL-6 production by IL-10. This abnormal regulation of IL-6 production is not secondary to a defect in IL-10 production, and is not associated with polymorphism of alleles at position –174 of the 5' flanking region or with mutations in the 3' untranslated region of the IL-6 gene. (J Rheumatol 2001;28:1670–6)

Key Indexing Terms:
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Systemic juvenile idiopathic arthritis (JIA) is a chronic inflammatory disease characterized by the association of chronic arthritis with systemic features, including high spiking fever, rash, hepatosplenomegaly, lymphoadenomegaly, serositis, and prominent laboratory evidence of inflammation. The genetic background of systemic JIA remains undetermined, as no clearcut associations of JIA with class I or class II HLA alleles have been reported[1].

As for pathogenic mechanisms, prominent production of interleukin 6 (IL-6) appears to be a characteristic feature of systemic JIA: patients with active systemic JIA have markedly elevated peripheral blood and synovial fluid levels of IL-6 that are significantly higher than in the other JIA onset types and in adult rheumatoid arthritis (RA)[2-7]. Evidence indicates that increased IL-6 production explains several clinical and biological manifestations of the disease, leading to the hypothesis that systemic JIA could be an IL-6 mediated disease[8]. Moreover, a recent observation provided a possible relation between prominent IL-6 production and genetic background. Fishman, *et al* described a novel single base G/C polymorphism at position –174 of the 5' flanking region of the IL-6 gene, and defined the functional role of this polymorphism, showing that the G and the C alleles predispose to higher and lower IL-6 production, respectively[9]. They reported a significant association of this polymorphism with systemic JIA with a possible protective role of the low producer CC genotype[9].

We evaluated *in vitro* production of IL-6 by peripheral blood mononuclear cells (PBMC) of patients with systemic JIA and its regulation by IL-10, a cytokine known to inhibit monocyte IL-6 production[10,11]. We report that PBMC from patients with systemic JIA produce elevated amounts of IL-6 in the absence of exogenous stimuli and show reduced inhibition of lipopolysaccharide (LPS) stimulated IL-6 production by IL-10. The elevated production of IL-6 and the reduced inhibition by IL-10 do not appear to be associated with alleles at position –174 of the 5' flanking region or

From the Clinica Pediatrica dell'Università di Pavia, IRCCS Policlinico San Matteo, Pavia, Italy.

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P. Pignatti, PhD, Research Fellow; M. Vivarelli, MD, Research Fellow; C. Meazza, PhD, Research Fellow; M.G. Rizzolo, MD, Research Fellow; A. Martini, MD, Professor of Pediatrics; F. De Benedetti, MD, PhD, Dirigente Medico Primo Livello.

Address reprint requests to Dr. A. Martini, Clinica Pediatrica, IRCCS Policlinico San Matteo, P. le Golgi 2, 27100 Pavia, Italy. E-mail: amartini@smatteo.pv.it

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with mutations in the 3' untranslated region (UTR) of the IL-6 gene.

MATERIALS AND METHODS

Patients and controls. Peripheral blood samples were obtained from 16 children (mean age 8.3 yrs, range 2–16) with systemic JIA[12]. All patients presented active disease defined by examination (mean disease duration 4.5 yrs, range 0.3–11.5). The extent and severity of joint involvement were measured using a joint swelling score[13]. At time of sampling, all patients were treated with nonsteroidal antiinflammatory drugs; about two-thirds were receiving weekly methotrexate (MTX), and one-third were taking prednisone (alternate day regimen for most). At time of sampling, 2 patients had active systemic features (daily spiking fever > 38°C). Ten healthy children comparable for age, hospitalized for minor surgical procedures or bone marrow donation, were used as controls. Permission to draw extra blood during routine venipuncture was obtained from the parents of all children. Synovial fluids were collected at time of intraarticular steroid injection, kept in ice, centrifuged, and stored at –70°C until tested for cytokine content.

Cell cultures. PBMC were separated from whole blood with Ficoll/Histopaque. To minimize *ex vivo* IL-6 production, PBMC for cellular lysates were separated and washed at 4°C in the presence of EDTA (4 mg/ml), with the exception of the last washing. Then cells were frozen and thawed 3 times and centrifuged. The supernatants were collected and stored at –70°C for future cytokine detection. For cell culture, PBMC were washed in RPMI 1640 (supplemented with glutamine, gentamicine, and 10% fetal calf serum), and 1×10^5 cells were cultured 24 h at 37°C in 5% CO_2 in 96 well plates in the absence or presence of 0.1 μg/ml LPS (Sigma, St. Louis, MO, USA) and in the absence or presence of increasing concentrations of recombinant human (rh) IL-10 (0.3, 1.0, 3.0 ng/ml) (R&D Systems, Minneapolis, MN, USA). The supernatants were harvested, centrifuged, and frozen at –70°C for future determinations of cytokines. The concentration of IL-10 able to induce 50% reduction of LPS stimulated IL-6 production was considered the 50% inhibitory dose.

IL-6 and IL-10 determinations. IL-6 and IL-10 levels were evaluated in cell culture supernatants, in serum and synovial fluid of patients and controls, with 2 commercial ELISA (R&D Systems and Endogen, Woburn, MA, USA, respectively). Assays were performed following the manufacturer's instructions. The immunoassay detection limits were 2.3 pg/ml for rhIL-6 and 3.7 pg/ml for rhIL-10.

IL-6 levels in lysates were measured using the hybridoma cell line B9 (kindly provided by Dr. L. Aarden, Netherland Red Cross, Amsterdam, The Netherlands)[2].

Polymerase chain reaction. For PCR, 1×10^6 PBMC from patients and controls were lysed in 300 μl of 50 mM Tris/HCl, 0.5% Np40, and 1 mg/ml proteinase K for 2 h at 60°C. After inactivation of proteinase K for 15 min at 95°C, lysates were centrifuged and 3 μl of supernatant were used for the amplification reaction. Primer set 1 (GACAACTCATCTCATTCTGC-CGTGACACACTCAAAGTTGC) amplified a sequence of 517 bp in the 3' untranslated region of the IL-6 gene; primer set 2 (CAGAAGAACTCA-GATGACTGG-GCTGGGCTCCTGGAGGGG) amplified a sequence of 611 bp in the 5' flanking region of the IL-6 gene. Cycling conditions for the first set of primers were: 95°C for 5 min followed by the addition of 0.5 U Taq polymerase (Boehringer Mannheim, Mannheim, Germany), then 30 cycles: annealing at 60°C for 1 min, extension at 72°C for 3 min, followed by a terminal elongation of 15 min at 72°C. The conditions of amplification were similar for primer set 2 except for the annealing temperature of 63°C. This reaction was performed for 25 cycles. The PCR products of primer set 1 were analyzed with an automated sequence analyzer. Then 10 μl of the product amplified with primer set 2 were digested with SfaNI (New England Biolabs, Beverly, MA, USA). After digestion the sample was denatured at 65°C for 5 min, and then visualized on 1% agarose gel.

Statistical analysis. Data were analyzed using the Mann-Whitney U test and the Spearman correlation test. A p value < 0.05 was considered statistically significant.

RESULTS

Increased spontaneous production of IL-6 in patients' PBMC. The amounts of IL-6 measured in the supernatants after LPS stimulation were comparable between patients and controls (8.48 ± 5.03 and 9.00 ± 2.93 ng/ml, respectively) (Figure 1), showing similar IL-6 production capacity with maximal stimulation. In the absence of exogenous stimuli, while PBMC of controls produced low amounts of IL-6 (0.16 ± 0.19 ng/ml), patients produced high amounts (3.06 ± 5.12 ng/ml) with a highly significant difference compared to controls (p = 0.002) (Figure 1). This finding shows that in the absence of stimulation PBMC of patients with systemic JIA produce elevated amounts of IL-6 *in vitro*. To evaluate whether this spontaneous *in vitro* secretion reflected an *in vivo* activation of IL-6 production, we analyzed the intracellular content of IL-6 in PBMC lysates obtained immediately after separation. We found that elevated intracellular amounts of IL-6 (1.58 ± 2.56 pg/5 × 10^6 PBMC) were present in patients, while controls had undetectable levels (< 0.15 pg/5 × 10^6 PBMC). Moreover, the amount of intracellular IL-6 estimated in patients correlated with serum IL-6 levels (r = 0.798, p = 0.001). These findings show that PBMC from patients with JIA are activated *in vivo* to produce high amounts of IL-6.

Reduced inhibition of IL-6 production by IL-10. To evaluate whether the elevated production of IL-6 in patients could be due to abnormal regulation of IL-6 production, we estimated the capacity of IL-10 to inhibit IL-6 production. PBMC from patients and controls were incubated in the absence or

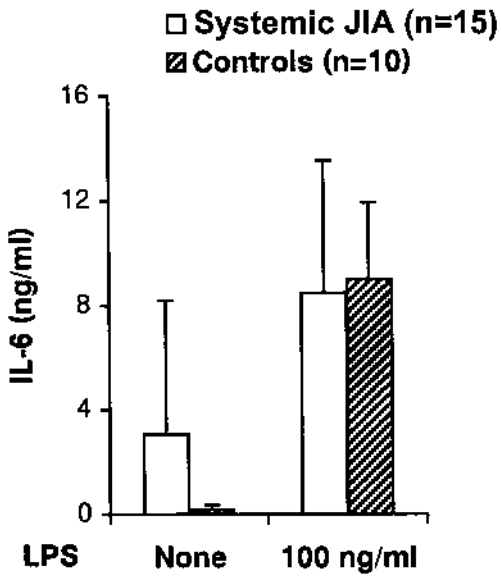

Figure 1. Unstimulated and LPS stimulated production of IL-6 in supernatants from PBMC cultures in patients with systemic JIA and controls. Results are mean + SD.

presence of LPS with the addition of increasing concentrations of recombinant human IL-10. The unstimulated production of IL-6 by patients' PBMC was inhibited by IL-10 with a mean percentage inhibition, at 0.3 ng/ml IL-10, of 55%. However, as described, the unstimulated production of IL-6 by controls' PBMC was very low or undetectable, making it impossible to measure inhibition by IL-10. Thus the comparison of the sensitivity of IL-6 production to IL-10 inhibition between patients and controls was evaluated after LPS stimulation. Among different subjects, we found different dose-response curves of inhibition of IL-6 production by IL-10 (Figure 2, panels A and B). To compare patients with controls, we calculated the concentration of IL-10 sufficient to obtain a 50% inhibition of the LPS stimulated IL-6 production. The 50% inhibitory dose was significantly higher in patients than controls (Figure 2C). Notably, we also found that the 50% inhibitory dose of IL-10 was directly correlated with spontaneous IL-6 production ($r = 0.665$, $p = 0.004$). Indeed, when we divided patients according to constitutive IL-6 production, patients with an IL-6 unstimulated production > 0.5 ng/ml had a 50% inhibitory dose of IL-10 that was significantly higher than patients with constitutive IL-6 production < 0.5 ng/ml (Figure 2C). *In vitro* IL-6 production and IL-10 50% inhibitory dose did not show significant association with disease activity variables (Table 1). In addition, when patients were divided according to treatment with MTX or prednisone at time of sampling, we found no significant differences in the production of IL-6, or in the 50% inhibitory dose of IL-10 (Table 2).

IL-10 production in patients. To rule out that high IL-6 production and reduced inhibition by IL-10 could be due to a defect in IL-10 production in patients, we first measured IL-10 levels in the supernatants of PBMC. As shown in Table 3, in the absence or presence of LPS the amount of IL-10 produced by patients' PBMC tended to be higher than that produced by controls' PBMC, but the difference was not statistically significant. Moreover, when we divided patients according to constitutive IL-6 production, patients with unstimulated IL-6 production > 0.5 ng/ml produced more IL-10 than patients with unstimulated IL-6 production < 0.5 ng/ml (Table 3). These data show that increased constitutive IL-6 production and reduced inhibition by IL-10 are not a direct consequence of a defect in IL-10 production *in vitro*. In addition, evaluation of synovial fluid IL-10 levels showed significantly ($p = 0.03$) higher levels of IL-10 (107 ± 68 pg/ml; n = 7) in patients with systemic JIA compared to patients with pauciarticular JRA (34 ± 42 pg/ml; n = 9). This finding also shows that *in vivo*, at the inflammatory sites, there is no absolute defect in IL-10 production.

Lack of association of polymorphisms at –174 and in the 3' UTR of IL-6 gene with abnormal regulation of IL-6 production. Since increased unstimulated production of IL-6 and reduced inhibition by IL-10 were not associated with disease activity variables, we evaluated whether the abnormal regulation of IL-6 production (i.e., high unstimulated production and decreased inhibition of IL-6 production by IL-10) could be related to polymorphisms in regulatory sequences of the IL-6 gene. Fishman, *et al* recently reported a novel, functionally relevant, single base polymorphism at position –174 of the IL-6 promoter with a significant association with systemic JIA[9]. As shown in Figure 3, when patients were divided according to their genotypes at position –174, the unstimulated or LPS stimulated IL-6 production as well as the 50% inhibitory dose of IL-10 do not appear to be related to the G/C polymorphism. These data suggest that the –174 G/C polymorphism is not directly

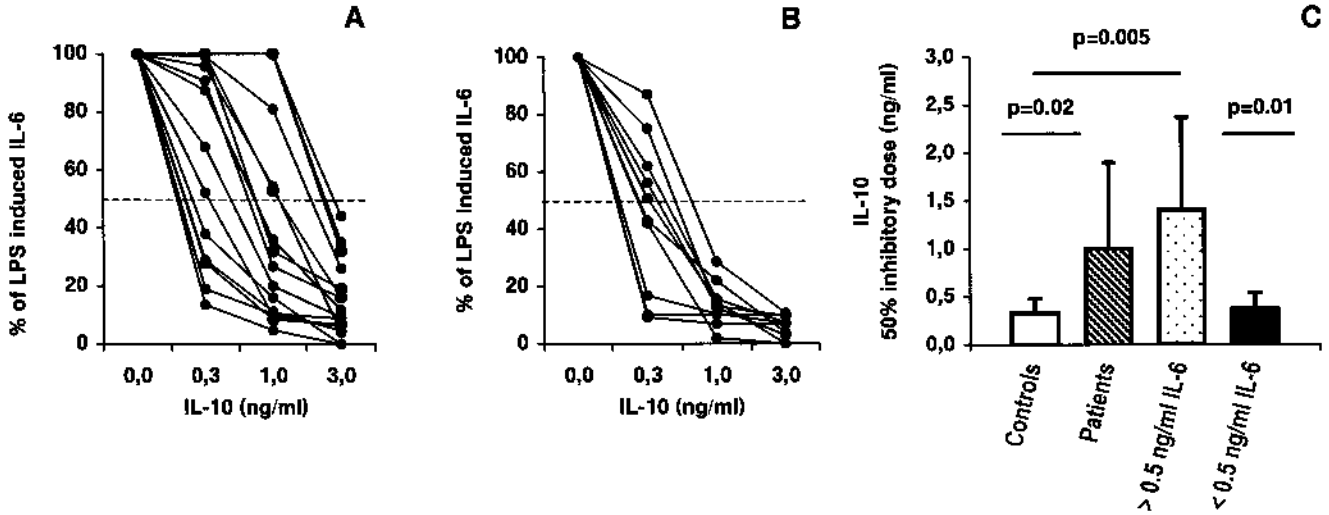

Figure 2. Percentage of LPS stimulated IL-6 production in the presence of increasing concentrations of rhIL-10 in patients (A) and controls (B). Panel C: concentration of IL-10 determining 50% inhibition of LPS stimulated IL-6 production in controls and in patients (shaded bar). Patients were subdivided according to their unstimulated IL-6 production, i.e., those with baseline production > 0.5 ng/ml (n = 8) or < 0.5 ng/ml (n = 8). Results are mean + SD.

Table 1. Correlation coefficients (Spearman's r) and significance levels of the associations of unstimulated IL-6 production and concentration of IL-10 determining a 50% inhibition of LPS induced IL-6 production (50% IL-10 inhibitory dose) with clinical and laboratory variables of disease activity in patients with systemic JIA.

	Unstimulated IL-6 Production		50% IL-10 Inhibitory Dose	
	r	p	r	p
C-reactive progein, n = 16	0.01	0.98	0.18	0.45
ESR, n = 16	0.10	0.69	0.22	0.39
Number of active joints, n = 16	–0.13	0.61	–0.07	0.79

Table 2. IL-6 levels in supernatant from PBMC cultures and concentration of IL-10 determining a 50% inhibition of LPS stimulated IL-6 production in patients with systemic JIA subdivided according to the presence or absence of steroid (PDN) or methotrexate (MTX) treatment at time of sampling. Results are mean ± SD.

	PDN		MTX	
	Yes, n = 8	No, n = 8	Yes, n = 8	No, n = 8
Unstimulated IL-6, ng/ml	3.11 ± 6.60	3.00 ± 3.07*	1.38 ± 1.55	4.75 ± 6.95*
LPS stimulated IL-6, ng/ml	8.72 ± 5.80	13.83 ± 15.8*	10.23 ± 5.27	6.52 ± 3.51*
50% inhibitory dose of IL-10, ng/ml	0.66 ± 0.63	1.28 ± 1.09*	0.85 ± 0.84	1.02 ± 1.00*

*p > 0.1 versus corresponding MTX or PDN treated.

Table 3. IL-10 levels in supernatants of unstimulated and LPS stimulated PBMC cultures. Results from patients with JIA shown together and subdivided according to their unstimulated IL-6 production (< 0.5 or > 0.5 ng/ml). Results are mean ± SD.

	IL-10, pg/ml	
	–LPS	+LPS
Controls, n = 10	6.8 ± 5.6	48.8 ± 30.1
JIA, n = 16	9.9 ± 7.7	66.5 ± 94.5
Unstimulated IL-6 < 0.5 ng/ml, n = 8	9.0 ± 7.5	72.5 ± 92.7
Unstimulated IL-6 > 0.5 ng/ml, n = 8	13.9 ± 7.8*	50.7 ± 81.9

*p < 0.05 vs controls and vs JIA unstimulated IL-6 < 0.5 ng/ml.

involved in the high unstimulated IL-6 production and the reduced inhibition by IL-10 observed in patients with systemic JIA.

IL-10 inhibits IL-6 production by decreasing IL-6 mRNA stability[14]. The exact molecular mechanism involved is not known. However, it has been shown that the IL-10 induced decreased stability of tumor necrosis factor-α (TNF-α) mRNA is mediated by the AUUUA elements present in the 3' UTR of the TNF message[15]. Since the 3' UTR of the IL-6 gene contains 8 AUUUA elements, we investigated whether reduced inhibition of IL-6 production by IL-10 could be due to mutations in or deletions of the AUUUA elements. A 517 bp sequence of the 3' UTR region

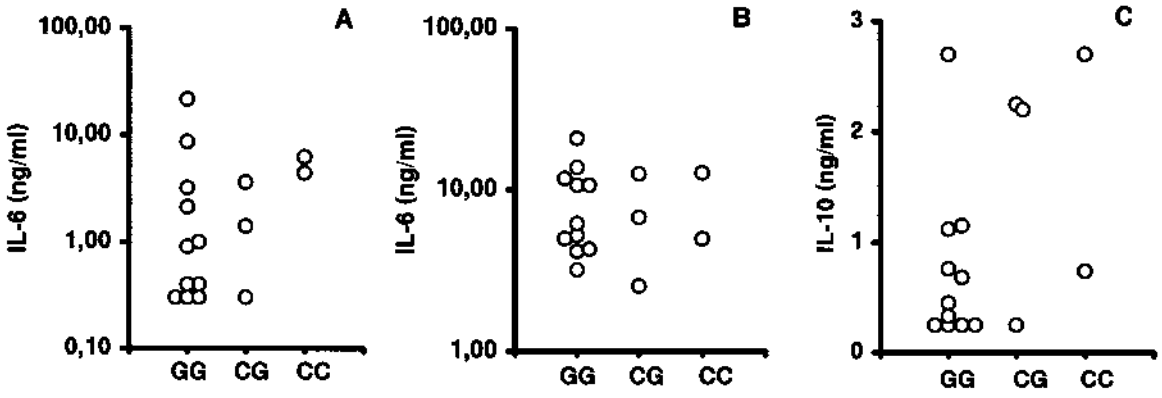

Figure 3. Unstimulated (A) and LPS stimulated (B) IL-6 production, and concentration of IL-10 determining a 50% inhibition of LPS stimulated IL-6 production (C) in patients according to their genotypes at position –174 in the 5' flanking region of the IL-6 gene.

of the IL-6 gene was amplified and sequenced. No differences were found in patients compared to controls and with respect to the published sequence (data not shown).

DISCUSSION

We report that PBMC from patients with systemic JIA are activated to produce elevated amounts of IL-6, as shown by markedly increased *ex vivo* unstimulated production and by the presence of detectable IL-6 in freshly isolated PBMC lysates. Of note, the amount of intracellular IL-6 correlated significantly with serum IL-6 levels. We have reported increased spontaneous production in patients with systemic JIA of a thymocyte costimulatory activity that we attributed to IL-1[16]. Since IL-1 levels are not significantly increased in systemic JIA[3,17] and since it has subsequently become apparent that IL-6 is a potent thymocyte costimulator[18], it is conceivable that this activity is due to IL-6. In addition to being present in peripheral blood, activation of IL-6 production may also be a feature of the synovial compartment in systemic JIA; indeed, Eberhard, *et al* demonstrated a higher frequency of IL-6 producing macrophages in synovial fluid from patients with systemic JIA compared to poly- or pauciarticular JIA[19].

To evaluate whether overproduction of IL-6 in systemic JIA could be secondary to an abnormal regulation of this cytokine, we investigated IL-10, which is known to be a potent inhibitor of the production of monocyte derived inflammatory cytokines[10,11]. We found a reduced inhibition by IL-10 of LPS induced IL-6 production in patients with systemic JIA compared to controls. IL-10 capacity to inhibit IL-6 production has been evaluated in other inflammatory autoimmune diseases. In systemic lupus erythematosus IL-10 inhibits IL-6 production by LPS stimulated PBMC to an extent comparable to that of controls[20]. To our knowledge, there are no reports comparing IL-10 inhibition of IL-6 production by PBMC of adult patients with RA and controls. However, Chomarat, *et al* reported that IL-10 inhibits IL-6 production by RA synovium less efficiently than by PBMC in RA[21]. Recently it has been reported that synovial fluid derived dendritic cells show resistance to the immunosuppressive effect of IL-10, due to low membrane expression of the IL-10 receptor 1 secondary to persistent internalization[22]. These results raise the possibility that decreased expression of IL-10 receptors might be responsible for the reduced inhibition of IL-6 production that we observed. Further studies are needed to clarify this issue.

The mechanism of the increased unstimulated production of IL-6 and the reduced inhibition by IL-10 in patients with systemic JIA remains to be established. To determine whether these could be secondary to polymorphisms in the regulatory regions of the IL-6 gene, we evaluated the relation of the abnormal regulation of IL-6 production with the –174 polymorphism and with possible polymorphisms in the 3' UTR of the IL-6 gene. A number of polymorphisms of the IL-6 region have been described[23-27]; however, the only polymorphism known to be disease associated is the single base G/C polymorphism at position –174 of the 5' flanking region of the IL-6 gene, reported to be specifically associated with systemic JIA[9]. Patients with systemic JIA, especially those with disease onset at or before 5 years of age, have a reduced prevalence of the CC genotype, which is associated with lower IL-6 production[9]. In agreement with the study from Fishman, *et al*[9], in our series the C allele was less frequent than the G allele. We found no significant difference in unstimulated or LPS stimulated IL-6 production or in the 50% inhibitory dose of IL-10 when patients were divided according to genotypes. However, the lack of statistically significant differences may be secondary to the small number of subjects with the CG or CC genotypes. Nevertheless, the data illustrated in Figure 3 strongly suggest that the –174 G/C polymorphism is not directly related to the high unstimulated IL-6 production and reduced inhibitory capacity of IL-10 that we observed in systemic JIA. In addition, the same analysis in healthy controls showed no relation between the G or C alleles with unstimulated IL-6 production or with inhibition by IL-10 (data not shown). Further studies are needed to establish whether other polymorphisms of the 5' flanking region of the IL-6 gene are involved.

The expression of several cytokines, including TNF and IL-6, as well as other early response genes, is regulated by the intracytoplasmic degradation of their mRNA, which appears to be mediated by the AUUUA elements present in the 3' UTR of their mRNA. It has been reported that IL-10 induces inhibition of IL-6 production by decreasing IL-6 mRNA stability[14]. Moreover, in the case of TNF-α production, it has clearly been demonstrated that IL-10 induced inhibition is secondary to decreased mRNA stability mediated through the AUUUA elements of the 3' UTR of the TNF message[15]. Since the 3' UTR of the IL-6 gene contains 8 AUUUA elements we evaluated the possible presence of mutations in or deletions of the AUUUA elements in systemic JIA. Sequencing of the 3' UTR of the IL-6 gene showed no differences from the published sequence, ruling out that reduced inhibition of IL-6 production by IL-10 could be due to mutations in or deletions of the AUUUA elements.

Reduced inhibition of IL-6 production by IL-10 could be secondary to a defect in IL-10 production resulting in a decreased total amount of IL-10 (i.e., endogenous plus exogenous) available for inhibition. However, we found that the amount of IL-10 released by PBMC of patients with JIA was comparable to that of controls, ruling out this possibility. It should be pointed out that contrasting results concerning *in vitro* IL-10 production in JIA have been reported, with both higher and lower production with respect to controls[28,29]. The different culture systems and stimuli used may account for these discrepancies. In addi-

tion, data on the *in vivo* expression of IL-10 in JIA are not consistent with a defect in IL-10 production. Murray, *et al* reported that the frequency of IL-10 mRNA expressing cells in systemic JIA synovial tissue was comparable to that of pauciarticular JIA[30]. We found that synovial fluid IL-10 levels were higher in systemic than in pauciarticular JIA.

Animal studies in murine collagen induced arthritis[31-33] provide support for the concept that IL-10 administration could ameliorate the signs and symptoms of arthritis. Trials in RA are in progress to evaluate the clinical efficacy and safety of IL-10 treatment[34]. Our results showing reduced inhibition of IL-6 production by IL-10 in patients with systemic JIA should be taken into account when planning therapeutic attempts with IL-10 in this disease.

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