Analysis of Leukemia Inhibitory Factor, Type 1 and Type 2 Cytokine Production in Patients with Eosinophilic Fasciitis

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ABSTRACT. ***Objective.*** Eosinophilic fasciitis (EF) is a scleroderma-like disease of unknown etiology characterized by skin induration, elevated immune globulins, and peripheral eosinophilia. The hallmarks of the chronic cutaneous involvement in this syndrome are inflammation and fibrosis of the fascia. To determine how the inflammatory process in EF may be regulated, we investigated the spontaneous and mitogen induced [lipopolysaccharide (LPS), phytohemagglutinin (PHA) or both LPS+PHA] syntheses of interleukins (IL)-2, 5 and 10, interferon−γ (IFN−γ), and leukemia inhibitory factor (LIF) cytokines by peripheral blood mononuclear cells (PBMC) from 4 patients with active EF and compared them to those of 10 healthy individuals.

Methods. We used a short term whole blood assay and culture supernatants were collected after 24 h to measure the IL-2 and IFN−γ contents and after 48 h to evaluate IL-5, IL-10, and LIF. Supernatant cytokine concentrations were determined by ELISA.

Results. All 4 patients had similar patterns of cytokine secretion. Cytokine production did not differ between patients and controls under basal conditions or when LPS was added to the cultures. In contrast, under PHA or LPS+PHA stimulation, significantly higher amounts of all 5 cytokines were detected in samples from patients compared to those from controls.

Conclusion. Overall, our data suggest that EF is characterized by an increased capacity of PBMC to produce IL-5 and IL-10, possibly leading to eosinophilia and immune globulin overexpression. In this context, the simultaneous elevations of type 1 cytokines (IL-2 and IFN−γ) and LIF production by the same cells may be an attempt by the immune system to limit the exacerbation of a type 2 dominant response. (J Rheumatol 2001;28:75–80)

Key Indexing Terms:
INTERLEUKIN-2 IL-10 INTERFERON−γ
LEUKEMIA INHIBITORY FACTOR EOSINOPHILIC FASCIITIS

Eosinophilic fasciitis (EF) was first described as an autonomous disorder by Shulman in 1974[1]. This disease is characterized by a scleroderma-like swelling and induration of the skin predominantly involving the arms and legs. It is associated with eosinophilia, immune globulin overproduction, and an elevated erythrocyte sedimentation rate[2,3]. Full thickness skin-to-muscle biopsies exhibit a predominantly sclerotic inflammatory process and contain mononuclear cell infiltrates of the fascia with a normal epidermis (occasional extension into the overlying subcutis and subjacent skeletal muscle may also be observed). Eosinophil infiltration of the fascia is common, particularly during the early stages of the disease.

The mechanisms responsible for eosinophilia and the excessive deposition of extracellular matrix components in the affected fascia of patients with EF are not known. The hypothesis of an autoimmune origin for this disease is supported by the successive detection of elevated immune globulins and circulating immune complexes in patients with active EF[4], the occurrence of EF in chronic graft-versus-host disease[5], and the association of EF with other autoimmune disorders[6].

The cytokine network could potentially play a role in the cascade of events leading to tissue fibrosis as suggested by several *in vitro* studies that showed that various cytokines are capable of regulating collagen-gene expression in fibroblast cultures[7,8]. Thus, it is generally accepted that transforming growth factor-ß plays a key role in the development of diverse fibrotic skin diseases, and its *in vivo* overexpression has been observed in systemic or localized scleroderma and EF[9-11].

Moreover, *in vivo* observations support the concept that cytokines participate in the development of tissue fibrosis,

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since serum samples from patients with scleroderma have been shown to contain elevated levels of several cytokines[12]. Similarly, the mechanisms underlying the activation and local infiltration of eosinophils in EF are not known nor is the role of eosinophilia in the disease. The observation that activated eosinophils produce a wide range of inflammatory cytokines that have the potential to modulate the fibrotic process supports their involvement in EF[13]. Interleukin (IL) 5, produced mainly by activated T cells[14,15], is important for the differentiation, proliferation, recruitment, and activation of eosinophils[16], and its potential overproduction could explain the eosinophilia observed in EF.

The respective contributions of the type 1 helper T cells, which produce IL-2, interferon–γ (IFN–γ), and tumor necrosis factor-α , and type 2 helper T cells, which synthesize IL-4, IL-5, IL-6, IL-10, and IL-13[17-21] in the pathogenesis of EF, have never been investigated.

We analyzed spontaneous and mitogen induced cytokine [IFN–γ , IL-2, IL-5, IL-10, and leukemia inhibitory factor (LIF)] production by peripheral blood mononuclear cells (PBMC) from 4 patients with Shulman's syndrome using a short term whole blood assay (WBA), which is reported to reproduce more accurately the natural environment and constitutes an appropriate milieu in which to evaluate cytokine production *in vitro*[22-24]. LIF, a cytokine thought to be involved in inflammatory processes and which is elevated in giant cell arteritis[25], rheumatoid arthritis[26,27], acute allograft rejection[28,29], and systemic lupus erythematosus[30], has never been investigated in fibrotic diseases.

MATERIALS AND METHODS

Patients. Four patients with a classic clinical picture of EF of recent onset (≤ 4 mo) who had received no treatment except analgesics were included in this study. Table 1 summarizes their clinical and biological characteristics. All had eosinophilia and denied consumption of L-tryptophan tablets or nutritional supplements containing L-tryptophan. Healthy controls included 4 men and 6 women unmatched with patients for age. All the patients and controls were Caucasian.

Samples from the edges of clinically active lesions were obtained by deep excisional skin biopsies that included the fascia. In all cases, the fascia was markedly indurated and thickened. Histological examinations of the biopsies of affected skin and fascia showed normal epidermis and papillary dermis. The thickened fascia contained accumulations of collagen and intense inflammatory cell infiltrates comprised lymphocytes, macrophages, and eosinophils. Inflammatory cells were scattered throughout the fascia and the deeper layers of the dermis.

Blood collection and short term WBA protocols. Blood samples were collected in sterile Vacutainer® tubes (Becton Dickinson, Grenoble, France) containing 100 IU/ml of sodium heparin (Choay, Paris, France). After a maximum storage period of 1 h at room temperature, blood was diluted 1:1 in RPMI-1640 (Gibco, Les Ullis, France), and 1 ml aliquots were deposited into 2 ml wells of a 24 well plate (Nunc, Roskilde, Denmark). Polyclonal activators were added as follows: phytohemagglutinin alone (PHA; Sigma, St. Louis, MO, USA) at a final concentration of 5 µg/ml; lipopolysaccharide alone (LPS from *Salmonella enteritidis*, Sigma) at a final concentration of 25 µg/ml; or both PHA + LPS at the same concentrations. An unstimulated control culture was run concomitantly for baseline determinations. The plates were incubated at 37°C in a humidified 5% CO_2 atmosphere. After 24 or 48 h of culture the contents of the wells were harvested, spun at 2000 × g for 2 min, and the supernatants were collected and stored frozen at –80°C until tested.

Cytokine assays. Culture supernatants were collected after 24 h to measure the IL-2 and IFN–γ contents and after 48 h to evaluate IL-5, IL-10, and LIF. Supernatant cytokine concentrations were determined by ELISA (Immunotech, Marseille, France). The positivity thresholds were: 10 pg/ml for IL-2, 0.08 IU/ml for IFN–γ , 5 pg/ml for IL-5, and 3 pg/ml for IL-10. LIF levels were measured by a 2-site sandwich ELISA using anti-LIF monoclonal antibodies (Mab) raised in our laboratory[31,32]. Mouse 1F10 anti-human LIF Mab was used for coating and biotinylated mouse 7D2 anti-human LIF Mab for detection. After subsequent incubation with a streptavidin-peroxidase complex (Amersham, Arlington Heights, UK) and vizualization with tetramethylbenzidine (Sigma), absorbance was measured at 450 nm and 570 nm. The threshold of positivity was calculated by adding 2 standard deviations to the mean of 6 blank wells and fell within the range of 5–25 pg/ml. Neither 1F10 nor 7D2 has any blocking activity in a functional assay, thereby ruling out any interference with the soluble receptor in this ELISA. For the remaining cytokines, all the ELISA used in this study were not influenced by the presence of natural soluble receptors, as tested by adding their recombinant soluble counterparts to the analyte. The interference of unknown components present in the sera of patients with inflammatory diseases in the cytokine measurements were ruled out by spiking those sera with a known amount of recombinant cytokine prior to the standard assay. No patient studied in this series had rheumatoid factors likely to interfere with the ELISA. For one sample determination using ELISA, duplicates were set up, and the mean of both optical densities taken for the calculation of the concentration for this particular determination. In addition, and for LIF only, 2 determinations per sample were carried out. The raw data were corrected for 10^6 PBMC, as determined with an automatic hemocytometer (H2, Bayer Diagnostics, Germany) the day of the test.

Statistical analysis. The distributions of control cytokine concentrations are reported as their median values, first and third quartiles, and ranges as recommended[33]. Comparisons between control and patient groups were performed using the nonparametric Mann-Whitney U test, with a level of significance set at 0.05. Correlations between IL-10 concentrations and immune globulins were determined with Spearman's correlation test. Test was performed with the Statistica software (Statsoft, Guyancourt, France).

RESULTS

Type 1 cytokine production in WBA. IFN–γ production. IFN–γ production did not differ markedly between patients and controls under basal conditions or LPS stimulation (Figure 1). In contrast, when PBMC were stimulated with PHA, the patients produced statistically much more IFN–γ than controls ($p = 0.004$). This trend was even more evident with LPS + PHA stimulation, which induced marked IFN–γ production by the cells from patients resulting in higher values than controls ($p = 0.001$).

IL-2 production. Spontaneous IL-2 production was equivalent and barely detectable between patients and controls (Figure 1). LPS, which is an activation stimulus mainly for peripheral blood monocytes and B cells, did not trigger significant IL-2 production, which was comparable between the 2 groups. In contrast, IL-2 levels increased dramatically in response to the polyclonal T cell activator PHA ($p = 0.004$) or LPS + PHA ($p = 0.004$) for patients compared with controls.

Type 2 cytokine production. IL-5 production. Spontaneous IL-5 production was low and equal between patients and controls

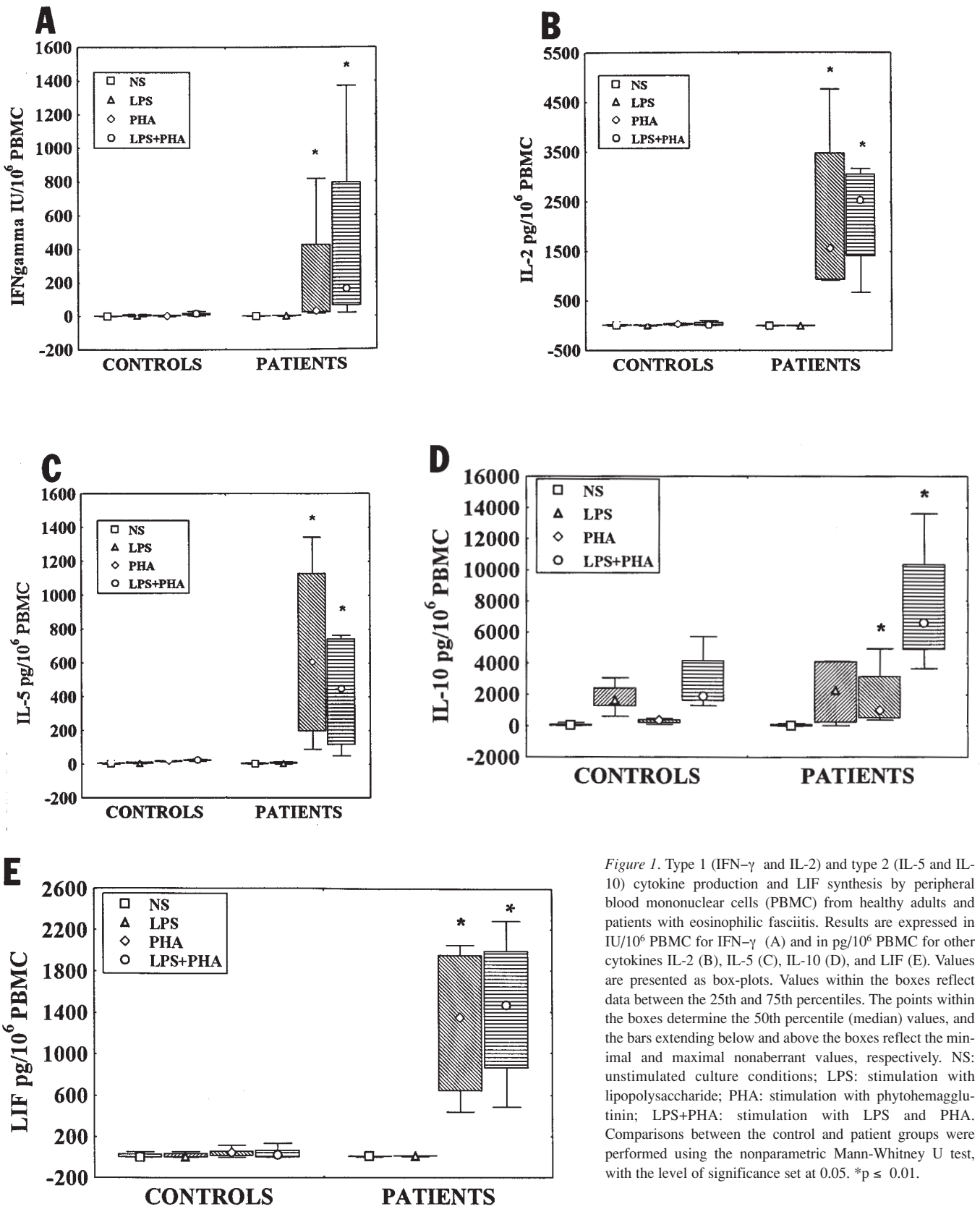

Figure 1. Type 1 (IFN–γ and IL-2) and type 2 (IL-5 and IL-10) cytokine production and LIF synthesis by peripheral blood mononuclear cells (PBMC) from healthy adults and patients with eosinophilic fasciitis. Results are expressed in IU/10^6 PBMC for IFN–γ (A) and in pg/10^6 PBMC for other cytokines IL-2 (B), IL-5 (C), IL-10 (D), and LIF (E). Values are presented as box-plots. Values within the boxes reflect data between the 25th and 75th percentiles. The points within the boxes determine the 50th percentile (median) values, and the bars extending below and above the boxes reflect the minimal and maximal nonaberrant values, respectively. NS: unstimulated culture conditions; LPS: stimulation with lipopolysaccharide; PHA: stimulation with phytohemagglutinin; LPS+PHA: stimulation with LPS and PHA. Comparisons between the control and patient groups were performed using the nonparametric Mann-Whitney U test, with the level of significance set at 0.05. *$p \leq 0.01$.

Table 1. Summary of the clinical and biological features of patients.

Variables	Patient 1	Patient 2	Patient 3	Patient 4
Age, yrs/sex	29/F	74/F	57/F	47/F
Time since onset of symptoms, mo	4	2	4	4
Cutaneous involvement				
Area	Arms, legs, hand, and back	Legs, thighs, and back	Arms and forearms	Legs, arms and hands
Type	Induration and hand edema	Induration and foot and leg edema	Induraton and forearm edema	Induration, leg and hand edema
Highest eosinophil count ($/mm^3$)	1410	1200	2800	1300
Immune globulins (g/l)	21	42	12	19
ESR (mm/h)	25	40	34	12

(Figure 1). LPS activation of PBMC from both populations did not enhance IL-5 release. In contrast, more IL-5 was detected in WBA of patient PBMC stimulated with PHA ($p = 0.01$) or LPS + PHA ($p = 0.01$) compared to those of controls.

IL-10 production. No difference was found between healthy individual and patient PBMC under baseline or LPS stimulated conditions, even though Patients 2 and 3 tended to produce more IL-10 than controls (Figure 1). Higher amounts of IL-10 were detected in WBA of patient PBMC stimulated with PHA ($p = 0.01$) or LPS + PHA ($p = 0.01$) than those of controls. LPS + PHA activation was the most potent for IL-10 production. It should be noted that the relationship between IL-10 levels and immune globulins approached significance ($p = 0.8$; $r = 0.2$).

LIF concentrations in the supernatants of short term WBA. Under basal conditions or LPS stimulation, LIF production did not differ markedly between patients and controls (Figure 1), suggesting that PBMC do not secrete LIF in the absence of exogenous stimulation, and/or that very little of this cytokine is circulating *in vivo*. In contrast, when stimulated with PHA ($p = 0.004$) or LPS + PHA ($p = 0.004$), PBMC of 4 patients increased LIF production compared to controls.

DISCUSSION

The concentrations of IFN−γ , IL-2, IL-5, IL-10, and LIF measurable by the available ELISA in WBA supernatants from 4 patients with EF or a group of healthy individuals were determined and compared. Until now, cytokine production by immunocompetent cells has been investigated either by detection of cytokine in peripheral blood or by examining *in vitro* secretion by isolated blood cells. In whole blood, natural cell to cell interactions are preserved, and circulating stimulatory and inhibitory mediators, such as soluble receptors, are also present at their physiological concentrations[22]. Recently, this method was applied to measure cytokine production by PBMC from patients with diverse autoimmune diseases or other diseases[34-37].

EF is a rare syndrome, giving rise to discussion in regards to its distinction from progressive systemic sclerosis. The pathological hallmarks of its chronic cutaneous involvement are inflammation and fibrosis of the fascia occasionally extending to the lower dermis and subjacent muscle. In addition, patients with Shulman's syndrome have increased circulating levels of immune globulins and eosinophils. IL-5, produced mainly by activated T cells[14,15], is thought to play a major role in the promotion of eosinophil differentiation and activation[16].

Diseases involving eosinophilia without an increase of other blood-cell lineages are usually accompanied by an overproduction of IL-5[16]. The mechanisms underlying this cytokine overproduction may involve an exacerbated response of the T helper lymphocytes of the Th2 phenotype. On the other hand, immune globulin production by B cells was also found to be controlled by other type 2 cytokines, e.g., IL-4 and IL-10.

Taken together, our present data support the notion of sustained overexpression of both type 1 and type 2 cytokines in EF, although wide variations were noted between individuals. However, they dealt with peripheral blood cells and not with local infiltrating cells from cutaneous lesions, which might change this conclusion.

High levels of Th2 (IL-5 and IL-10) cytokines may explain eosinophilia and excess immune globulin production. In agreement with a previous study in which IL-5 was not found in serum but was found in synovial fluid of a patient with EF[38], basal levels of IL-5 produced by PBMC from our patients were not different from those of controls. However, PHA stimulation triggered increased IL-5 production, suggesting a T cell dependent process. This skewing of the cytokine response in Shulman's syndrome is not readily explained and may be the result of an unknown antigen driven oligoclonal expansion. The links between eosinophilia and T cells and other components of cell mediated immunity are already documented, since primed T lymphocyte clones in the presence of their specific antigens are capable of producing a local or systemic eosinophil response[39,40]. Once activated and attracted into the inflamed tissue, eosinophils may also be able to perpetuate local inflammatory responses and, consequently, the disease. In this context, eosinophil extracts can induce fibroblast proliferation[41]. In addition, eosinophil activation

has been found to contribute to the inflammatory process in scleroderma[42].

Similarly, IL-10 is a potent stimulator of B lymphocytes[43,44], and probably plays a major role in the polyclonal B lymphocyte hyperactivity seen in patients with rheumatoid arthritis or Sjögren's syndrome and in the development of autoimmunity. Our patients' cultured PBMC secreted abnormally high amounts of IL-10 in the presence of PHA but surprisingly not in the presence of LPS, suggesting that T lymphocytes, and not monocytes or B lymphocytes, synthesized IL-10.

Type 1 cytokine concentrations were also elevated in patient PHA stimulated WBA compared to those of normal individuals. IFN–γ is known to be a potent inhibitor of collagen synthesis acting at the transcriptional level[45] and a repressor of type 2 cytokine-secreting T helper lymphocytes. It is therefore possible that the high IFN–γ levels released by EF T cells could represent either a negative regulatory feedback response to counter a full-blown eosinophil mediated immune response, leading to tissue damage or another expression of T cell involvement as pointed out above.

The sharply increased LIF synthesis noted in the WBA supernatants of our patients was a new finding and might be part of an even more complex regulatory mechanism. Indeed, although the biological roles of LIF have been defined *in vitro*, its *in vivo* status as a proinflammatory or antiinflammatory mediator remains controversial[26,46-49]. In addition to its capacity to trigger an acute phase protein production by hepatocytes, it has recently been implicated as a bridge between the endocrine and immune systems participating in neuroendocrine stress responses[50-52]. Mouse pituitary LIF and its receptor mRNA are induced dose-dependently by intraperitoneal injection of LPS *in vivo*[53], thereby supporting a paracrine function for LIF in mediating an immuno-neuroendocrine interaction within the pituitary.

LIF appears to act mainly on pituitary corticotrophs. Primary cultures of mouse pituitary cells respond to exogenous LIF by enhanced adrenocorticotrophic hormone (ACTH) secretion[54], as do AtT20 murine corticotrophs[55]. In addition, LIF potentiates the ability of corticotropin-releasing hormone (CRH) to induce ACTH secretion in AtT20 cells[55]. The LIF effect on the corticotrophs is blocked by antibodies directed against gp130 and the LIF receptor subunits, and is also attenuated by dexamethasone. The LIF overexpression found in EF patients could therefore be interpreted as an attempt by the organism to overcome, via increased corticosteroid production, a full-blown cell mediated immune response leading to tissue damage responsible for clinical symptoms. This interpretation is corroborated by the usual efficacy of corticotherapy in Shulman's syndrome.

In summary, we showed that all 4 EF patients had similar cytokine secretion patterns, characterized by increased syntheses of type 1 and type 2 cytokines. However, we cannot draw firm conclusions from 4 patients not matched for age and sex with controls. The high productions of IL-5 and IL-10 might explain eosinophilia and elevated immune globulins that characterize this syndrome. The elevated production of type 1 cytokines by T cells is not specific to Shulman's disease, but rather attests to T cell activation and recruitment by an unknown antigen. In this context, LIF might be part of a regulatory loop intended to control the inflammation process and limit the tissue damage responsible for the clinical symptoms.

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