



Brief report

# Cytokine production and modulation: Comparison of patients with chronic fatigue syndrome and normal controls

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## Abstract

We studied cytokine production in 15 patients with chronic fatigue syndrome (CFS) and 23 controls. CFS patients' peripheral blood mononuclear cells were cultured with lipopolysaccharide or phytohemagglutinin. Enzymatic immunoassay indicated cytokine concentration in culture supernatants. CFS patients showed significantly lower mRNA levels and transforming growth factor-beta1 (TGF- $\beta$ 1) production. Cytokine dysregulation affects CFS pathogenesis. TGF- $\beta$ 1 may aid treatment because it affects CFS inflammatory characteristics.

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## 1. Introduction

Chronic fatigue syndrome (CFS) is a condition that is characterized by unexplained, disabling fatigue; low-grade fever and subtle indications of immune system activation (Komaroff and Buchwald, 1991) often accompany it. In addition, unrefreshing sleep, impaired concentration and memory, and depressed

mood and anxiety are common symptoms (Miike et al., 2003). Although the pathogenesis of CFS remains a subject of intense study, some research suggests that a persistent viral infection is of etiologic importance (Archard et al., 1988; Yousef et al., 1988); one recent report of a variety of immunologic abnormalities in a high proportion of patients suggests involvement of an immunoregulatory dysfunction (Lloyd et al., 1989).

Cytokines have been implicated in the pathogenesis and clinical manifestations of CFS via their effects on the central nervous system (Moutschen et al., 1994; Levy, 1994). Moreover, one striking feature of CFS is its sudden onset following an acute,

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presumably viral, illness and the persistence or recurrence of flu-like symptoms. Such symptoms have been attributed to persistent cytokine production (Komaroff, 1988; Komaroff and Buchwald, 1991). To date, there is no clearly effective therapy for this disorder (Miike et al., 2003), but conflicting data from placebo-controlled studies suggest that IV $\gamma$ -globulin may be beneficial (Lloyd et al., 1990; Peterson et al., 1990).

This study investigates the immunoactivity depending on the evaluation of cytokine production. This report describes cytokine production in culture supernatants and in plasma of patients with CFS because cytokine dysregulation might be an important factor that is involved in pathogenesis of this syndrome. Furthermore, such knowledge might facilitate the evaluation of the effectiveness of  $\gamma$ -globulin treatment.

## 2. Methods

### 2.1. Subjects

This study evaluated 15 patients (8 men, 7 women) with CFS, aged  $20.3 \pm 9.47$  years, and 23 normal controls (14 men, 9 women), aged  $23 \pm 2.27$  years. All patients were referred to our hospital because of CFS symptoms. These patients met Center for Disease Control (CDC) diagnostic criteria for CFS. The period of CFS symptoms was longer than 12 months (avg. 1.3 years; range 1–3 years).

### 2.2. Cell culture

For this study, peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation, washed three times, and then adjusted to  $1 \times 10^6$  cells/ml with RPMI 1640 medium containing 10% heat-inactivated fetal calf serum (FCS) (FCS-free medium for TGF- $\beta$ 1 study), and 2 mM L-glutamine. Cells were then cultured with 5  $\mu$ /ml phytohemagglutinin (PHA) or 50 ng/ml lipopolysaccharide (LPS) and incubated 3 days at 37 °C under 5% CO<sub>2</sub>.

### 2.3. Cytokine measurement

IL-6, TNF- $\alpha$ , IL-1 $\beta$ : Concentration of these cytokines in the culture supernatant of LPS-stimulated

PBMC was measured by two-step (sandwich) enzyme immunoassay from Immunotech (Marseille, France). Samples and standards were incubated in microtitre plate wells, then coated with the first monoclonal antibody. The wells were emptied and washed; then a second monoclonal antibody linked to acetylcholinesterase was added. After incubation, the wells were emptied and washed; then bound enzymatic activity was measured by adding a chromogenic substrate. The resultant color intensity was proportional to cytokine concentration. Sensitivity of this ELISA assay is 3 pg/ml for IL-6, 5 pg/ml for TNF- $\alpha$ , and 15 pg/ml for IL-1 $\beta$ .

Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1): Concentration in cell culture supernatants of LPS-stimulated PBMC was assayed using the quantitative sandwich enzyme immunoassay technique (R and D Systems, Minneapolis, MN). The TGF- $\beta$ 1 soluble receptor type II, which binds TGF- $\beta$ 1, was pre-coated onto a microplate. Standards and samples were pipetted into wells; consequently, all TGF- $\beta$ 1 was bound by the immobilized receptor. The minimum detectable dose of TGF- $\beta$ 1 was < 7 pg/ml.

IFN- $\gamma$ : from PHA-stimulated PBMC culture supernatants. This assay employs enzyme-linked immunosorbent assay (ELISA; R and D Systems) with sensitivity of 8 pg/ml.

IL-4 and IL-18: IL-4 concentration in the culture supernatant of PHA stimulated PBMCs was measured using ELISA (Biosource International, Inc., Camarillo, CA) with sensitivity of <2.0 pg/ml. The IL-18 concentration in the culture supernatant of LPS-stimulated PBMC was measured using an ELISA kit (Medical and Biological Laboratories Co. Ltd., Nagoya, Japan) with minimum sensitivity of 12.5 pg/ml.

#### 2.3.1. Study of TGF- $\beta$ 1 mRNA expression

For RNA extraction,  $5 \times 10^6$  PBMCs were cultured with LPS for 3 h; then mRNA was isolated using the RNeasy Mini Kit (Qiagen, Inc.). Samples were hybridized with gene-specific biotin-labeled capture oligonucleotide probes and digoxigenin-labeled detection probes on streptavidin-coated microplate (Colorimetric mRNA Quantitation Kit; R and D Systems) according to the manufacturer's instructions. The standard curve was linear from 6.25 to 400 attomole/ml (amol/ml) of TGF- $\beta$ 1 mRNA.

#### 2.4. Statistical analysis

The Mann–Whitney *U*-test was used to compare study groups for significant differences. Probability (*P*) values of less than 0.05 were considered statistically significant.

### 3. Results

TGF- $\beta$ 1 protein production in the culture supernatant of LPS-stimulated PBMC from CFS subjects ( $5473.341 \pm 2749.808$ ) was significantly lower than that of normal controls ( $6698.338 \pm 2248.446$ )  $P=0.018$ . TGF- $\beta$ 1 mRNA production in LPS-stimulated PBMC was significantly lower in CFS patients ( $44.856 \pm 26.011$ ) than in normal controls ( $97.976 \pm 34.766$ ) ( $P < 0.001$ ). However, no statistically significant differences were detected when IL-10, IL-18, IL-4, TNF- $\alpha$ , IFN- $\gamma$ , and IL-6 were measured. Table 1 shows those results.

### 4. Discussion

The present study elucidated cytokine production pattern in patients with CFS, and provided a comparative evaluation of cytokine production. Abnormal regulation of cytokine activity may contribute to pathophysiology and clinical manifestations of CFS (Moutschen et al., 1994; Levy, 1994).

The present results demonstrate that production of TGF- $\beta$ 1 was significantly depressed in cell culture supernatants of LPS-stimulated PBMC from patients

with CFS, although some previous studies showed that serum levels of TGF- $\beta$ 1 or the intracellular TGF- $\beta$ 1 positive-cell counts in the blood were higher in CFS patients than in control subjects (Chao et al., 1991; Peterson et al., 1994; Bennett et al., 1997). Another study found normal serum levels of TGF- $\beta$ 1 (MacDonald et al., 1996). These conflicting findings may have resulted from differences in experimental approaches. Discrepancies often arise between data derived from levels of cytokines produced in vitro by cultured PBMC and in vivo levels of cytokines in the blood (Chao et al., 1991).

As a mechanism of TGF- $\beta$ 1 enhancement following such IV gamma globulin therapy, TGF- $\beta$ 1 is considered to function as an inhibiting antibody production (immunosuppressive factor). In short, IV $\gamma$ -globulin therapy enhances TGF- $\beta$ 1 secondary in vivo and/or in vitro from the viewpoint of suppressing immunoglobulin synthesis systems (Kekow et al., 1991; Bouchard et al., 1995). The important aspect of our study is that TGF- $\beta$ 1 might be considered to be an indicator for improvement of CFS patients' conditions. Therefore, TGF- $\beta$ 1, as a multifunctional anti-inflammatory cytokine, might be involved in immune functions; deficiency of this cytokine reported in CFS patients in the present study may contribute to inflammation that characterizes CFS syndrome, such as myalgia and muscular fatigue. This finding supports the anti-inflammatory and immunosuppressive functions of TGF- $\beta$ 1. The current study also investigated in vitro production of the anti-inflammatory cytokine IL-10, which has been reported to inhibit IFN- $\gamma$  and IL-4 production by T lymphocytes, IL-1 $\beta$ , IL-6 and TNF- $\alpha$  by mononuclear phagocytes (Yssel et

Table 1  
Cytokine levels in culture supernatants and plasma

Cytokine (pg/ml)	Controls ( <i>n</i> =23)	Patients ( <i>n</i> =14)	<i>P</i>
TGF- $\beta$ 1 (in vitro)	6698.338 $\pm$ 2248.446	5473.341 $\pm$ 2749.808	=0.018
TGF- $\beta$ 1 mRNA (amol/ml)	97.976 $\pm$ 34.766	44.856 $\pm$ 26.011	<0.001
TGF- $\beta$ 1 (plasma)	5.969 $\pm$ 3.554	7.836 $\pm$ 348	
IL-6 (in vitro)	11420.203 $\pm$ 3978.625	18181.78 $\pm$ 29360.645	
IL-6 (plasma)	0.873 $\pm$ 0.449	0.963 $\pm$ 0.803	
TNF- $\alpha$ (in vitro)	817.033 $\pm$ 684.887	946.333 $\pm$ 594.160	
IL-1- $\beta$ (in vitro)	506.119 $\pm$ 328.702	420.554 $\pm$ 269.412	
IL-10 (in vitro)	3744.514 $\pm$ 2159.093	4908.869 $\pm$ 2902.178	
IL-18 (in vitro)	68.252 $\pm$ 31.955	64.009 $\pm$ 23.338	
IFN- $\gamma$ (in vitro)	723.286 $\pm$ 1508.999	1234.083 $\pm$ 2348.071	

al., 1992; D'Andrea et al., 1993). In addition, we examined the cytokines IL-4, IFN- $\gamma$ , IL-1 $\beta$ , TNF- $\alpha$ , and IL-18. However, these cytokines were found to have comparable values for CFS patients and normal controls. This similarity concurs with findings by Borish et al., (1998). Previous studies and the results presented herein demonstrate the need for further detailed studies of these cytokines to clarify their roles in CFS pathogenesis.

In conclusion, results of this study suggest that cytokine dysregulation is not a singular or dominant factor in the pathogenesis of CFS: immunological, autonomic, and neuroendocrine abnormalities may be mutually dependent and mutually reinforcing factors.

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