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CYTOKINE PRODUCTION BY ADHERENT AND NON-ADHERENT MONONUCLEAR CELLS IN CHRONIC FATIGUE SYNDROME‡

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Summary—It has been suggested that cytokines play a role in certain clinical manifestations of chronic fatigue syndrome (CFS). In this study adherent (monocytes) and non-adherent (lymphocytes) mononuclear cells were stimulated in the presence or absence of phytohemagglutinin (PHA) or lipopolysaccharide (LPS), respectively, and supernatants were assayed for IL-6, TNF-α, and IL-10 by ELISA. IL-6 was also measured at the mRNA level by polymerase chain reaction. The levels of spontaneously (unstimulated) produced TNF-α by non-adherent lymphocytes and spontaneously produced IL-6 by both adherent monocytes and non-adherent lymphocytes were significantly increased as compared to simultaneously studied matched controls. The abnormality of IL-6 was also observed at mRNA level. In contrast, spontaneously produced IL-10 by both adherent and non-adherent cells and by PHA-activated non-adherent cells were decreased. This preliminary study suggests that an aberrant production of cytokines in CFS may play a role in the pathogenesis and in some of the clinical manifestations of CFS. © 1997 Elsevier Science Ltd.

Introduction

Fatigue is the major feature of chronic fatigue syndrome (CFS). Cytokines have been suggested to play a role in the pathogenesis and clinical manifestation of CFS via their effects on CNS (Moutschen et al., 1994; Levy, 1994). Abnormalities in the regulation of the hypothalamic-pituitary-adrenal (HPA) are a well-recognized feature of endogenous depression and associated fatigue. It is suggested that the mechanism underlying this phenomenon is altered activity of corticotrophin releasing hormone (CRH) at the level of hypothalamus, (Levy, 1994). Demitrack et al., 1991, have reported alteration activation of HPA in patients with CFS. Therefore, alterations in the secretion of any of the cytokines in combination with altered HPA axis may play a role in the fatigue and depression associated with CFS. Moutschen et al., 1994 have proposed a role of TNF-α in the asthenia associated with CFS. IL-1, IL-6 and TNFα are proinflammatory cytokines that are produced by a number of central loci and are potent stimulators of HPA (Dinarello,

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1988, Gupta, 1988 Kishimoto et al., 1990). A large number of immune abnormalities, including cytokine production in CFS, have been reported (Buchwald & Komaroff, 1991, Gupta & Vayuvegula, 1991, Gupta, 1992, Jone, 1991, Kalimas et al., 1990, Lloyd et al., 1989, Straus et al., 1989). The published data on cytokine levels are contradictory (Cheney et al., 1989, Lever et al., 1988, Straus et al., 1989 Lloyd et al., 1994, Linde et al., 1992). Furthermore, no study has been done on separated adherent and non-adherent cells, and none has been reported on IL-10 production in CFS. In the present study, we examined spontaneous and phytohemagglutinin-(PHA, for lymphocytes) and lipopolysaccharide (LPS, for monocytes)-induced IL-6, IL-10 and TNF-α production in patients with CFS and matched healthy controls. Our data demonstrate increased IL-6 and TNF-α production and decreased production of IL-10 in CFS.

MATERIALS AND METHODS

Subjects

Six patients with CDC-defined CFS (male:female; 1:5; ages 32–42 years) and six age-(36–44 years) and sex-matched (male: female 1:5) healthy, normal volunteers were studied. The protocol for the study was approved by the Institutional Review Board of the University of California, Irvine, CA, U.S.A.

Materials

ELISA kits for IL-6, TNF-α, and IL-10 were purchased from Genzyme, Cambridge, MA, U.S.A. Primers for IL-6 and β-actin were synthesized by Genechem, Inc. Waltham, MA, U.S.A. The primers for IL-6 were: 5'-ATGAACTCCTTCTCCACAAGC and 3'-CTACATTTGCCGAAGAGCCCTCAGGCTGGACTG. PHA-P and lipopoly-saccharide (LPS) were purchased from SIGMA Chemicals, St. Louis, MO, U.S.A. GenAmp PCR kit was purchased from Perkin Elmer, CO, U.S.A.).

Methods

Peripheral blood mononuclear cells (MNC) were separated on Ficoll-Hypaque density gradient. MNC were washed three times with phosphate buffer saline (PBS) and resuspended in RPMI-1640 medium supplemented with 20% heat-inactivated fetal bovine serum (FBS). Adherent and non-adherent cells were separated by plastic adherence by incubating MNC at 37^{0}C for 45 min. Non-adherent (lymphocytes) were decanted and adherent (monocytes) cells were removed by rubber policeman. Adherent cells were >92% CD14+ monocytes, whereas non-adherent cells (lymphocytes) contained <2% CD14+ monocytes and were >92% CD3+ lymphocytes. Cells were resuspended in RPMI-1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2mM L-glutamine, and 10% FBS. Adherent cells ($2x10^6$/ml) were incubated in the presence or absence of lipopolysachharide (1 μg/ml) for 4 h and lymphocytes were incubated in the presence or absence of phytohemagglutinin (PHA 10μg/ml) for 24 h at 37°C in a 5% CO_2 atmosphere. At the end of culture, supernatants were collected and stored frozen at −20°C until assayed. Cytokine levels were assayed by ELISA technique, using serial dilutions of test supernatants

against a series of standards. Cytokine levels in cell culture supernatants stimulated in the absence of LPS or PHA were termed "spontaneous". Stimulated levels of cytokines were calculated by substrating spontaneously produced cytokines from stimulated cultures. All samples were done in triplicate and at each occasion, patients and controls were done in pairs (i.e., same number of patients and controls of same age and sex). Data are expressed for cytokine levels as mean pg/ml. Time and concentration of LPS and PHA kinetics for peak cytokine production by monocytes and lymphocytes were similar in CFS and control groups (data not shown).

Polymerase chain reaction

IL-6 at the mRNA level was also measured by reverse transcriptase polymerase chain reaction (RT-PCR), using a RT-PCR kit. In brief, total cellular RNA was extracted by guanidium thiocynate-phenol chloroform method. Two hundred nanogram of total RNA was used as a template for cDNA synthesis and amplification by PCR. A known amount of pAW109, a positive control template transcribed from the plasmid pAW109 in the GeneApm RNA PCR kit was added to the cellular RNA prior to first strand cDNA synthesis, and then co-amplified along with the cytokine message. Specific DNA sequences were amplified by PCR, using a Perkin-Elmer Thermal Cycler. The temperature giving optimal results were: denature at 94°C for 1 min, primer annealing at 60°C, and primer extension at 72°C for 2 min. β-actin was used as internal control.

Results

IL-6 production in CFS

The data of IL-6 production by adherent (AD) monocytes and non-adherent (NAD) lymphocytes in CFS and controls are shown in Figure 1. Spontaneous IL-6 production in CFS by NAD lymphocytes (54 ± 4 pg/ml) and by AD monocytes (59 ± 6 pg/ml) was significantly ($P < 0.005$) higher than the spontaneous IL-6 produced by NAD lymphocytes (2 ± 0.9 pg/ml) and AD monocytes (26 ± 3 pg/ml) from healthy controls. In CFS, IL-6 production by PHA-activated NAD lymphocytes (2396 ± 436 pg/ml) and by LPS-activated AD monocytes (3100 ± 487 pg/ml) was similar ($P > 0.5$) to that produced by PHA-activated NAD lymphocytes (1780 ± 723 pg/ml) and by LPS-stimulated AD monocytes (2234 ± 498) from controls. To determine whether the increased IL-6 production was also at the mRNA level, RT-PCR was performed. A representative experiment is shown in Figure 2. An increased IL-6 mRNA was observed in both AD and NAD cells.

IL-10 production in CFS

Data of spontaneous and PHA-induced IL-10 by NAD lymphocytes and by LPS-activated monocytes from patients with CFS and controls are shown in Figure 3. Spontaneously produced IL-10 by both NAD lymphocytes (12.2 ± 2 pg/ml) and AD monocytes (7.1 ± 1.4 pg/ml) in CFS was significantly lower ($P < 0.05$) than that produced by NAD lymphocytes (23.3 ± 4 pg/ml) and by AD monocytes (17.5 ± 2.2 pg/ml) from controls. PHA-induced IL-10 production by NAD lymphocytes in CFS (405 ± 56 pg/ml) was significantly

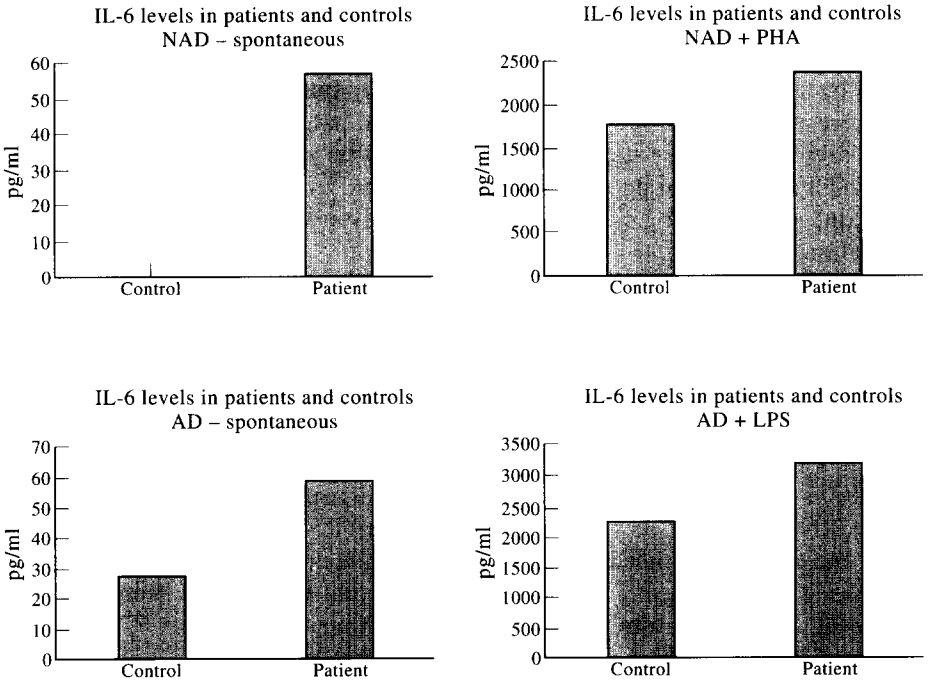

Figure 1. IL-6 production in CFS.Adherent (AD, monocytes) and non-adherent (NAD, lymphocytes) cells from six each of CFS patients and controls were incubated in the presence or absence of LPS (for adherent monocytes) or PHA (for non-adherent lymphocytes) respectively for 4 h (monocytes) and 18-24 hours (for lymphocytes). Supernatants were collected and assayed for IL-6, using ELISA kits. Results are expressed as mean pg/ml. Spontaneously produced cytokines were those present in supernatants from cell cultured in the absence of PHA or LPS. Induced cytokine levels (net levels) were calculated by subtracting the spontaneously produced cytokines from induced cytokines.

($P < 0.05$) lower than that produced by controls (904 ± 34 pg/ml). However, no significant ($P > 0.1$) difference was observed in LPS-induced IL-10 production by AD monocytes from CFS (601 ± 81 pg/ml) from that produced by controls (794 ± 102 pg/ml).

TNF-α production in CFS

Results of TNF-α production in CFS and controls are shown in Figure 4. Spontaneous TNF-α produced by AD monocytes from CFS (182 ± 23 pg/ml) was significantly higher ($P < 0.001$) than that produced by lymphocytes from controls (5 ± 2 pg/ml). Spontaneously produced TNF-α by NAD lymphocytes in CFS (65 ± 7 pg/ml) was similar ($P > 0.05$) to that produced by controls (104 ± 35 pg/ml). No significant difference ($P > 0.05$) was observed in PHA-induced TNF-α production by NAD lymphocytes (704 ± 89 pg/ml) and LPS-induced TNF-α production by AD monocytes (1820 ± 345 pg/ml) from patients with CFS from that produced by PHA-activated NAD lymphocytes (1020 ± 205 pg/ml) and AD monocytes (1234 ± 342 pg/ml).

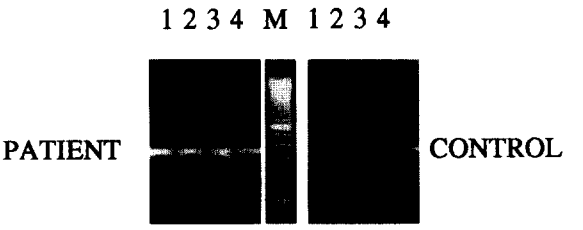

Figure 2. A representative experiment of IL-6 mRNA in CFS and control. Total RNA was isolated from unstimulated and stimulated adherent monocytes and non-adherent lymphocytes. IL-6 mRNA was assayed by RT-PCR, using specific primers. β-actin was used as an internal control. Lane 1 is adherent monocytes. Lane 2 is LPS-activated monocytes. Lane 3 is non-adherent lymphocytes, and the Lane 4 is PHA-activated lymphocytes. M is the marker.

Discussion

It has been hypothesized that abnormal production of cytokines may play a role in the pathogenesis and clinical manifestations of CFS (Moutschen et al., 1994). A number of investigators have reported levels of certain cytokines in serum and in culture supernatants. Cheney et al., 1989 reported elevated levels of interleukin 2 (IL-2) in sera of patients with CFS. In contrast, Straus et al., 1989, in 25 patients with CFS, observed normal serum levels of interleukin 1-β (IL-1β), IL-2, interferon-γ (IFN-γ), IFN-α, and tumor necrosis factor (TNF). The reason for these discrepancies could be due to differences in capture antigens used in coating of ELISA plates used in these assays and effect of certain serum blocking

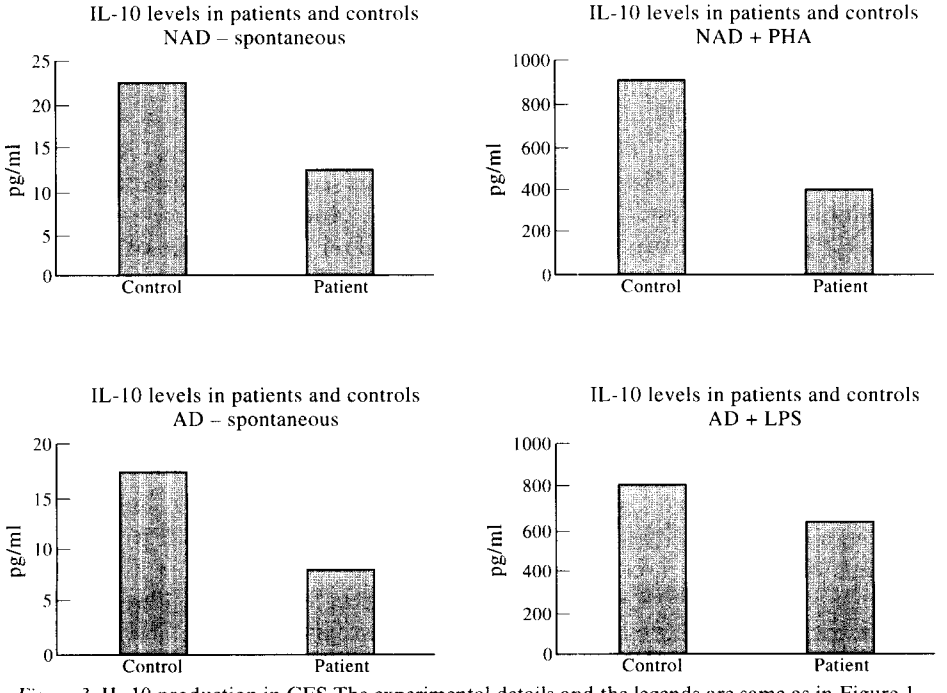

Figure 3. IL-10 production in CFS.The experimental details and the legends are same as in Figure 1.

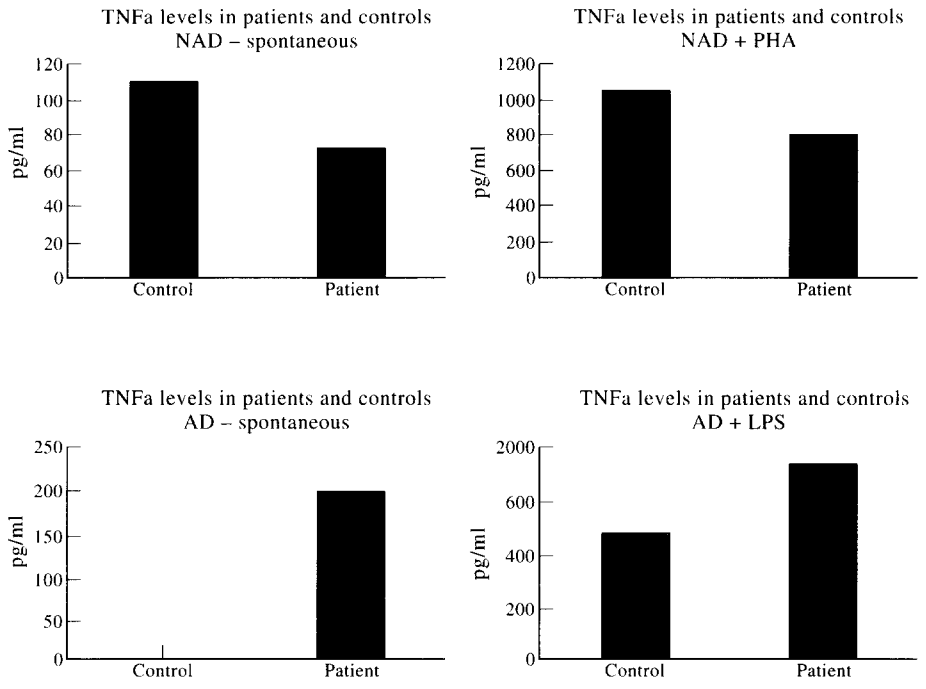

Figure 4. TNF-α production in CFSExperimental conditions and legends are same as in Figure 1.

factors. A number of investigators observed decreased *in vitro* production of IL-2 and IFN-γ by peripheral blood MNC upon stimulation with mitogens (PHA, Con A or PWM) or phorbol ester (Kalimas et al., 1990, Straus et al., 1993, Chao et al., 1991a, Gold et al., 1990, Milton et al., 1991). In contrast, Morte et al., 1988 observed normal interferon production, and Altmann et al., 1988 and Rasmussen et al., 1991 observed increased interferon production in CFS. The latter two studies were performed on small samples of patients.

Chao et al., 1991b, observed increased serum levels of IL-6 and neopterin in patients with CFS, suggesting an activation of mononuclear phagocytic cells. Patarca et al., 1991 observed no significant difference in serum IL-6 levels between patients with CFS and controls. However, in their study there was a marked variability among subjects with large standard deviations. Furthermore, these investigators did detect IL-6 mRNA in unstimulated MNC from CFS and suggested that there may be latent activation of MNC in CFS. In the present study, we observed that spontaneous IL-6 production by both adherent monocytes ($P < 0.05$) and non-adherent lymphocytes ($P < 0.001$) was significantly increased in CFS as compared to controls. We have also observed increased IL-6 mRNA in unstimulated adherent monocytes and non-adherent lymphocytes in CFS, suggesting increased IL-6 in CFS at both transcriptional and translational levels. This would suggest an *in vivo* activation of both monocytes and lymphocytes in CFS. Chao et al., 1991a reported increased IL-6 production by unfractionated MNC in response to PHA or LPS. In the present study, although there was a modest increase in IL-6 production by lym-

phocytes and monocytes stimulated with PHA and LPS respectively, the differences were not statistically significant ($P > 0.1$). However, the sample size of our present study is small.

Tumor necrosis factor-α is a proinflammatory molecule that appears to play a role in the pathogenesis of AIDS and multiple sclerosis, both associated with chronic fatigue (Matsuyama et al., 1991, Brosnan et al., 1988). Lloyd et al., 1994 observed normal levels of serum TNF-α in CFS and there was no further increase following exercise-induced fatigue. In contrast, Chao et al., 1991b reported increased TNF-α production by MNC of CFS in response to LPS. In the present study, we observed significantly increased ($P < 0.001$) spontaneous production of TNF-α by adherent monocytes from CFS; although there was a trend towards increased LPS-induced TNF-α production by adherent cells, the differences were not statistically significant ($P > 0.05$). The differences between our study and those of Chao et al., 1991a could be due to use of fractionated adherent and non-adherent cells and the small number of patients examined in the present study.

IL-10 is a cytokine that is produced by a variety of cell types, including TH2 type T cells (Howard et al., 1992). IL-10 inhibits cytokines produced by TH1 type T cells, including IL-2 and IFN-γ. Furthermore, IL-10 inhibits monokine production by activated macrophages, including IL-6 and TNF-α (Fiorentino et al., 1991). There are no published reports of IL-10 in CFS. In the present study, we observed a significant decrease ($P < 0.05$) in spontaneous IL-10 production by monocytes and lymphocytes and PHA-induced IL-10 production by non-adherent cells in CFS. A decreased IL-10 production could explain increased IL-2 and IFN-γ production described in previous studies (Kalimas et al., 1990, Straus et al., 1989, Chao et al., 1991b) and increased IL-6 and TNF-α production in the present study of patients with CFS.

In summary, patients with CFS demonstrate abnormal cytokine (IL-6, TNF-α, and IL-10) production by adherent monocytes and non-adherent lymphocytes. IL-6 and TNF-α are proinflammatory cytokines and, therefore, their increased production may be responsible for many flu-like symptoms of CFS, including myalgia and muscular fatigue.

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