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Normal production of inflammatory cytokines in chronic fatigue and fibromyalgia syndromes determined by intracellular cytokine staining in short-term cultured blood mononuclear cells

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SUMMARY

It has been proposed that cytokines play a role in the pathogenesis of chronic fatigue syndrome (CFS) and fibromyalgia syndrome (FMS). However, different studies have reported conflicting results using enzyme-linked immunosorbent assay or polymerase chain reaction to detect cytokines in these conditions. In the present study, for the first time, the production of inflammatory [interleukin (IL)-1α, IL-6, and TNF-α] and anti-inflammatory (IL-10) cytokines by $CD14^+$ and $CD14^-$ peripheral blood mononuclear cells (PBMC) from chronic fatigue syndrome (CFS) and fibromyalgia syndrome (FMS) patients and sex- and age-matched normal subjects was investigated at the level of individual cells using the technique of intracellular cytokine staining and flow cytometry. Cultures were carried out in the presence of polymyxin B to inhibit the effect of endotoxins on cytokine production by monocytes. The mean intensity of fluorescence (MIF) and percentage of $CD14^+$ (monocytes) and $CD14^-$ (lymphocytes) cytokine-producing mononuclear cells were comparable in patients and controls in either unstimulated or IFN-γ-stimulated conditions. Our study indicates that dysregulation of cytokine production by circulating monocytes or non-monocytic cells (lymphocytes) is not a dominant factor in the pathogenesis of CFS/FMS.

Keywords chronic fatigue syndrome fibromyalgia syndrome intracellular cytokines monocyte

INTRODUCTION

The currently preferred terms for patients with unexplained fatigue and/or chronic pain are chronic fatigue syndrome (CFS) and fibromyalgia syndrome (FMS). To fulfil the criteria for FMS, an individual must have both chronic widespread pain and the presence of 'tender points' on examination. The current definition of CFS requires that the affected individual displays severe persistent fatigue without a defined cause, as well as the presence of four of the eight following symptoms: myalgia, arthralgia, sore throat, tender nodes, cognitive difficulty, headache, postexertional malaise or sleep disturbances [1].

There is a significant body of opinion which believes that CFS and FMS are part of a spectrum of conditions classified as neuroendocrine immune dysfunction [2]. It is likely that these chronic widespread fatigue/pain syndromes arise from interactions between the autonomic central nervous system [3,4], hypothalamic–pituitary–adrenal (HPA) axis [5–7] and the immune system [8].

Cytokines are considered to play a role in the pathogenesis and clinical manifestations of CFS/FMS. Specifically, tumour necrosis factor (TNF)-α, interleukin (IL)-1 and IL-6 are important regulators of inflammation, and may also be involved in the regulation of the HPA and the sympathetic nervous system [9]. Investigation into circulating cytokines levels has not indicated changes in CFS or FMS compared to controls [10,11]. However, the published data on the levels of cytokines secreted by cultured cells are contradictory. In fact, studies have reported increased [11–14], normal [5,10] or even decreased [15] production of inflammatory cytokines in the stimulated or unstimulated culture supernatants of peripheral blood mononuclear cells (PBMC) from CFS/FMS patients using enzyme-linked immunosorbent assay (ELISA) or polymerase chain reaction (PCR).

To analyse the quantity and quality of inflammatory and anti-inflammatory cytokine production by $CD14^+$ (monocytes) and $CD14^-$ PBMC (mainly lymphocytes) from CFS/FMS patients, we employed intracellular cytokine staining and flow cytometric analysis to facilitate the detection of cytokine production by

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individual cells. This is the first time this technique has been applied to the analysis of cytokine production in CFS/FMS.

MATERIALS AND METHODS

Study subjects

The present study comprised 22 CFS/FMS patients (six males/16 females, aged 18–55 years) and 19 age- and sex-matched healthy controls (five males/14 females, aged 18–55 years). Controls and patients were divided into two groups based on whether their cells were cultured in presence or absence of polymyxin B. Three patients and four controls were analysed for cytokine production in both the presence and absence of polymyxin B. The study protocol was approved by the Ethics Committee of University Hospital Nottingham and all subjects gave informed consent. The CFS/FMS outpatients were recruited in a specialist CFS/FMS clinic at University Hospital, Nottingham, UK. All patients fulfilled either the American College of Rheumatology criteria for the classification of FMS [16] or the Oxford criteria for CFS [17], and their clinical profiles were assessed by comprehensive semi-structural biopsychosocial interview conducted by D.S. Twenty-two patients were examined; two patients presented the symptoms of CFS only, four patients presented with the symptoms of FMS, while 14 patients presented with the symptoms for both CFS and FMS. This is consistent with a recently reported study in which 58% of female FMS and 80% of male FMS patients met the full criteria for CFS [18].

The controls were 19 healthy adults, with negative past, present or family history for psychiatric disorders, and all were free of any medications known to affect the immune system.

Cell separation and culture

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized whole blood of normal healthy donors and CFS/FMS patients by standard density gradient centrifugation with Histopaque 1077 (Sigma, Poole, UK). PBMC were harvested from the interface, washed once with Hanks's balanced salt solution (Sigma) and once with RPMI-1640 medium (Invitrogen, Paisley, UK), centrifuging at 400 ***g*** for 10 min. The PBMC were resuspended at 1×10^6 cells/ml in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) (Harlan, Loughborough, UK), 10 mM HEPES buffer (Sigma), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (all from Invitrogen).

The above cell suspensions (1-ml aliquots) were dispensed into 5 ml sterile tubes (Elkay, Hampshire, UK) with or without 1500 U/ml human recombinant (hr) IFN-γ (R&D Systems, Oxon, UK) for 6 h at 37°C in 5% CO_2 with tubes at a 5° slant from the horizontal position in the presence of 10 μg/ml brefeldin A (Sigma) and with or without 50 μg/ml polymyxin B sulphate (Sigma). Cells without hrIFN-γ were cultured in the presence of brefeldin A only and considered as unstimulated cells. At the end of the incubation, 20 μl of 100 mM EDTA were added to each tube and incubated for 10 min at room temperature. Cells were washed with RPMI-1640/2% FCS, resuspended in 0·5 ml of 0·5% formaldehyde in azide-free balanced electrolyte solution (Beckman Coulter, High Wycombe, UK) and stored at 4°C overnight. We found that 6 h stimulation was the optimal time for the detection of cytokines within $CD14^+/CD14^-$ cells, as culturing of cells for more than 6 h resulted in severe down-regulation of CD14 expression. The percentage of $CD14^+$ cells was greatly reduced by 12 and 24 h of culture without stimulation in the presence of brefeldin A only. Reduction of the monocyte population was also observed in the light scatter cytogram, indicating that cell death rather than CD14 marker down-regulation was responsible for this reduction. The same results were obtained with PBMC from the patient group (data not shown).

Antibodies

The following monoclonal antibodies were used: phycoerythin (PE)-rat antihuman IL-10, fluorescein isothiocyanate (FITC)-rat antihuman IL-6 and FITC-mouse antihuman IL-1α (all from Becton Dickinson, Oxford, UK); PE-mouse antihuman TNF-α and allophycocyanin (APC)-mouse antihuman CD14 (Beckman Coulter); mouse IgG_1-PE and mouse IgG_1-FITC (Beckman Coulter) were used as isotype controls.

Intracellular cytokine staining

Intracellular cytokine staining was performed as described previously [19]. Briefly, the fixed cells were washed three times with cold PBA [phosphate buffered saline (PBS) with 0·5% bovine serum albumin and 0·1% sodium azide], saponin buffer [PBA/0·04% saponin (Sigma)] and 10% FCS in saponin buffer, respectively, at 300 ***g*** for 5 min at 4°C. Fluorochrome-conjugated antibodies were mixed and added to the cells after the last wash, followed by incubation at 4°C in the dark for 2 h with occasional shaking. Fluorochrome-conjugated, isotype-matched IgG_1 was used as a control for detecting non-specific binding. Cells were then washed three times with saponin buffer and fixed with 0·5 ml of 0·5% formaldehyde in azide-free balanced electrolyte solution (Beckman Coulter).

Flow cytometric analysis

Cells were analysed using a Coulter EPICS Altra flow cytometer (Beckman Coulter). For each tube, 150 000 events were collected in a gate created around the viable lymphocyte and monocyte populations by forward and side scatter characteristics. Dot plots of CD14 *versus* cytokine staining (TNF-α, IL-1α, IL-6 and IL-10) were created. Quadrants were applied to the isotype control dot plots and medium-control dot plots to exclude nonspecific staining of cells. The same quadrants were used for the dot plots of the stimulated cells. The reproducibility of the method was confirmed by analysing cells from the same individual on separate occasions.

Statistical analysis

Comparisons between two sample populations were made with the non-parametric Mann–Whitney *U*-test. Comparison within one sample population was assessed by Wilcoxon signed rank test. Correlation between cytokine-producing cells and disease activity were tested using Spearman's correlation analysis.

RESULTS

Viable PBMCs were gated electronically in the flow cytometer and cytokine production by $CD14^+$ and $CD14^-$ cells was ascertained in CFS/FMS patients and normal individuals. Preliminary experiments revealed that when cells were cultured in medium supplemented with 10% FCS in the presence of brefeldin A, a high proportion of intracellular cytokine-positive $CD14^+$ cells were detected in both patients and controls (Table 1). This was particularly the case for expression of TNF-α and IL-1α, with

Table 1. MIF and percentage of cytokine-producing $CD14^+$ and $CD14^-$ cells from controls and CFS/FMS patients cultured for 6 h in the absence of polymyxin B. PBMC were cultured in medium and stained for intracellular cytokines as indicated in the Materials and Methods. Results, given as medians and (25th and 75th percentiles), are percentage of cytokine producing $CD14^+$ and $CD14^-$ cells (upper row) and MIF (lower row)

	Controls ($n = 10$)		CFS/FMS ($n = 10$)	
Cytokines	$CD14^+$	$CD14^-$	$CD14^+$	$CD14^-$
TNF-α				
%cells	43 (28–77)*	0·98 (0·52–1·7)*	44 (33–60)	0·98 (0·77–1·4)
MIF	327 (174–447)	143 (83–191)	192 (129–389)	168 (81–200)
IL-1α				
%cells	31 (25–86)*	0·10 (0·04–0·82)*	44 (24–70)	0·03 (0·01–0·6)
MIF	254 (182–416)	190 (150–228)	225 (184–346)	130 (48–198)
IL-6				
%cells	3·1 (0·9–18)*	0·08 (0·02–0·23)*	2·5 (1·7–9·4)	0·01 (0·0–0·40)
MIF	126 (98–157)	89 (0·0–141)	98 (79–148)	41 (0·0–122)
IL-10				
%cells	3·9 (2·5–6·0)*	1·0 (0·65–1·8)*	4·0 (2·7–8·3)	0·6 (0·3–1·4)
MIF	47 (43–61)	55 (42–56)	51 (45–69)	56 (52–62)

*$P > 0·05$ comparing percentage and MIF of $CD14^+$ and $CD14^-$ cells between the normal control group and the CFS/FMS group.

Table 2. MIF and percentage of inflammatory (TNF-α, IL-1α and IL-6) and anti-inflammatory (IL-10) cytokine-producing $CD14^+$ cells from controls and CFS/FMS patients cultured for 6 h in presence of polymyxin B. PBMC were cultured for 6 h with (stimulated) or without (unstimulated) human recombinant IFN-γ. Results, given as medians (means) and [25th and 75th percentiles], are the percentage of cytokine-producing $CD14^+$ cells (upper row) and MFI (lower row) in whole PBMC

	Controls ($n = 13$)		CFS/FM ($n = 15$)	
Cytokines	Unstimulated	Stimulated	Unstimulated	Stimulated
TNF-α				
%cells	1·2 (1·3)[0·78–1·65]*	7·0 (7·0)[5·0–9·0]**	1·4 (1·7)[0·8–2·5]	6·7 (7·0) [4·2–10·8]
MIF	77 (75)[62–88]	83 (87)[73–103]	76 (77)[62–89]	81(89)[68–123]
IL-1α				
%cells	0·18 (0·22)[0·05–0·38]*	0·16 (0·33)[0·14–0·57]**	0·22 (0·22)[0·08–0·34]	0·21(0·35)[0·08–0·84]
MIF	122 (118)[106–170]	189 (200)[153–245]	146 (153)[122–183]	183 (186)[113–278]
IL-6				
%cells	ND	ND	ND	ND
MIF	ND	ND	ND	ND
IL-10				
%cells	1·4 (4·1)[0·45–2·1]*	0·8 (2·2)[0·13–2·0]**	1·7 (2·3)[0·49–4·1]	2·0 (2·4)[0·09–4·77]
MIF	35 (39)[33–47]	45 (41)[32–45]	40 (42)[33–50]	37 (42)[31–54]

ND = not detectable. *$P > 0·05$ comparing percentage of cells and MIF in the unstimulated normal group with the unstimulated CFS/FMS group. **$P > 0·05$ comparing percentage of cells and MIF in the hrIFN-γ-stimulated normal group with the hrIFN-γ-stimulated CFS/FMS group.

lower expression of IL-6 and IL-10. Culturing the cells in presence of polymyxin B dramatically reduced the proportion of cytokine positive cells and the mean intensity of fluorescence (MIF) of staining in both patients and controls, particularly the production of inflammatory cytokines (IL-1α, TNF-α and IL-6) in the $CD14^+$ monocyte population (Table 2). Following 6 h culture with polymyxin B, the median percentage of cytokine-positive cells and (MIF) for intracellular TNF-α, IL-1α and IL-6 produced by $CD14^+$ cells reduced from 43% (327), 31% (254) and 3·1% (126) to 1·2% (77), 0·18% (122) and non-detectable levels in the normal control group; and from 44% (194), 44% (225), and 2·5% (98) to 1·4% (76), 0·22% (146) and non-detectable levels in the patient group, respectively (Tables 1 and 2). However, the MIF and percentage of $CD14^+$ and $CD14^-$ cytokine-producing cells were comparable in patients and controls when cells were cultured for 6 h without stimulation either in presence or absence of polymyxin B ($P > 0·05$) (Tables 1–3).

Stimulation with phorbol myristate acetate and ionomycin (PMA/I) resulted in severe cell death and down-regulation of CD14 expression (data not shown). Therefore, a more physiological stimulator, namely hrIFN-γ, was chosen. A summary of the production of inflammatory and anti-inflammatory cytokines by

Table 3. MIF and percentage of inflammatory (TNF-α, IL-1α and IL-6) and anti-inflammatory (IL-10) cytokine-producing CD14$^-$ cells from controls and CFS/FMS patients cultured for 6 h in the presence of polymyxin B. PBMC were cultured for 6 h with (stimulated) or without (ustimulated) human recombinant IFN-γ. Results, given as medians (means) and [25th and 75th percentiles], are the percentage of cytokine-producing CD14$^-$ cells (upper row) and MIF (lower row) in whole PBMC.

	Controls ($n = 13$)		CFS/FM ($n = 15$)	
Cytokines	Unstimulated	Stimulated	Unstimulated	Stimulated
TNF-α				
%cells	0·6 (0·6)[0·4–0·68]*	0·8 (0·9)[0·62–1·14]**	0·62 (0·6)[0·54–0·68]	0·91 (0·93)[0·63–1·1]
MIF	97 (103)[73–120]	93 (92)[82–104]	77 (88)[72–94]	84 (83)[77–90]
IL-1α				
%cells	0·0 (0·02)[0·0–0·06]*	0·0 (0·06)[0·0–0·15]**	0·01 (0·17)[0·0–0·08]	0·01(0·33)[0·0–0·93]
MIF	0·0 (41)[0·0–115]	0·0 (56)[0·0–145]	114 (103)[0·0–202]	64 (102)[0·0–235]
IL-6				
%cells	0·08 (0·2)[0·0–0·38]*	0·33 (0·33)[0·1–0·6]**	0·02 (0·07)[0·0–0·08]	0·01 (0·07)[0·0–0·11]
MIF	111 (92)[0·0–140]	117 (92)[50–135]	84 (69)[0·0–127]	40 (69)[0·0–137]
IL-10				
%cells	0·63 (0·74)[0·17–1·0]*	0·45 (0·83)[0·16–1·32]**	0·30 (1·0)[0·17–2·2]	0·57 (1·7)[0·05–3·5]
MIF	44 (46)[38–53]	48 (46)[40–53]	52 (50)[39–57]	41 (47)[39–59]

*$P > 0·05$ comparing percentage of cells and MIF in the unstimulated normal group with the unstimulated CFS/FMS group. **$P > 0·05$ comparing percentage of cells and MIF in the hrIFN-γ-stimulated normal group with the hrIFN-γ-stimulated CFS/FMS group.

CD14$^+$ and CD14$^-$ cells in CFS/FMS patients and controls following 6 h stimulation with hrIFN-γin the presence of polymyxin B is shown in Tables 2 and 3, respectively. MIF and percentage of intracellular TNF-α and IL-1α-producing monocytes increased significantly after 6 h stimulation in both patients and controls ($P < 0·05$) (Table 2). However, hrIFN-γ stimulation significantly increased production of only TNF-α, but not IL-1α, in the CD14$^-$ cells of both patients and controls (Table 3). Intracellular IL-6 was undetectable in either stimulated or unstimulated CD14$^+$ cells of the patient and control groups in presence of polymyxin B and IL-6 production by CD14$^-$ cells was very low (Tables 2 and 3). Examination of the IL-10 response of CD14$^+$ and CD14$^-$ cells to hrIFN-γ showed that cells did not respond in either CFS/FMS patients or controls ($P > 0·05$). The MIF and percentage of cytokine-producing CD14$^+$ and CD14$^-$ cells were comparable between patients and controls for all the cytokines measured in hrIFN-γ stimulated conditions ($P > 0·05$).

There was no significant correlation between the frequency of cytokine-producing cells and fatigue/pain severity or the impact of disease within the patient group, which were measured by the Chalder Fatigue Scale [20], the Brief Pain Inventory [21] and the Work and Social Adjustmant Scale [22].

DISCUSSION

In the present study, using flow cytometry, the percentage of cells and MIF of inflammatory and anti-inflammatory cytokines from CFS/FMS patients and healthy controls were determined and compared at the individual cell level. The main findings are that the MIF and percentage of CD14$^+$ (monocytes) and CD14$^-$ (lymphocytes) cytokine-producing cells were comparable between patients and controls when cells were cultured for 6 h with or without hrIFN-γstimulation and either in the presence or absence of polymyxin B.

It has been hypothesized that abnormal production of cytokines may play a role in the pathogenesis of CFS/FMS. However, several studies have reported conflicting results with respect to the levels of certain cytokines in the circulation and in culture supernatants using ELISA or PCR. Chao *et al*. [13] reported an increase in LPS-stimulated release of IL-1β, IL-6 and TNF-α from PBMC, but not in serum, from CFS patients *versus* control subjects. In contrast, Swanink *et al*. [15] showed that endotoxin-stimulated *ex vivo* production of TNF-α and IL-1β was significantly lower in CFS; however, there was no obvious difference in circulating cytokines and *ex vivo* production of IL-1α. Gupta *et al*. [14] investigated the levels of cytokines in the supernatant of adherent (monocytes) and non-adherent (lymphocytes) mononuclear cells cultured in the presence or absence of LPS or phytohaemagglutinin (PHA). The levels of IL-6 produced spontaneously by both monocytes and lymphocytes from CFS patients were significantly increased, while those of IL-10 decreased. Furthermore, a recent report from the National Institute of Health on 13 FMS patients and eight healthy controls showed no differences in IL-6 serum levels [5]. Finally, no sign of monocyte activation, as a main source of inflammatory cytokines, was reported in FMS patients by measuring the levels of neopterin [23].

Although these conflicting finding may have resulted from differences in experimental approaches (e.g. differences in capture antibodies or effects of serum-derived blocking agents in ELISA) or differences in patients' conditions, patients consistently show normal levels of cytokines in the circulation compared to PBMC culture supernatants. In fact, discrepancies always arise between data derived from the levels of cytokines produced by cultured mononuclear cells either in stimulated or unstimulated conditions [10–12]. Overall, a review of the literature suggests that *in vivo* levels of proinflammatory cytokines in CFS/FMS are normal, but that the PBMC of these patients can, in some circumstances, produce increased levels of these cytokines *in vitro*. This could be a result of other factors associated with the

pathophysiology of CFS/FMS affecting PBMC, but in a way that becomes manifest only in the artificial *in vitro* culture environment. Because our protocol involved a very brief *ex vivo* culture of 6 h, it is not surprising that we found normal levels of cytokine production by PBMC of CFS/FMS patients, as observed by others for circulating cytokines *in vivo*.

Monocytes/macrophages are the main source of inflammatory cytokines IL-1, IL-6 and TNF-α. We found that culturing monocyte for more than 6 h dramatically reduced the frequency of these cells as a result of apoptosis. Therefore, an accurate evaluation of increased or decreased levels of cytokines in PBMC cultured for more than 6 h is highly problematical.

The dramatic reduction of MIF and percentage of intracellular cytokine positive cells (particularly for TNF-α and IL-1α) in the monocytes of both patients and controls in the presence of polymyxin B indicates that LPS contaminated the cultures: this was despite all possible efforts to ensure that the cell cultures were performed in endotoxin-free conditions. However, it is notable that LPS at very low concentrations is able to bind to CD14 on the surface of monocytes [24] within 1 min of exposure, and is internalized within 5 min. Binding is dependent on the presence of LPS-binding protein, which is a normal plasma component. Therefore, 5–10 min of exposure to physiologically relevant concentrations of LPS are sufficient to trigger maximal TNF-alpha release by monocytes [25]. Polymyxin B, an inhibitor of LPS-mediated activation, essentially abrogated the LPS-binding protein- and CD14-dependent binding of LPS to monocytes. Our data suggest that the total removal of LPS from our system was not possible and, by considering the predominant effect of LPS on monocytes, culturing of monocytes in the absence of polymyxin B may have resulted to false positive cytokine production. This was magnified by the highly sensitive nature of the intracellular cytokine staining technique, which is able to detect very low quantities of cytokines within individual cells.

Although some CFS/FMS symptoms may be due directly to inflammatory cytokines [26], our study indicates that dysregulation of cytokines derived from circulating monocytes or nonmonocytic cells (lymphocytes) is not a dominant factor in the pathogenesis of these syndromes. However, it does not exclude totally a role for cytokines in the pathogenesis of CFS/FMS for the following reasons: first, the results obtained in either *ex vivo* or *in vitro* studies do not necessarily represent the *in vivo* conditions. Secondly, some neuropsychiatric symptoms in patients with CFS/FMS could be related more closely to disordered cytokine production within tissues, e.g. by glial cells within the CNS [27], than to production of cytokines in the circulation.

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REFERENCES

1 Clauw DJ, Chrousos GP. Chronic pain and fatigue syndromes: overlapping clinical and neuroendocrine features and potential pathogenic mechanisms. Neuroimmunomodulation 1997; **4**:134–53.

2 Coetzer P, Lockyer I, Schorn D, Boshoff L. Quantitative disability evaluation of syndromes presenting with chronic fatigue. SAMJ 2000; **90**:1034–52.

3 Cohen H, Neumann L, Shore M, Amir M, Cassuto Y, Buskila D. Autonomic dysfunction in patients with fibromyalgia: application of power spectral analysis of heart rate variability. Semin Arthritis Rheum 2000; **29**:217–27.

4 Rowe PC, Bou Holaigah I, Kan JS, Calkins H. Is neurally mediated hypotension an unrecognised cause of chronic fatigue? Lancet 1995; **345**:623–4.

5 Torpy DJ, Papanicolaou DA, Lotsikas AJ, Wilder RL, Chrousos GP, Pillemer SR. Responses of the sympathetic nervous system and the hypothalamic–pituitary–adrenal axis to interleukin-6: a pilot study in fibromyalgia. Arthritis Rheum 2000; **43**:872–80.

6 Scott LV, The J, Reznek R, Martin A, Sohaib A, Dinan TG. Small adrenal glands in chronic fatigue syndrome: a preliminary computer tomography study. Psychoneuroendocrinology 1999; **24**:759–68.

7 Demitrack MA, Dale JK, Straus SE *et al.* Evidence for impaired activation of the hypothalamic–pituitary–adrenal axis in patients with chronic fatigue syndrome. J Clin Endocrinol Metab 1991; **73**:1224–34.

8 Buskila D. Fibromyalgia, chronic fatigue syndrome, and myofascial pain syndrome. Curr Opin Rheumatol 2001; **13**:117–27.

9 Kishimoto T, Akira S, Narazaki M, Taga T. Interleukin-6 family of cytokines and gp 130. Blood 1990; **86**:1243–54.

10 Lloyd A, Gandevia S, Brockman A, Hales J, Wakefield D. Cytokine production and fatigue in patients with chronic fatigue syndrome and healthy control subjects in response to exercise. Clin Infect Dis 1994; **1**:142–6.

11 Wallace DJ, Linker-Israeli M, Hallegua D, Silverman S, Silver D, Weisman MH. Cytokines play an aetiopathogenetic role in fibromyalgia: a hypothesis and pilot study. Rheumatology 2001; **40**:743–9.

12 Cannon JG, Angel JB, Ball RW, Abad LW, Fagioli L, Komaroff AL. Acute phase responses and cytokine secretion in chronic fatigue syndrome. J Clin Immunol 1999; **19**:414–21.

13 Chao CC, Janoff EN, Hu SX *et al.* Altered cytokine release in peripheral blood mononuclear cell cultures from patients with the chronic fatigue syndrome. Cytokine 1991; **3**:292–8.

14 Gupta S, Aggarwal S, See D, Starr A. Cytokine production by adherent and non-adherent mononuclear cells in chronic fatigue syndrome. J Psychiat Res 1997; **31**:149–56.

15 Swanink CM, Vercoulen JH, Galama JM *et al.* Lymphocyte subsets, apoptosis, and cytokines in patients with chronic fatigue syndrome. J Infect Dis 1996; **173**:460–3.

16 Wolfe f Smythe HA, Yunus MB *et al.* The American college of rheumatology 1990 criteria for the classification of fibromyalgia. Report of the multicenter criteria committee. Arthritis Rheum 1990; **33**:160–72.

17 Sharpe MC, Archard LC, Banatvala JE *et al.* A report – chronic fatigue syndrome: guidelines for research. J Roy Soc Med 1991; **84**:118–21.

18 White KP, Speechley M, Harth M, Ostbye T. Co-existance of chronic fatigue syndrome with fibromyalgia syndrome in the general population. A controlled study. Scand J Rheumatol 2000; **29**:44–51.

19 Amel Kashipaz MR, Huggins ML, Lanyon P, Robins A, Todd I, Powell RJ. Quantitative and qualitative analysis of the balance between type 1 and type 2 cytokine-producing $CD8^-$ and $CD8^+$ T cells in systemic lupus erythematosus. J Autoimmunity 2001; **17**:155–63.

20 Chalder T, Berelowitz G, Pawlikowska T, Watts L, Wessely S, Wright D, Wallace EP. Development of a fatigue scale. J Psychosom Res 1993; **37**:147–53.

21 Daut RL, Cleeland CS, Flanery RC. Development of the Wisconsin Brief Pain Questionnaire to assess pain in cancer and other diseases. Pain 1983; **17**:197–210.

22 Mundt JC, Marks IM, Shear MK, Greist JM. The Work and Social Adjustment Scale: a simple of impairment in functioning. Br J Psychiatry 2002; **180**:461–4.

23 Bonaccorso S, Lin AH, Verkerk R *et al.* Immune markers in fibromyalgia: comparison with major depressed patients and normal volunteers. J Affect Disord 1998; **48**:75–82.

24 Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science 1990; **249**:1431–3.
25 Gallay P, Jongeneel VV, Barras C *et al.* Short time exposure to lipopolysaccharide is sufficient to activate human monocytes. J Immunol 1993; **150**:5086–93.
26 Yabuchi K, Maruta E, Yamamoto J *et al.* Intracerebroventricular injection of isoproterenol produces its analgesic effect through interleukin-1 beta production. Eur J Pharmacol 1997; **334**:133–40.
27 Vollmer-Conna U, Lloyd A, Hickie I, Wakefield D. Chronic fatigue syndrome: an immunological perspective. Aust NZ J Psychiatry 1998; **32**:523–7.