CVI
Journals.ASM.org

Exercise and Sleep Deprivation Do Not Change Cytokine Expression Levels in Patients with Chronic Fatigue Syndrome

Toru Nakamura,[a,b] Stephan Schwander,[c]* Robert Donnelly,[c] Dane B. Cook,[d] Felix Ortega,[c] Fumiharu Togo,[e] Yoshiharu Yamamoto,[e] Neil S. Cherniack,[a,c]† Marc Klapholz,[c] David Rapoport,[f] Benjamin H. Natelson[a,b]*

Pain & Fatigue Study Center[a] and Departments of Neurosciences[b] and Medicine,[c] UMDNJ-New Jersey Medical School, Newark, New Jersey, USA; Department of Kinesiology, University of Wisconsin School of Education, Madison, Wisconsin, USA[d]; Educational Physiology Laboratory, Graduate School of Education, The University of Tokyo, Tokyo, Japan[e]; Department of Medicine, Division of Pulmonary and Critical Care Medicine, NYU School of Medicine, New York, New York, USA[f]

A major hypothesis regarding the cause of chronic fatigue syndrome (CFS) is immune dysregulation, thought to be reflected in upregulated proinflammatory cytokines leading to the symptoms that are characteristic of this illness. Because the symptoms worsen with physical exertion or sleep loss, we hypothesized that we could use these stressors to magnify the underlying potential pathogenic abnormalities in the cytokine systems of people with CFS. We conducted repeat blood sampling for cytokine levels from healthy subjects and CFS patients during both postexercise and total sleep deprivation nights and assayed for protein levels in the blood samples, mRNA activity in peripheral blood lymphocytes (PBLs), and function in resting and stimulated PBLs. We found that these environmental manipulations did not produce clinically significant upregulation of proinflammatory cytokines. These data do not support an important role of immune dysregulation in the genesis of stress-induced worsening of CFS.

Chronic fatigue syndrome (CFS) is a medically unexplained illness characterized by new onset of fatigue that lasts for at least 6 months and is severe enough to produce a substantial decrease in physical activity. The fatigue must be accompanied by at least four of eight infectious, rheumatological, or neuropsychiatric symptoms (1). Because the illness onset is characterized as influenza-like in many patients, an early hypothesis that has continued to galvanize the research community is that CFS is caused by immune activation. That hypothesis was strengthened by an earlier report of high levels of immune activation markers in CFS (2). The hypothesis was further strengthened by the now-well-known fact that the administration of proinflammatory cytokines produces sickness behaviors consisting of fever, severe fatigue, disturbed sleep, difficulty with cognitive processing, and musculoskeletal achiness (3), all of which are symptoms seen with CFS.

We have spent considerable effort evaluating the hypothesis that CFS is caused by immune activation. In our initial study, we were unable to replicate the early finding of increased levels of immune activation markers in CFS (4). Subsequently, we reported that the levels of cytokine message (mRNA) were not different between CFS patients and matched controls and increased similarly for both groups after exhaustive exercise (5). In contrast to the hypothesis related to the upregulation of proinflammatory cytokines, we used advanced statistical modeling with neural networks and found evidence for increases in the daytime levels of interleukin 4 (IL-4), an anti-inflammatory Th2 cytokine (6).

This finding led us to an alternative hypothesis. One of the most common complaints of both CFS and fibromyalgia (FM) patients is unrefreshing sleep. Cytokines are known to influence sleep, as proinflammatory cytokines increase sleep and anti-inflammatory cytokines interfere with sleep (7). We hypothesized that some patients with CFS have an unbalanced cytokine network with upregulated anti-inflammatory cytokines that lead to disturbed sleep and then to fatigue. Our first study of this hypothesis (6) supported this; that study was done on patients with FM, a diffuse pain syndrome with symptoms that overlap with those of CFS. In that study, we tracked pro- and anti-inflammatory cytokine levels across the 24-h day. We found that levels of IL-10, an anti-inflammatory cytokine, were significantly higher during nighttime sleep in patients than in matched controls but not during the period prior to sleep (8). We recently completed a large study using multiple methods to assay for cytokines during the sleep of CFS patients with and without FM (9). That study also found increases in nocturnal IL-10 in patients with CFS only but not in patients with both CFS and FM. However, the magnitude of the increase was small and might have been outside the range of clinical significance.

To evaluate further the immunological hypothesis for CFS, we extended our studies to include a night following exhaustive exercise and a night during which subjects were not allowed to sleep. One of the symptoms that differentiates both CFS and FM from medically explained causes of severe fatigue and widespread pain is that even minimal exertion in someone with CFS or FM produces a dramatic worsening of the entire symptom complex. We reasoned that symptom exacerbation in CFS might be accompanied by further abnormalities in cytokines. Similarly, we reasoned that a night of sleep deprivation would act as a stressor to produce a further shift in the cytokine balance away from that seen in healthy control subjects.

Received 15 August 2013 **Accepted** 4 September 2013
Published ahead of print 11 September 2013
Address correspondence to Benjamin H. Natelson, bnatelson@bethisraelny.org.
* Present address: Stephan Schwander, Rutgers School of Public Health, Department of Environmental and Occupational Health & Center for Global Public Health, Piscataway, New Jersey, USA; Benjamin H. Natelson, Department of Pain Medicine and Palliative Care, Beth Israel Medical Center, New York, New York, USA.
† Deceased.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/CVI.00527-13.

doi:10.1128/CVI.00527-13

MATERIALS AND METHODS

The subjects studied here were 26 women with CFS and 24 healthy female controls, ranging in age from 25 to 55 years, whose sleep we characterized previously during a night in the sleep laboratory (10, 11). The subjects with CFS were either physician-referred or self-referred in response to media reports about our study. The healthy controls were acquaintances of the patients or responded to the recruitment flyers. The patients fulfilled the 1994 case definition for CFS (1) and thus had no medical explanation for their symptoms based on history, physical examination, and laboratory tests, and they had no serious psychiatric diagnoses, including schizophrenia, eating disorders, or substance abuse. We checked for psychiatric diagnoses according to the *Diagnostic and Statistical Manual of Mental Disorders*, fourth edition (DSM-IV), criteria using the computerized version of the Diagnostic Interview Schedule (DIS-IV) (12). This diagnostic interview allowed us to identify study candidates with major depressive disorder ($n = 5$) who were not included in this analysis, because depression by itself is known to relate to sleep morphology (13) and to affect cytokines (14). The existence of comorbid FM ($n = 11$) was diagnosed based on the American College of Rheumatology's 1990 criteria (15). All subjects provided informed consent, approved by the institutional review board of the University of Medicine and Dentistry of New Jersey (UMDNJ) to participate in this research. If menstruating, the subjects were studied during the follicular phase of their menstrual cycles.

Procedures. Following a night to habituate subjects to sleeping in the sleep lab and to eliminate from further study the subjects with sleep pathology (see our earlier paper [9] for details), subjects returned for a normal night of sleep. Upon arrival at the sleep lab, subjects underwent venous catheterization to allow remote blood sampling without disturbance, were instrumented for polysomnography, and went to bed at their usual bedtime. Within 1 h after installation of the venous catheter and while still awake, subjects had approximately 2 ml of blood removed, with replacement of the volume using heparinized saline. Three additional blood samples were taken at approximately 1:00 a.m., 3:00 a.m., and 5:00 a.m. The subjects then awoke between 7:15 and 8:00 a.m., after which a final blood sample was taken. The sleep and cytokine data from this normal night have been published elsewhere (9).

At least 1 week later, the subjects were asked to return to the laboratory in the late afternoon to undergo maximal exercise testing (see reference 16 for details). On the evening after the exercise test, the subjects reported to the sleep lab, underwent venous catheterization to allow for remote blood sampling without disturbance, were instrumented for polysomnography, and went to bed at their usual bedtime. Blood was sampled at the same times as during the normal sleep night.

At least 1 week later, subjects returned to the sleep laboratory where, following venous catheterization, they remained awake throughout the night, interacting as needed with the sleep nurse to ensure a wakeful state. Blood was sampled using the same time schedule as on the normal sleep night.

Using a visual analog scale (VAS), perceived sleepiness, pain, feeling blue, anxiety, and fatigue before and after the normal night and the nights following exercise or sleep deprivation were estimated (on a scale of 0 to 15.5 cm).

Treatment of blood and cytokine analyses. Whole venous blood was collected in heparinized tubes (Vacutainer) and in PAXgene tubes (Qiagen Inc., Valencia, CA) for cytokine analysis.

Cytokine protein and mRNA analysis in blood plasma and by mRNA expression. One of the heparinized tubes was centrifuged, and blood plasma was collected and frozen at −80°C for later analysis of cytokine concentrations by Luminex technology. The samples were thawed, diluted 1:1 with assay buffer, and assayed with Millipore's Milliplex human multicytokine detection system (Billerica, MA) according to the instructions in the kit. This kit simultaneously measured the proinflammatory cytokines IL-1β, IL-6, IL-8, and tumor necrosis factor alpha (TNF-α) and the anti-inflammatory cytokines IL-4 and IL-10. In order to increase the detection limits of the kits, we modified the standard curve to include the following concentrations of each cytokine: 0, 0.49, 0.98, 1.95, 3.90, 7.80, 15.60, 31.25, and 62.5 pg/ml.

The PAXgene tubes were stored frozen (at −80°C) until further processing for cytokine message (mRNA isolation for quantitative real-time PCR [qRT-PCR]). RNA was isolated from the PAXgene tubes according to the manufacturer's guidelines. cDNA synthesis was performed using the following method: for each reaction mixture, 10 μl of RNA was added to the following reagents in 0.2-ml PCR tubes: 2.0 μl reaction buffer (10×), 0.8 μl of deoxynucleoside triphosphate mix, 2.0 μl random primers, and 1.0 μl reverse transcriptase. The volume was brought to 20 μl with PCR-grade water. The samples were incubated at 25°C for 10 min and then at 37°C for 120 min, followed by 85°C for 5 s. All samples were then stored at −70°C until further use. For the real-time PCR assay, the TaqMan universal PCR was performed in 96-well optical plates using an ABI 7500 sequence detection system (Applied Biosystems, Foster City, CA) in a volume of 20 μl. All real-time PCR data were analyzed using the delta-delta threshold cycle (C_T) method using human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a normalization control. mRNA expression was measured for IL-1β, TNF-α, IL-4, and IL-10.

Preparation of peripheral blood mononuclear cells. Another tube of heparinized whole blood was used to isolate peripheral blood mononuclear cells (PBMCs) within 6 h of venipuncture. The PBMCs were prepared by centrifugation (300 × *g*, 45 min, at room temperature) of heparinized venous whole blood diluted 1:1 with RPMI medium over a Ficoll-Paque (Axis-Shield PoC As, Oslo, Norway) density gradient (17). The PBMCs were removed from the interface, washed twice in RPMI medium, and resuspended in complete cell culture medium (RPMI with L-glutamine, penicillin, and streptomycin), counted in a hemacytometer and adjusted at 10^6/ml. The viability of the PBMCs was 100% by trypan blue exclusion.

Cytokine analysis by ELISPOT assay. PBMCs were plated on enzyme-linked immunosorbent spot assay (ELISPOT) plates for immediate analysis of cytokine production under the baseline conditions and also following *in vitro* stimulation with lipopolysaccharide (LPS) (1 μg/ml final concentration) or phytohemagglutinin (PHA) (5 μg/ml final concentration). The ELISPOT assays were performed using capture and biotinylated detection antibodies for IL-1β, IL-6, and IL-8 (Cell Sciences, Canton, MA), gamma interferon (IFN-γ), TNF-α (Endogen/Pierce, Rockford, IL), and for IL-10 and IL-4 (BD Pharmingen, San Diego, CA), and streptavidin-peroxidase (Sigma-Aldrich, St. Louis, MO), chromogen 1% 3-amino-9-ethylcarbazole (Pierce, Rockford, IL), and MultiscreenHTS high protein-binding 96-well plates (Millipore, Bedford, MA). In the 96-well plates, the optimal PBMC numbers (densities) per well for the detection of the cytokines were as follows: 20,000 for IL-6 and TNF-α, 100,000 for IL-1β and IL-8, and 200,000 for IL-4, IL-10, and IFN-γ. The optimal short-term cell culture (ELISPOT assay) incubation periods were 4 h for TNF-α, 20 h for IL-1β and IL-8, 24 h for IL-6 and IFN-γ, and 48 h for IL-4 and IL-10. The cell culture incubation periods and cell densities for the various cytokine ELISPOT assays were chosen on the basis of results from previously published studies (18–20) and the recommendations of the manufacturer of each kit. We also provided extensive methodological details about the three assays used to determine cytokine concentrations (9).

Data preprocessing. Previously, we developed a preprocessing method that systematically removes extreme outlier values from cytokine data in order to improve the Gaussianity of their distributions (9). To do this, the outliers were defined as the data that exceeded the confidence area estimated from a locally weighted regression model. We removed these outliers from the data set. The total rate of the data points eliminated by this procedure was <0.1% for all three different assays under the three experimental conditions. Next, the data were log transformed after adding a value of 1 to each numerical value to improve the normality of the data. No evidence was found for either a temporal pattern for the outliers in any of the data sets or the number of outliers and patterns between healthy control subjects and patients with CFS.

We analyzed data from the three experimental conditions in this study: the normal night, the night following exercise, and the night of

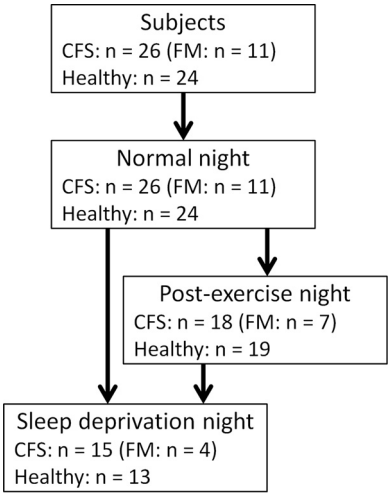

FIG 1 Schematic showing flow of studies. Analysis of data following exercise or during sleep deprivation was done using data from subjects who had already provided data on the normal night.

sleep deprivation. Having data from the baseline (normal) sleep conditions allowed us to determine if exercise or sleep deprivation changed any cytokines from that baseline sleep state. Not every subject volunteered to participate in all three experiments. For comparisons of the effects of these conditions on cytokine levels, we therefore selected only pairwise samples from those subjects providing data for all three conditions to allow for testing statistical differences between the conditions. Thus, the number of subjects whose samples were analyzed is not the same as the number of subjects who entered the study (see Fig. 1).

Statistical analysis. Throughout our study, we adopted a multilevel modeling approach (21) for hypothesis testing for several reasons: the existence of missing data points (<0.1% of total data points), induction of hierarchical structures by the repeat blood sampling within each subject, and participation in three experimental conditions. Therefore, to deal with these issues in a statistically valid way, we fitted multilevel models to our data sets. All the multilevel model analyses were completed using SAS Proc Mixed (SAS 9.1; SAS Institute Inc., Cary, NC). Tukey-Kramer multiple comparison adjustments were applied to evaluate the *P* values. *P* values of <0.05 were considered statistically significant throughout this study. The formulas used in generating this multilevel modeling approach are available in the supplemental material.

Mean cytokine levels. We fitted an unconditional multilevel model, commonly referred to as the null model (see model 1 in the supplemental material), to examine the group mean of each cytokine level. In this model, the value of cytokine level is the dependent variable with no predictor. Note that the mean cytokine levels shown in Tables 1 and 2 and models 1 to 6 in the supplemental material were estimated from the raw data using the null model, but the statistical tests were conducted with the log-transformed data.

Group effects on cytokine levels at each sampling time. In order to examine the effects of sampling time, we included in the null model the time of day at which observations were taken as a predictor of cytokine levels (see model 2 in the supplemental material). In this model, we considered the time of day to be a categorical variable, and its effect was constrained to be a fixed value across the subjects (a fixed effect). The improvement of the model's goodness of fit was evaluated by calculating the difference in the deviance statistics and testing it with a chi-square test with the degrees of freedom being equal to the difference in the number of parameters. Therefore, since we confirmed the improvement of the model for all cytokines, we included this term in the successive models, but its exclusion did not significantly change our results.

Next, we extended this model to test the group effects on cytokine levels at each sampling time and the mean cytokine levels (see model 3 in the supplemental material); we tested whether the coefficients of time of day and intercept of the above model varied from group to group by including the categorical variable representing groups (healthy controls or CFS group) into them. We fitted this model to each cytokine data set for each study night, separately.

Effects of experimental conditions on cytokine levels. In order to achieve the primary study goals, the levels of cytokines measured during sleep in patients with CFS and in healthy control subjects were compared following sleep deprivation and after a maximal exercise stress test. To test them, we included a categorical value representing study nights (normal, sleep deprivation, and postexercise stress) into the model and tested whether its coefficient varied across groups (see model 4 in the supplemental material).

Associations with both sleep indices and VAS scores. The sleep indices (sleep efficiency [SE] and sleep latency [SL]) were also examined using a multilevel model to see the effects of exercise on the sleep properties across groups. In this model, we assumed that the sleep index is the dependent variable with the categorical variable of study conditions as a predictor, and the intercept has the random effect. To test the group

TABLE 1 Significant effects of sleep deprivation on cytokine levels measured by three different assays

Assay type and cytokine tested	Data for each group[a]			
	Control		CFS	
	Normal	Sleep deprivation	Normal	Sleep deprivation
ELISPOT assay (*n*)	12		13	
IL-1β[b]	94.2 ± 70.0	140.1 ± 94.1	**25.7 ± 13.1**	**40.7 ± 13.2**[e]
IL-8[b]	**37.2 ± 11.5**	**56.1 ± 16.3**[e]	60.1 ± 12.2	69.8 ± 11.7
Luminex assay (*n*)	9		10	
IL-1β[c]	**0.03 ± 0.02**	**0.08 ± 0.04**[e]	0.18 ± 0.07	0.14 ± 0.05
qRT-PCR assay (*n*)	13		15	
IL-4[d]	**0.76 ± 0.33**	**0.29 ± 0.11**[e]	0.42 ± 0.13	1.34 ± 0.92
IL-10[d]	0.55 ± 0.15	0.50 ± 0.18	**0.36 ± 0.06**	**0.25 ± 0.03**[e]

[a] The statistical tests were conducted with log-transformed data. Values in bold type indicate significant cases.
[b] No. of cytokine-producing cells (mean ± SE) (raw data).
[c] Cytokine level (mean ± SE) (pg/ml) (raw data).
[d] Cytokine level (relative quantity) (mean ± SE) (raw data).
[e] Significant within-group difference from normal night.

TABLE 2 Significant effects of exercise stress on cytokine levels measured by three different assays

Assay type and cytokine tested[a]	Data for each group[b]							
	Control		CFS		CFS only		CFS+FM	
	Normal	Exercise	Normal	Exercise	Normal	Exercise	Normal	Exercise
ELISPOT assay	17		15		9		6	
IL-1β[c]	**74.4 ± 50.2**	**109.9 ± 62.6**[f]	28.4 ± 11.9	26.4 ± 8.0	37.3 ± 18.3	29.0 ± 10.7	15.0 ± 8.9	22.5 ± 12.0
IL-4[c]	6.9 ± 1.5	8.4 ± 2.6	**11.6 ± 2.4**	**7.7 ± 1.8**[f]	**12.5 ± 2.9**	**7.4 ± 2.4**[f]	10.2 ± 4.1	8.2 ± 2.7
IL-6[c]	47.2 ± 11.4	69.2 ± 16.2	**74.7 ± 21.7**	**51.0 ± 20.4**[f]	**50.9 ± 18.2**	**8.1 ± 3.2**[f]	110.0 ± 43.0	**115.2 ± 38.0**[g]
ST IL-6[c]	96.8 ± 14.1	117.2 ± 16.3	**118.5 ± 16.7**	**95.1 ± 16.7**[f]	**108.4 ± 11.8**	**67.9 ± 16.9**[f]	134.0 ± 36.6	135.7 ± 25.5
IL-8[c]	36.2 ± 8.5	54.9 ± 13.6	**57.8 ± 10.1**	**24.6 ± 5.4**[f]	58.9 ± 14.0	25.8 ± 5.9	**56.1 ± 13.9**	**22.8 ± 10.2**[f]
ST IL-8[c]	50.1 ± 10.0	62.9 ± 13.5	**72.2 ± 7.8**	**32.0 ± 7.5**[f]	**60.6 ± 11.1**	**31.4 ± 10.0**[f]	**89.0 ± 6.4**	**33.0 ± 11.1**[f]
TNF-α[c]	**145.1 ± 28.0**	**182.6 ± 30.3**[f]	**130.0 ± 33.4**	**151.3 ± 22.5**[f]	157.6 ± 46.5	148.7 ± 27.3	**88.6 ± 40.7**	**155.0 ± 38.6**[f]
Luminex assay	17		15		9		6	
IL-1β[d]	**0.09 ± 0.04**	0.11 ± 0.05	0.33 ± 0.11	0.34 ± 0.12	0.16 ± 0.07	0.16 ± 0.07	**0.57 ± 0.22**[h]	0.60 ± 0.25
IL-4[d]	**0.18 ± 0.08**	**0.10 ± 0.03**[f]	0.07 ± 0.03	0.06 ± 0.02	0.02 ± 0.02	0.06 ± 0.03	0.15 ± 0.07	0.08 ± 0.05
IL-10[d]	**2.22 ± 0.39**	**1.40 ± 0.25**[f]	2.37 ± 0.34	2.24 ± 0.34	2.02 ± 0.43	2.07 ± 0.49	2.89 ± 0.54	2.49 ± 0.37
qRT-PCR assay (*n*)[e]	18		17		10		7	

[a] ST, stimulant-induced data.
[b] The statistical tests were conducted with log-transformed data. Values in bold type indicate significant cases.
[c] No. of cytokine-producing cells (mean ± SE) (raw data).
[d] Cytokine level (mean ± SE) (pg/ml) (raw data).
[e] There were no significant cases for the qRT-PCR assay.
[f] Significant within-group difference from normal night.
[g] Significant difference between CFS only and CFS+FM.
[h] Significant difference between control subjects and patients with CFS+FM.

differences in the effects of the different study nights, we tested whether the coefficient and intercept of the model varied across groups.

A limited sample size and the existence of missing data made it possible for us to evaluate only the postexercise data set for correlations between the sleep indices and mean cytokine levels. To assess these relations, we used both Pearson's (ρ) and Spearman's (*r*) correlation coefficients and regarded the correlations to be valid if both coefficients were significant. We also evaluated the correlations between the changes in VAS scores and the cytokine levels before and after each of the studies, where the differences in the cytokine levels were calculated by subtracting the first sample data from the last ones.

RESULTS

Mean cytokine levels. We estimated the average level of each cytokine for healthy control subjects and patients with CFS during the three study nights (see Tables S1 to S6 in the supplemental material). These levels were evaluated from the log-transformed data by using the null model. We furthermore stratified the CFS data into two subgroups (CFS only and CFS plus FM) for the exercise study and evaluated their cytokine levels; we were unable to do this analysis for the night following sleep deprivation due to a small sample size. In Tables 1 and 2, we show the cases only.

Group effects on cytokine levels at each sampling time. We fit the multilevel model for the tests of the group effect on cytokine levels at each sampling time. We did not find any group differences between the control and CFS groups for any cytokine at any time point during any study night. However, an analysis of stratified data from the exercise study demonstrated that the number of IL-1β-producing cells measured by the ELISPOT assay in the stimulated condition for the CFS only group was significantly lower than that of healthy control subjects at 5 a.m. ($P < 0.05$) (Fig. 2).

Effects of study conditions on cytokine levels. (i)Effects of sleep deprivation. Table 1 summarizes the effects of sleep deprivation on the cytokine levels as measured by three different assays. The data for the nonsignificant cases are shown in Tables S1 to S3 in the supplemental material.

(a) ELISPOT assay. Compared to the results for the normal night, the healthy controls showed increases in the number of cells secreting IL-8 ($P < 0.05$) during sleep deprivation and the CFS group showed increases in the number of cells secreting IL-1β ($P < 0.05$). However, there were no statistically significant differences between the CFS and control groups in measures of stimulated or unstimulated cytokine production on either the normal sleep or sleep-deprived nights.

(b) Luminex assay. The only cytokine to show any significant

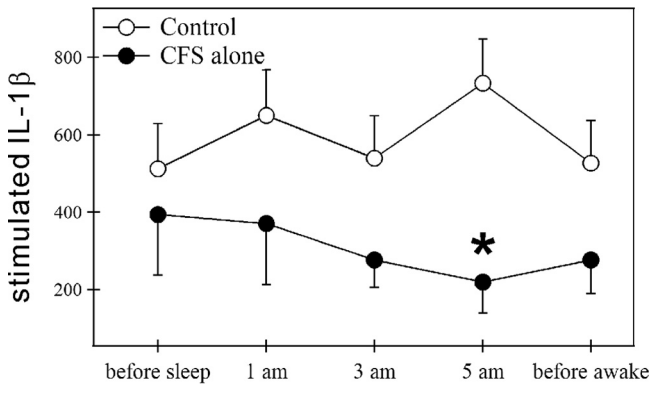

FIG 2 Group-averaged IL-1β in the stimulated condition (the number of producing cells) from ELISPOT assay for the control and CFS-only groups during the postexercise night. The error bars indicate the standard error of the means. *, significant difference from control; note that the statistical tests were conducted with log-transformed data.

TABLE 3 VAS scores before and after sleep deprivation and exercise nights

Type of night and group	VAS scores (mean ± SD) by category[a]				
	Sleepiness	Pain	Feeling blue	Anxiety	Fatigue
Normal night					
Control					
Before sleep	**4.7 ± 4.0**	0.1 ± 0.2	0.2 ± 0.7	1.7 ± 3.6	**3.1 ± 4.2**
After awake	**2.5 ± 3.1**[c]	0.3 ± 0.8	0.3 ± 0.8	0.7 ± 1.3	**1.4 ± 2.1**[c]
CFS					
Before sleep	8.8 ± 4.0[b]	7.1 ± 3.9[b]	1.1 ± 1.9[b]	1.5 ± 2.0	9.4 ± 3.0[b]
After awake	7.8 ± 3.6[b]	7.1 ± 3.8[b]	0.9 ± 1.6	0.9 ± 1.7	8.2 ± 3.2[b]
Night of sleep deprivation					
Control					
Before deprivation	3.6 ± 5.3	0.9 ± 2.1	0.1 ± 0.2	0.8 ± 1.7	2.2 ± 4.6
After deprivation	4.7 ± 4.5	0.9 ± 1.8	0.2 ± 0.5	0.2 ± 0.5	3.5 ± 4.5
CFS					
Before deprivation	9.6 ± 3.7[b]	**8.3 ± 2.6**[b]	0.9 ± 1.2[b]	0.9 ± 1.1	**10.5 ± 2.7**[b]
After deprivation	11.2 ± 4.4[b]	**9.8 ± 3.4**[b,c]	0.6 ± 0.9	0.9 ± 0.9[b]	**11.9 ± 3.2**[b,c]
Night following exercise					
Control					
Before sleep	3.7 ± 3.6	0.0 ± 0.0	0.04 ± 0.14	0.7 ± 1.8	2.4 ± 3.6
After awake	1.2 ± 1.4	0.0 ± 0.0	0.1 ± 0.2	0.05 ± 0.17	0.6 ± 1.3
CFS					
Before sleep	10.4 ± 3.8[b]	8.7 ± 4.5[b]	**1.0 ± 1.4**[b]	1.8 ± 3.0	10.5 ± 3.4[b]
After awake	8.3 ± 4.0[b]	8.1 ± 5.2[b]	**0.5 ± 0.8**[b,c]	0.7 ± 1.0[b]	9.5 ± 3.7[b]

[a] Values in boldface indicate the cases where the experimental effects on subjective symptoms were significant (repeated-ANOVA, $P < 0.05$; Bonferroni multiple comparison adjustment was used).
[b] Significant difference from healthy controls.
[c] Significant difference between before and after sleep deprivation/exercise tests.

change was IL-1β, which increased significantly for the control group during sleep deprivation relative to the normal night. There were no significant group differences among any of the cytokines for patients compared to controls on both nights.

(c) qRT-PCR assay. For the healthy controls, IL-4 mRNA expression on the sleep deprivation night significantly decreased compared with the result for the normal night; for the CFS group, IL-10 mRNA expression decreased. There were no statistically significant group differences in the mRNA cytokine expression levels for patients compared to controls on both nights.

(ii) Effects of exercise stress. Table 2 summarizes the effects of exercise stress on cytokine levels measured by three different assays. The data for the nonsignificant cases are shown in Tables S4 to S6 in the supplemental material.

(a) ELISPOT assay. For the healthy controls, the number of unstimulated cells expressing IL-1β and TNF-α increased significantly on the night following exercise compared to levels during the normal night. For the CFS group, the number of unstimulated cells expressing IL-4, IL-6, and IL-8, as well as the number of stimulated cells expressing IL-6 and IL-8, decreased significantly ($P < 0.05$), while the number of cells expressing TNF-α increased. There were no significant differences between the CFS and control groups for the numbers of cells expressing any of the cytokines under stimulated and unstimulated conditions on both nights, except for a tendency toward a lower IL-1β cell count in patients with CFS ($P = 0.10$) on the night following exercise.

To further evaluate the data, we stratified the CFS data into two subgroups (CFS with FM [$n = 9$] and CFS only [$n = 6$]) and compared the group differences between the groups. The number of cells secreting IL-6 for the CFS only subgroup significantly decreased on the night following exercise compared to the results for the normal night. The CFS plus FM subgroup showed a significant decrease in IL-8 and an increase in TNF-α compared to the results for the normal night. In addition, the number of cells secreting IL-6 was significantly higher for CFS patients with FM than for patients with CFS only on the night following exercise. These findings suggest different responses of the immunological cytokine network to exercise stress between CFS patients with and without FM.

(b) Luminex assay. For the healthy controls, IL-4 and IL-10 production significantly decreased on the night following exercise compared to the normal night. There were no significant differences in any of the cytokines between the subjects with CFS and the healthy controls on either night.

(c) qRT-PCR assay. There were no statistically significant group differences in any of the cytokines for subjects with CFS compared to healthy controls on either night.

Associations with both sleep indices and VAS scores. Table 3 summarizes the sleep indices and VAS scores before and after the three nights. The subjective scores of sleepiness, pain, and fatigue in patients with CFS were significantly higher than those of the controls before and after sleep deprivation, as well as during the night following exercise stress. In addition, sleep deprivation significantly worsened both pain and fatigue in the CFS patients.

(i) ELISPOT assay. Following exercise, sleep efficiency in the subjects with CFS was significantly lower than that of the controls in this paired data set (see Table S4 in the supplemental material). However, using the ELISPOT assay, we found no significant correlation between sleep efficiency and mean numbers of cells pro-

ducing cytokines. Sleep latency for the controls on the normal sleep night correlated negatively with mean cell count producing IL-6 ($\rho = -0.55$, $r = -0.53$) on the normal night. On the other hand, for CFS, the differences in the mean cell counts producing both basal and stimulated IL-6 before sleep and after awakening on the postexercise night showed a significant negative correlation with the changes in the subjective scores of pain (for IL-6, $\rho = -0.77$, $r = -0.86$, and for stimulated IL-6, $\rho = -0.73$, $r = -0.70$), fatigue (for IL-6, $\rho = -0.75$, $r = -0.65$, and for stimulated IL-6, $\rho = -0.76$, $r = -0.75$), and sleepiness (for IL-6, $\rho = -0.72$, $r = -0.67$, and for stimulated IL-6, $\rho = -0.67$, $r = -0.67$), indicating that the counts of cells producing IL-6 decreased with worsening of these subjective symptoms.

(ii) Luminex assay. Although there were no significant differences in the sleep indices between the groups under any of the three conditions studied (see Table S5 in the supplemental material), the sleep efficiency of healthy controls on the normal night showed highly significant negative correlations between the mean levels of IL-10 ($\rho = -0.75$, $r = -0.74$) and TNF-α ($\rho = -0.65$, $r = -0.57$). In addition, differences in the levels of TNF-α for the controls showed a significant negative correlation with the changes in fatigue scores ($\rho = -0.71$, $r = -0.81$). For the CFS group on the postexercise night, there was a significant positive correlation between sleep efficiency and mean levels of IL-4 ($\rho = 0.56$, $r = 0.57$), while no significant correlations were found between the subjective symptoms.

(iii) qRT-PCR assay. No significant correlations were found between sleep efficiency and the levels of cytokines using the qRT-PCR assay (see Table S6 in the supplemental material). The sleep latency of the healthy controls on the night following exercise showed statistically significant positive correlations with the levels of IL-6 ($\rho = 0.51$, $r = 0.54$), and the difference in IL-1β levels showed a significant negative correlation with changes in the sleepiness ($\rho = -0.65$, $r = -0.63$) and fatigue scores ($\rho = -0.61$, $r = -0.66$).

DISCUSSION

A major hypothesis for the cause of CFS is cytokine dysregulation, which is usually thought to reflect upregulated proinflammatory cytokines. Our prior research did not confirm this hypothesis, but we did find evidence for some increases in anti-inflammatory cytokines, possibly explaining the common CFS patient complaint of disturbed sleep (6, 9, 22). The purpose of this experiment was to try to push the cytokine system in order to magnify the differences between the patients and controls using processes that patients report as worsening their condition, namely, sleep deprivation and exercise. In a prior study where we used maximal exercise and tracked cytokines using one method only—RT-PCR—we found no differences between the patients and controls (23). In this study, we found that these environmental manipulations, known to influence cytokine systems within the groups, did not produce any consistently significant differences between the patients and controls.

Because there is no clear gold standard for assessing cytokines in the resting state or following environmental perturbation, we used three available methods which looked at the monocellular expression of cytokines basally and following cellular stimulation, the cytokine levels within the blood plasma, and the semiquantitative amounts in the cytokine mRNA expression in cells. In fact, we found only one difference between the patients and controls related to the sleep conditions, assay methods, and multiple sampling during the night: CFS patients had lower blood plasma IL-1β levels than controls at 5 a.m. following exercise the afternoon before.

There were, however, some differences in the response patterns of the patients and controls following maximal exercise compared to data collected on the normal night. While the number of cells expressing TNF-α increased for both patients and controls, the patients showed decreases in the number of cells expressing IL-4, IL-6, and IL-8 without cellular stimulation and in cells expressing IL-4 and IL-6 with cellular stimulation, while the healthy controls showed increases in cells expressing IL-1β.

White et al. (24) found differences between the patients and controls after stratifying the patients by whether they developed an exercise-induced symptom flare-up. Patients who developed symptom worsening 48 h after exercise showed increases in a number of cytokines, while those in whom symptoms did not worsen showed decreases in others. We used a different stratification strategy based on our having shown differences between the patients with CFS only and those with CFS plus FM (25); thus, we stratified patients by CFS only and CFS plus FM groups. While this stratification strategy did not produce differences between the illness groups and the controls, we did find differences in the specific cytokines that were affected by exercise for these groups compared to the results for the normal night (i.e., the CFS only group had increases and decreases, respectively, in the number of cells expressing IL-4 and IL-6, while the CFS plus FM group had increases and decreases, respectively, in the number of cells expressing TNF-α and IL-8). The differences in these patterns manifested themselves in the CFS plus FM group showing a significantly higher number of cells expressing IL-6 than the CFS only group. A recent meta-analysis found the only cytokine difference between FM patients and controls to be elevated IL-6 levels (26).

Sleep deprivation also did not produce a difference in cytokine levels between patients and controls across the 3 methods and using multiple sampling compared to the normal night. Also, the effects of sleep deprivation on cytokine levels were less dramatic than for exercise. PCR testing revealed decreases in mRNA cytokine expression of IL-4 for the controls and in that of IL-10 for the CFS group. Luminex analysis revealed an increase in the number of cells expressing IL-1β for the controls. These changes, which are consistent with a small but significant proinflammatory response, have been previously reported to occur following sleep deprivation in healthy women (27).

The major outcome of these studies is our finding of an inconsistency in the results across the analytical methods, a finding which has been noted by others (28). The patterns of cytokine mRNA expression may not necessarily reflect the patterns of cytokine protein production due to posttranscriptional regulation of these genes by mechanisms, such as mRNA decay, translation, nuclear export, and cytoplasmic localization (29). This variability in the results raises questions about which method researchers should use in the future to try to define cytokine abnormalities in illness populations.

We did find a number of significant differences between the sleep latency data and cytokines in the healthy controls. Using data from the normal night, sleep latency correlated negatively with the number of cells expressing IL-6 and with the blood plasma levels of IL-10 and TNF-α. Also, on the night following exercise, a significant correlation was found for the controls between sleep la-

tency and IL-6 production. These data suggest a relationship between sleep structure and cytokine production that we are currently exploring and that has been suggested to exist previously (30). Of interest were several correlations among the individual cytokine levels and changes in fatigue, sleepiness, or pain after sleep. Since these results were found more often in the controls than in the patients, they may suggest an element of cytokine dysfunction in the patients.

The data reported here in combination with those published from a night of normal sleep (9) do not support an important role of cytokine alterations in the genesis of chronic fatigue syndrome. Negative studies are always problematic because of the possibility of our having found more dramatic effects had we waited longer to sample and prolonged the period of sleep deprivation for another 24 h. Moreover, looking for individual cytokine abnormalities may not reveal discrete abnormalities compared to what using a more integrated network analysis can show (31). However, we designed this study based on the current state of knowledge on the topic by using a maximum exercise demand and overnight sleep deprivation, both of which can make worsen symptoms in CFS patients. Moreover, we used three different methods of assessing cytokines and were careful to control for multiple statistical comparisons. Under those conditions, none of the assays produced significant differences in cytokine production between the patients and controls.

ACKNOWLEDGMENTS

This work was supported by NIH grant no. AI-54478. Additional support to extend the work to IL-6 and IL-8 was provided by the American Fibromyalgia Syndrome Association.

REFERENCES

1. **Fukuda K, Straus SE, Hickie I, Sharpe MC, Dobbins JG, Komaroff A.** 1994. The chronic fatigue syndrome: a comprehensive approach to its definition and study. International Chronic Fatigue Syndrome Study Group. Ann. Intern. Med. **121:**953–959.
2. **Landay AL, Jessop C, Lennette ET, Levy JA.** 1991. Chronic fatigue syndrome: clinical condition associated with immune activation. Lancet **338:**707–712.
3. **Smedley H, Katrak M, Sikora K, Wheeler T.** 1983. Neurological effects of recombinant human interferon. Br. Med. J. (Clin. Res. Ed.). **286:**262–264.
4. **Zhang Q, Zhou X, Denny T, Ottenweller J, Lange G, LaManca JJ, Lavietes MH, Pollet C, Gause WC, Natelson BH.** 1999. Changes in immune parameters in Gulf War veterans but not in civilians with chronic fatigue syndrome. Clin. Diagn. Lab. Immunol. **6:**6–13.
5. **LaManca JJ, Sisto S, Zhou XD, Ottenweller JE, Cook S, Peckerman A, Zhang Q, Denny TN, Gause WC, Natelson BH.** 1999. Immunological response in chronic fatigue syndrome following a graded exercise test to exhaustion. J. Clin. Immunol. **19:**135–142.
6. **Hanson SJ, Gause WC, Natelson BH.** 2001. Detection of immunologically significant factors for chronic fatigue syndrome using neural-network classifiers. Clin. Vaccine Immunol. **8:**658–662.
7. **Krueger JM, Obál F, Fang J, Kubota T, Taishi P.** 2000. The role of cytokines in physiological sleep regulation. Ann. N. Y. Acad. Sci. **917:**211–221.
8. **Togo F, Natelson BH, Adler GK, Ottenweller JE, Goldenberg DL, Struzik ZR, Yamamoto Y.** 2009. Plasma cytokine fluctuations over time in healthy controls and patients with fibromyalgia. Exp. Biol. Med. (Maywood) **234:**232–240.
9. **Nakamura T, Schwander SK, Donnelly R, Ortega F, Togo F, Broderick G, Yamamoto Y, Cherniack NS, Rapoport D, Natelson BH.** 2010. Cytokines across the night in chronic fatigue syndrome with and without fibromyalgia. Clin. Vaccine Immunol. **17:**582–587.
10. **Togo F, Natelson BH, Cherniack NS, Fitzgibbons J, Garcon C, Rapoport DM.** 2008. Sleep structure and sleepiness in chronic fatigue syndrome with or without coexisting fibromyalgia. Arthritis Res. Ther. **10:**R56. doi:10.1186/ar2425.
11. **Kishi A, Natelson BH, Togo F, Struzik ZR, Rapoport DM, Yamamoto Y.** 2011. Sleep-stage dynamics in patients with chronic fatigue syndrome with or without fibromyalgia. Sleep **34:**1551–1560.
12. **Robins LN, Cottler LB, Bucholz KK, Compton WM, North CS, Rourke KM.** 2000. Diagnostic interview schedule for the DSM-IV (DIS-IV). Washington University School of Medicine, St Louis, MO.
13. **Ford DE, Cooper-Patrick L.** 2001. Sleep disturbances and mood disorders: an epidemiologic perspective. Depress. Anxiety **14:**3–6.
14. **Maes M, Smith R, Scharpe S.** 1995. The monocyte-T-lymphocyte hypothesis of major depression. Psychoneuroendocrinology **20:**111–116.
15. **Wolfe F, Smythe HA, Yunus MB, Bennett RM, Bombardier C, Goldenberg DL, Tugwell P, Campbell SM, Abeles M, Clark P, Fam AG, Farber SJ, Fiechtner JJ, Franklin CM, Gatter RA, Hamaty D, Lessard J, Lichtbroun AS, Masi AT, McCain GA, Reynolds WJ, Romano TJ, Russell IJ, Sheon RP.** 1990. The American College of Rheumatology 1990 criteria for the classification of fibromyalgia. Report of the Multicenter Criteria Committee. Arthritis Rheum. **33:**160–172.
16. **Togo F, Natelson BH, Cherniack NS, Klapholz M, Rapoport DM, Cook DB.** 2010. Sleep is not disrupted by exercise in patients with chronic fatigue syndromes. Med. Sci. Sports Exerc. **42:**16–22.
17. **Bøyum A.** 1976. Isolation of lymphocytes, granulocytes and macrophages. Scand. J. Immunol. **Suppl 5:**9–15.
18. **Schwander SK, Torres M, Sada E, Carranza C, Ramos E, Tary-Lehmann M, Wallis RS, Sierra J, Rich EA.** 1998. Enhanced responses to *Mycobacterium tuberculosis* antigens by alveolar lymphocytes during active pulmonary tuberculosis. J. Infect. Dis. **178:**1434–1445.
19. **Schwander SK, Torres M, Carranza C, Escobeda D, Tary-Lehmann M, Andersen P, Toossi Z, Ellner JJ, Rich EA, Sada E.** 2000. Pulmonary mononuclear cell responses to antigens of *Mycobacterium tuberculosis* in healthy household contacts of patients with active tuberculosis and healthy controls from the community. J. Immunol. **165:**1479–1485.
20. **Guerkov RE, Targoni OS, Kreher CR, Boehm BO, Herrera MT, Tary-Lehmann M, Lehmann PV, Schwander SK.** 2003. Detection of low-frequency antigen-specific IL-10-producing CD4(+) T cells via ELISPOT in PBMC: cognate vs. nonspecific production of the cytokine. J. Immunol. Methods **279:**111–121.
21. **Raudenbush SW, Bryk AS.** 2002. Hierarchical linear models: applications and data analysis methods, 2nd ed. Sage Publications, Inc., Newbury Park, CA.
22. **Natelson BH, Weaver SA, Tseng CL, Ottenweller JE.** 2005. Spinal fluid abnormalities in patients with chronic fatigue syndrome. Clin. Diagn. Lab. Immunol. **12:**53–55.
23. **LaManca J, Sisto S, DeLuca J, Johnson SK, Lange G, Pareja J, Cook S, Natelson BH.** 1998. The influence of exhaustive treadmill exercise on cognitive function in patients with chronic fatigue syndrome. Am. J. Med. **105:**59S–65S.
24. **White AT, Light AR, Hughen RW, Bateman L, Martins TB, Hill HR, Light KC.** 2010. Severity of symptom flare after moderate exercise is linked to cytokine activity in chronic fatigue syndrome. Psychophysiology **47:**615–624.
25. **Abbi B, Natelson BH.** 2013. Is chronic fatigue syndrome the same illness as fibromyalgia: evaluating the 'single syndrome' hypothesis. QJM. **106:**3–9.
26. **Uçeyler N, Häuser W, Sommer C.** 2011. Systematic review with meta-analysis: cytokines in fibromyalgia syndrome. BMC Musculoskelet. Disord. **12:**245. doi:10.1186/1471-2474-12-245.
27. **Irwin MR, Carrillo C, Olmstead R.** 2010. Sleep loss activates cellular markers of inflammation: sex differences. Brain Behav. Immun. **24:**54–57.
28. **Favre N, Bordmann G, Rudin W.** 1997. Comparison of cytokine measurements using ELISA, ELISPOT and semi-quantitative RT-PCR. J. Immunol. Methods **204:**57–66.
29. **Stoecklin G, Anderson P.** 2006. Posttranscriptional mechanisms regulating the inflammatory response. Adv. Immunol. **89:**1–37.
30. **Thomas KS, Motivala S, Olmstead R, Irwin MR.** 2011. Sleep depth and fatigue: role of cellular inflammatory activation. Brain Behav. Immun. **25:**53–58.
31. **Broderick G, Fuite J, Kreitz A, Vernon SD, Klimas N, Fletcher MA.** 2010. A formal analysis of cytokine networks in chronic fatigue syndrome. Brain Behav. Immun. **24:**1209–1217.