Plasma Cytokine Fluctuations over Time in Healthy Controls and Patients with Fibromyalgia

FUMIHARU TOGO,*,† BENJAMIN H. NATELSON,*,[1] GAIL K. ADLER,‡ JOHN E. OTTENWELLER,* DON L. GOLDENBERG,§ ZBIGNIEW R. STRUZIK,|| AND YOSHIHARU YAMAMOTO||

*Pain & Fatigue Study Center, Department of Neurosciences, UMDNJ-New Jersey Medical School, Newark, New Jersey 07103; †Department of Work Stress Control, Japan National Institute of Occupational Safety and Health, Kawasaki 214-8585, Japan; ‡Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115; §Department of Medicine, Tufts University School of Medicine, Boston, Massachusetts 02111; and ||Graduate School of Education, University of Tokyo, Tokyo 113-0033, Japan

We examined the pattern of cytokine secretion across the 24-hr day for women with widespread pain and tenderness having the diagnosis of fibromyalgia (FM) and matched healthy controls. Subjects were given time to habituate to being in a clinical research laboratory environment and then were sampled for cytokines without their being disturbed for a 24-hr period including an 8-hr sleep period. Cytokine levels were uniformly low but characterized by bursts of secretion. Bursting occurred either in singlets or in doublets with a range from 88 to 131 mins between doublet bursts. There was an element of synchronization of these bursts with most occurring at the beginning of sampling. FM patients showed a shift to increased IL-10 in the nighttime compared to controls. The relation between this anti-inflammatory cytokine to the pro-inflammatory cytokines studied also differed between groups: FM patients showed an increased ratio of IL-10 burst amplitude to that of pro-inflammatory cytokines IL-1β, IL-8, and TNF-α. We interpret this to indicate a skew away from the normal balance favoring pro-inflammatory cytokines in controls toward one favoring an anti-inflammatory response in FM. These changes toward anti-inflammatory predominance in FM may explain their common complaint of disturbed sleep because these cytokines are known to disrupt sleep. Exp Biol Med 234:232–240, 2009

Key words: biological rhythms; ultradian; cytokines; pain

Introduction

Homeostatic control of basal cytokine activity in the human immune system is not well understood. So, for example, it is not known if discrete, different cell types or lineages exist to produce pro-inflammatory cytokines and anti-inflammatory cytokines independent of one another or whether release of one of these types of cytokines activates release of the other type of cytokine. We reasoned we might accrue new knowledge addressing this question by adapting a method used to understand the endocrine system: frequent plasma sampling over time. This technique has shown that hormonal secretion occurs in bursts, rather than being a smooth continuous process. Data on how cytokines fluctuate over time do not exist. However, based on the ubiquity of fast-frequency or episodic secretion of hormones and other physiological variables (1–3), we expected to find that independent cell types or lineages exist to produce pro-inflammatory or anti-inflammatory cytokines; if these lineages were truly independent, the bursting patterns of pro-inflammatory compared to anti-inflammatory cytokines should also be independent and not show similarity in phase over time. Alternatively, if release of one type of cytokine produces release of the other by a feed forward mechanism suggesting dependence between the systems, one might expect that fluctuations in levels of each class of cytokines will be in phase.

In addition to addressing these questions in healthy people, we extended our study to include patients with

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[1] To whom correspondence should be addressed at UMDNJ-New Jersey Medical School, 1618 ADMC, 30 Bergen Street, Newark, NJ 07103. E-mail: natelsbe@umdnj.edu

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fibromyalgia (FM) to determine whether this patient population showed similar or different relations between pro-inflammatory and anti-inflammatory cytokines. FM is a medically unexplained illness characterized by four quadrant pain lasting at least 3 months and accompanied by multiple areas of tenderness on palpation of the body using 4 kg of force (4). FM occurs more often in women than men but is quite common in both sexes, occurring in approximately 4% of the population (5). Because activation of the body's immune system by infection or infusion of exogenous cytokines is known to produce a syndrome resembling FM (6), one hypothesis for the genesis of FM is cytokine dysregulation as manifested by upregulated inflammatory cytokines. However, increases in pro-inflammatory cytokines would not explain the common complaint of unrefreshing sleep in FM because this class of cytokines is sleep enhancing (7). In contrast, data exist to indicate that anti-inflammatory cytokines are sleep disrupting (8, 9). Therefore an alternative hypothesis would predict a shift toward anti-inflammatory cytokines—a result reported to occur during the disordered sleep of alcoholics (10). These different lines of reasoning led to two possible outcomes in FM: either a frank increase in pro-inflammatory cytokines or exactly the opposite result—an increase in anti-inflammatory cytokines.

We had a unique opportunity to address all these issues related to cytokine biology in healthy people and in others with FM. We brought both healthy subjects and FM patients into a clinical research center for a several-day protocol during which we sampled plasma every 20 mins for a 24-hr period. This study design allowed us to explore the hypotheses noted above as well as look at the possibility of a disrupted sleep-wake cytokine rhythm in FM. Such a disruption is known to exist in chronic insomnia (11), and one could consider the complaint of unrefreshing sleep in FM as a variant of insomnia.

Methods and Procedures

Subjects. Twenty-four hour plasma cytokine patterns were determined in seven premenopausal women with FM as defined by American College of Rheumatology criteria (4) and nine healthy premenopausal women; a previous paper reported their hormonal fluctuations across time (12). None of the subjects had current psychiatric diagnoses as detected by a Structured Clinical Interview from the fourth edition of the Diagnostic and Statistical Manual of Mental Disorders. All subjects underwent a detailed history, physical exam, and measurement of blood and urine chemistries, including thyroid function studies. All had normal laboratory studies, a body mass index below 29.9 kg/m^2, and no current medical problems other than FM.

Prescription and nonprescription medications (except for thyroid hormone and acetaminophen, the latter until 48 hrs before admission) were discontinued for 2 weeks before the studies reported here. No woman had received any form of glucocorticoids within the year before study or estrogen/progesterone within the previous 4 months.

All studies took place at the General Clinical Research Center of Brigham and Women's Hospital, and each subject gave her written informed consent to participate. All study procedures were reviewed and approved by the Human Research Committee of the Partners HealthCare System and approval for analysis of collected samples for cytokines by that of UMDNJ.

Protocol. Starting two days before admission to the General Clinical Research Center, subjects consumed a controlled nutrient, isocaloric diet consisting of 125 mEq sodium, 100 mEq potassium, 1000 mg calcium, and 2500 cc fluid; this diet continued throughout the in-patient protocol. Following admission to the research center, subjects slept for 8-hr periods on their usual sleep schedule for 3 consecutive nights. The first two nights were to allow for habituation to the facility.

Starting with night 2 and continuing through the end of the study, subjects were kept in an environment devoid of time cues (no clock, watch, radio, or TV) and in dim light ($<$15 lux). Beginning 7 hrs before the third sleep night and continuing through the end of the protocol, subjects remained in bed, either at a 30-degree head-up tilt during scheduled wake periods or supine during scheduled night sleep periods. After the third night of sleep, subjects consumed a constant small snack at hourly intervals until the end of the protocol. Starting 7 hrs before the third night of sleep, blood was sampled every 20 mins for cytokines (see below for details) for a 24-hr period through a one-inch iv catheter inserted into a large forearm vein 2 hrs before initiation of blood sampling. To avoid disrupting sleep, blood sampling was performed from outside the subject's room and was done without touching the subject. A total of 336 ml of blood was withdrawn over 24 hrs and approximately twice this volume was replaced in flushes of the catheter with warmed 0.45% heparinized sodium chloride.

Assay Methods. Plasma samples which had been stored frozen at –80°C were shipped overnight on dry ice and kept frozen at –80°C. They were thawed, diluted 1:1 with assay buffer, and assayed with Millipore Inc.'s Beadlyte Human Multi-Cytokine Detection System 2 (Bellerica, MA) according to the instructions in the kit with some modifications. This kit simultaneously measures the pro-inflammatory cytokines IL-1β, IL-6, IL-8, and TNF-α and the anti-inflammatory cytokine IL-10. There were two modifications to the assay protocol. To stop the reaction and remove the unbound luminescence, the wells were rinsed twice with assay buffer instead of using the stop solution. In addition, the standard solution was serially diluted so the standard curve had concentrations of 0, 0.49, 0.98, 1.95, 3.90, 7.80, 15.60, 31.25 and 62.5 pg/ml. Data reduction was performed using the MasterPlex-QT software (Miraibio, Alameda, CA); the standard curves were fit with a linear model using those standards that encompassed the values

Table 1.

Cyto-kine	Minimal detectable dose (pg/ml)	Concentration of pool (pg/ml)	Intra-assay variability (% C.V.)	Inter-assay variability (% C.V.)
IL-1β	0.3	1.03	1.1	1.6
IL-6	0.9	1.83	1.0	2.4
IL-8	0.5	3.02	2.5	2.7
IL-10	0.3	2.23	1.0	2.4
TNF-α	0.3	0.98	0.9	1.3

for each cytokine obtained from the samples in each assay. The r^2 for the linear fits was always above 0.95 and very often above 0.99. In each 96-well plate, the standards and a quality control pool were run in triplicate, and 33 samples were run in duplicate.

Assay characteristics are described in Table 1. The minimum detectable dose was calculated as the concentration that was 2 standard deviations of the mean above the zero standards. All values below the minimum detectable dose were assigned concentrations of zero. The mean concentrations of the pool are presented along with the coefficients of variability (standard deviation divided by the mean) that were calculated from the pool run with each of 24 assays.

Cross-reactivity in a multiplex system can arise from both the antibody and the luminescent reporter used for each cytokine. The detailed data for cross-reactivity in this assay system are available from Millipore Inc. (Bellerica, MA). When 2,500 pg/ml of each standard was run with the assay protocol, the cross-reactivity with the antibodies for the other cytokines was less than 1.0%, and often undetectable. When examining the cross-reactivity of the reporters by adding 1,000 pg/ml of all the standards except the one specific for a given reporter, the cross-reactivity was less than 0.2%. Given the cytokine concentrations in our samples, cross-reactivity among them should not have contributed meaningfully to any of the values in our data, and particularly to any correlations among the cytokines.

Data Analysis. We used the continuous wavelet transform (CWT) to evaluate spatio-temporal characteristics of 24-hr cytokine secretion patterns, considered to be suitable especially for episodic and locally periodic data like those in this study (13–15). The original data were linearly interpolated and resampled every minute. The CWT was then applied to each resampled data record. The CWT of a discrete sequence $x\ (t)$ is defined as

$$W_x(n,s) = \sum_{t=0}^{N-1} x(t)\psi*\left[\frac{(t-n)}{s}\right],$$

where the $(*)$ indicates the complex conjugate. The decomposition basis is obtained by translation and scaling of the mother wavelet Ψ using time/position n and scale s. By increasing or decreasing s, the frequency contents of the basis function are changed. The modulus and phase of the wavelet transform is defined as

$$|W_x(n,s)| = \sqrt{[\Re(W_x(n,s))]^2 + [\Im\ (W_x(n,s))]^2}$$

and

$$\phi_x(n,s) = \tan^{-1}\frac{\Im\ (W_x(n,s))}{\Re(W_x(n,s))},$$

respectively, where the $\Im$ and $\Re$ respectively stand for the imaginary and real part of the wavelet transform. Phase difference between two sequences ($x_1\ (t)$ and $x_2\ (t)$) is computed as

$$\phi_{x_1,x_2}(n,s) = |\phi_{x_1}(n,s) - \phi_{x_2}(n,s)|.$$

For the analysis of instantaneous frequency of signals, we used the Morlet wavelet which is one of the most commonly used wavelets (16). In particular, the Morlet wavelet is most suitable for assessing transient frequency characteristics of time series. The Morlet wavelet can be expressed in a following form:

$$\psi(t) = \pi^{-\frac{1}{4}} e^{i\omega_0 t} e^{-\frac{t^2}{2}}$$

with its Fourier transform (in frequency domain ω):

$$\hat{\psi}(\omega) = \pi^{-\frac{1}{4}} e^{-\frac{(\omega-\omega_0)^2}{2}}$$

for $\omega_0 > 5$. The Fourier wavelength λ of the basis function can be calculated as

$$\lambda = \frac{2\pi s}{\omega_c}$$

where ω_c is the mother wavelet center frequency (the frequency where the energy of Ψ reaches maximum) and $\omega_c \approx \omega_0$ for $\omega_0 > 5$. In our analysis, we used $\omega_0 = 2\pi$, which guarantees balanced time and frequency localization. In this case, the relationship between scale and (angular) frequency is given by $\omega \approx 2\pi/s$ and frequency (in Hz) is $f \approx s^{-1}$.

In order to test whether the observed $W_x\ (n,s)$ was significantly above the level of random fluctuations, the null hypothesis of significance above the background spectrum was tested following Torrence and Compo (17); if a peak in the wavelet power spectrum is significantly above the background spectrum, it can be assumed to probe a true (locally) periodic feature with a certain degree of confidence. We generated 100 randomly shuffled data sequences $y_k\ (t)$ $(k = 1,2, \ldots 100)$ from each original data record. As in the original data, each shuffled data record was linearly interpolated and resampled every minute. We then estimated for the randomly shuffled data records the distribution of the local wavelet power spectrum, defined as

$$\frac{|W_y(n,s)|^2}{\sigma^2} \Rightarrow \frac{1}{2} P_k \chi_2^2$$

at each time n and scale s, where "⇒" indicates "is distributed as." The 1/2 factor removes the degree of

Table 2. Means and Variances of Each Cytokine During Awake and Sleep for Healthy Controls and Fibromyalgia (FM) Patients[a]

	Healthy ($n = 9$)		FM ($n = 7$)	
	Awake	Sleep	Awake	Sleep
Mean (pg/ml)				
IL-10	0.95 ± 0.38	0.84 ± 0.38	1.05 ± 0.30	1.45 ± 0.55*,#
IL-1β	1.24 ± 1.17	1.16 ± 1.33	0.79 ± 0.64	1.33 ± 1.32
IL-6	16.8 ± 15.3	12.2 ± 11.5	14.0 ± 10.9	16.5 ± 18.0
IL-8	11.8 ± 5.4	12.5 ± 6.4	8.4 ± 1.9	7.9 ± 1.7
TNF-α	0.70 ± 0.61	0.72 ± 0.83	0.24 ± 0.13	0.31 ± 0.19
Variance (pg^2/ml^2)				
IL-10	0.21 ± 0.14	0.27 ± 0.38	0.40 ± 0.46	0.53 ± 0.96
IL-1β	2.39 ± 4.38	1.06 ± 1.21	1.00 ± 1.39	1.73 ± 3.39
IL-6	245 ± 468	57 ± 59	109 ± 129	147 ± 302
IL-8	25.8 ± 37.1	29.2 ± 30.3	8.7 ± 14.9	4.7 ± 8.0
TNF-α	3.98 ± 10.92	1.38 ± 3.02	0.10 ± 0.10	0.14 ± 0.16

[a] Values are means ± SD.
* $P < 0.05$ from healthy controls.
# $P < 0.05$ from awake.

freedom factor from the X^2 distribution. The value of P_k is the mean spectrum of 100 randomly shuffled data at the Fourier frequency k corresponding with the wavelet scale s. In order to estimate the power spectrum density for the randomly shuffled data, we first extracted from each shuffled record 10 time-shifted, equally overlapping intervals with 1,024 data points. A fast Fourier transformation was then applied to each interval. Finally, results for the 10 intervals were averaged over frequency. Thereafter, the W_x (n,s) from the original data was compared with the 95% confidence interval of W_y (n,s) from the randomly shuffled data.

Statistical Analyses. Interrelationships between the value of the anti-inflammatory cytokine IL-10 during every statistically significant secretory bursts and the values of the pro-inflammatory cytokines, IL-1β, IL-6, IL-8, and TNF-α were tested by the Pearson or Spearman correlation coefficients. The interrelationships between anti- and pro-inflammatory cytokines were tested by least squares regression analyses. Differences in regression slopes were tested by interaction effects between healthy controls and FM patients in ANCOVA. Differences in the mean and variance of each cytokine between awake and sleep and between healthy controls and FM patients were assessed using ANOVA. Differences in the mean of peak values of statistically significant secretory bursts for each cytokine between healthy controls and FM patients were assessed using non-paired Student's t tests. Phase differences in cytokine secretion between IL-10 and pro-inflammatory cytokines for awake and sleep and for healthy controls and FM patients were assessed using ANOVA. Post hoc analyses used Bonferroni tests to adjust for multiple comparisons.

Results

Mean IL-10 concentration during sleep was significantly higher in FM patients than healthy controls ($P < 0.05$), while mean concentrations for IL-10 during awake and for pro-inflammatory cytokines during awake and sleep did not differ ($P > 0.05$) between groups (see Table 2). FM patients showed a significant increase ($P < 0.05$) in mean IL-10 concentration during sleep compared to awake; no other day-night differences were found. There were no significant differences in means for any of the other cytokines between the groups, and variances for all of the cytokines did not differ significantly ($P > 0.05$) between healthy controls and FM patients during either wake or sleep conditions.

There was no general pattern across cytokines that was consistent either for patients or controls. Figure 1 shows the data from three cytokines plotted over time for one healthy control. IL-1β and TNF-α were at low levels except when burst secretion occurred; IL-10 levels were relatively stable at low levels, dipping even further in late evening. Figure 2 provides examples of raw data and time series for levels of anti-inflammatory (IL-10) and pro-inflammatory cytokines (IL-1β and IL-6) sampled over a 24-hr period with the 8 hrs lights-out sleep period (420–900 mins) demarcated by vertical dotted lines for 2 healthy controls (Subj. 5 and 6) and 2 FM patients (Subj. 11 and 13). As was the case in Figure 1, visual inspection reveals rather flat levels of cytokines throughout the entire sampling period with episodic large, but brief, increases (e.g., Subject 6′s data).

To identify such intermittent variations statistically, we applied the CWT to examine the amplitude spectrum with respect to time. The lower part of each panel shows moduli, or amplitudes, of CWT encoded with color levels; the reddish regions have higher amplitudes, and the vertical axis

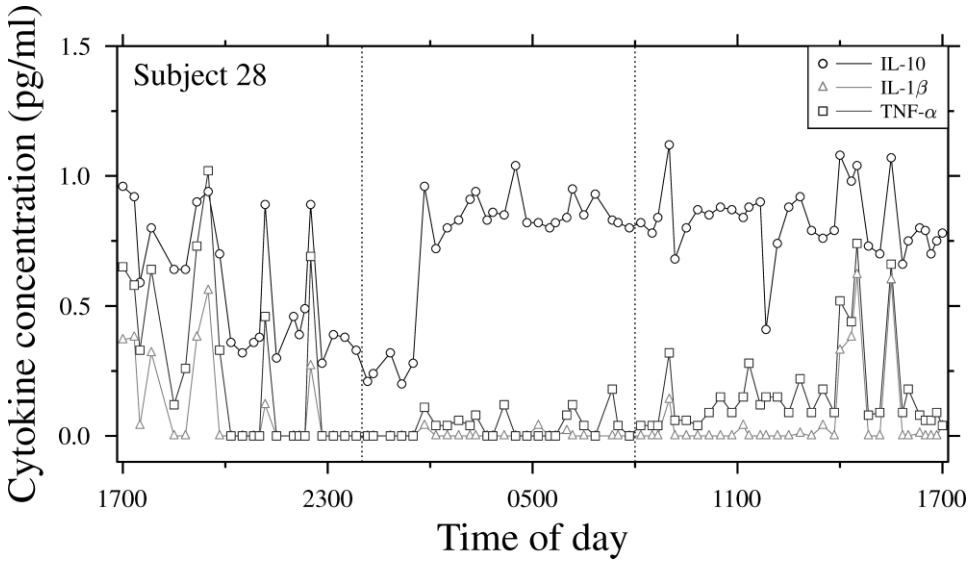

Figure 1. Twenty-four hour profile of IL-10, IL-1β, and TNF-α in a healthy control. Lights out period is demarcated by vertical dotted lines.

is scale of the wavelet (in minutes). The amplitude of the moduli in the region surrounded by a black line is significantly ($P < 0.05$) higher than those obtained for randomly shuffled data (see Methods), indicating that the increase did not occur by chance. Time periods with moduli between 30 to 150 mins in scale that were significantly higher than baseline are indicated by red bars in the upper part of each panel. The analytic method revealed episodic bursts to occur either singly or as doublets. The shape of the moduli reflects whether the burst is singular or doublet. This is because, for doublets, the regions with higher moduli are mostly observed in the higher frequency (or small scale) range (30–150 mins), suggestive of the locally "periodic" nature, while, for singular bursts, the region is narrow and extends downwards to the low frequency region. Over 24 hrs, healthy controls averaged 2.3 ± 0.9 [SD] single bursts and 2.6 ± 1.8 doublets while FM patients averaged 3.3 ± 2.2 single bursts and 2.0 ± 1.9 doublets ($P > 0.05$ for all comparisons between groups). Table 3 provides information on peak levels attained during these bursts. Secretory bursts for all of the cytokines occurred from 8.5% to 16.4% (median = 13%) of the 24-hr data collection period with no differences between FM and controls. The average time

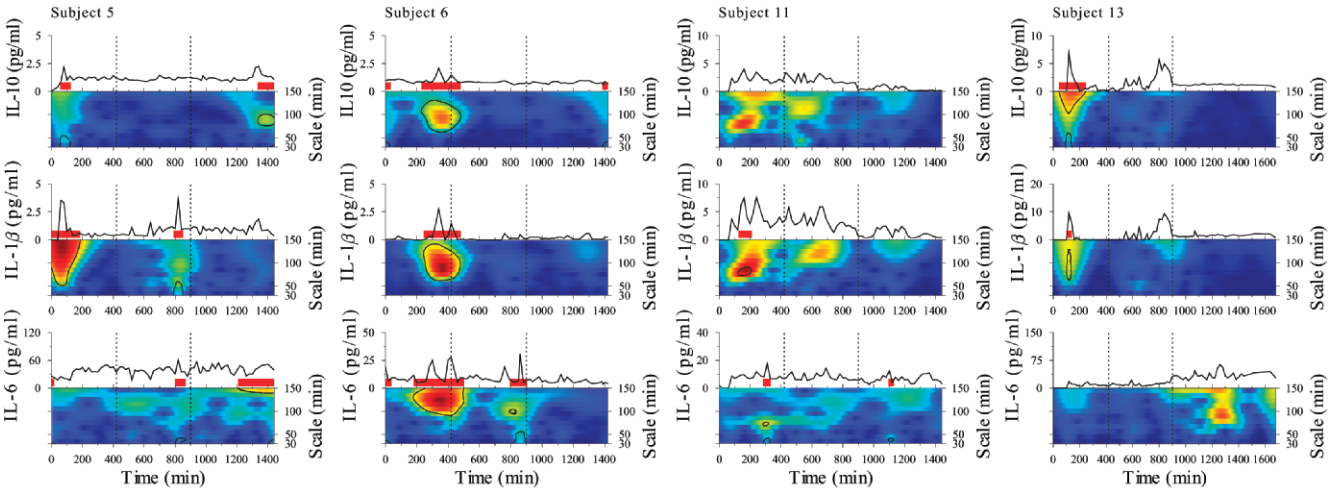

Figure 2. Examples of raw data and time series for levels of anti-inflammatory (IL-10) and pro-inflammatory cytokines (IL-1β and IL-6) sampled over a 24-hr period for 2 healthy controls (subjects 5 and 6) and 2 fibromyalgia (FM) patients (subjects 11 and 13). Lights out indicated by vertical dotted lines (420–900 mins). The lower part of each panel shows moduli of CWT encoded with color levels; the reddish regions have higher amplitudes. The moduli in the region surrounded by a black line are significantly ($P < 0.05$) higher than those obtained for randomly shuffled data. Time periods with moduli between 30 to 150 mins in scale that were significantly higher than randomly shuffled data are indicated by red bars in the upper part of each panel.

Table 3. Mean of Peak Values of Statistically Significant Secretory Bursts for Each Cytokine for Healthy Controls and Fibromyalgia Patients[a]

Peak (pg/ml)	Healthy	FM
IL-10	1.93 ± 0.98 ($n = 7$)	2.47 ± 2.26 ($n = 5$)
IL-1β	5.54 ± 4.72 ($n = 9$)	4.11 ± 3.21 ($n = 7$)
IL-6	39.6 ± 41.6 ($n = 9$)	33.8 ± 46.3 ($n = 5$)
IL-8	22.8 ± 14.8 ($n = 8$)	14.0 ± 8.7 ($n = 6$)
TNF-α	4.96 ± 7.29 ($n = 8$)	1.37 ± 0.91 ($n = 7$)

[a] Number of subjects having statistically significant bouts in parentheses. Values are means ± SD.

Table 4. The Inverse of Frequency (Min) at Which the Doublet Bursts Are Observed (Means ± SD)[a]

	Healthy	FM
IL-10	95 ± 32	—
IL-1β	94 ± 22	93 ± 29
IL-6	126 ± 17	108 ± 39
IL-8	127 ± 41	131 ± 21
TNF-α	88 ± 38	101 ± 48

[a] Values are means ± SD. No doublet bursts were observed in IL-10 for FM patients.

between secretory bursts in a doublet ranged from 88 mins to 131 mins (i.e., the doublet period, see Table 4).

Figure 3 shows the temporal location of each statistically significant secretory burst for each cytokine studied (IL-10, IL-1β, IL-6, IL-8, and TNF-α) and for every subject (black bars for healthy and gray bars for FM). The lower right panel aggregates the data for each cytokine for healthy and FM subjects. Synchronization across subjects occurred least often for IL-10 and IL-6. In contrast, synchronization was seen for the other cytokines occurring in the first 200 mins of sampling and then again toward the end of night. Synchronization across subjects was greatest for IL-8 in the patient group—occurring early during sample collection.

Figure 4 depicts scatter plots showing the relation between the value of the anti-inflammatory cytokine IL-10 during every statistically significant secretory burst compared to values of the pro-inflammatory cytokines, IL-1β, IL-6, IL-8, and TNF-α occurring at the same time. Data from all subjects in each group are shown. Finding significant correlations between a pair of cytokines would indicate consistency in secretory pattern between that pair. During the awake state, the magnitude of secretory bursts of IL-10 correlated significantly with the level of pro-inflammatory cytokines, IL-1β, IL-8, and TNF-α, both for healthy controls and FM patients. For IL-6, a significant correlation was found for controls but not for patients. These pairs of cytokines that showed significant correlations maintained them irrespective of whether Pearson's or Spearman's correlation coefficients were evaluated. Regression slopes of IL-1β, IL-8, and TNF-α, against IL-10 were significantly ($P < 0.05$) greater for healthy controls than for FM patients, indicating that the ratio of levels of pro-inflammatory cytokines against the anti-inflammatory cytokine IL-10 was higher for controls than for patients; we interpret this to indicate a skew away from the normal balance favoring pro-inflammatory cytokines in controls toward one favoring an anti-inflammatory response in FM.

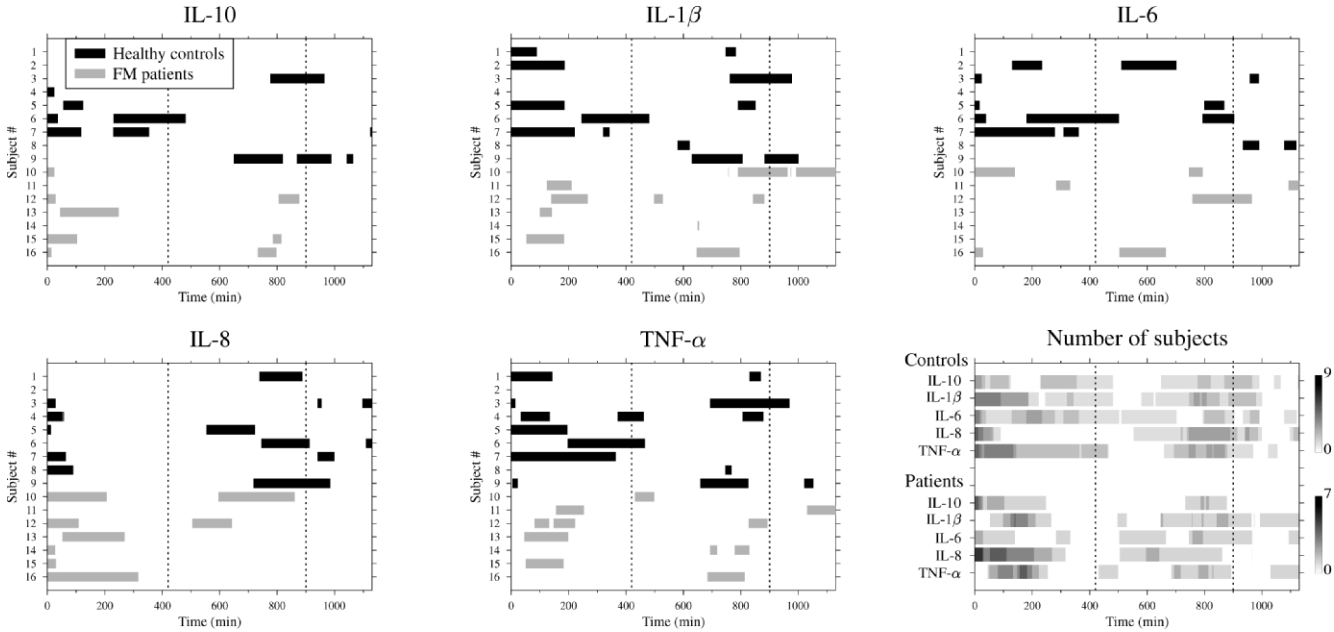

Figure 3. The temporal location of each statistically significant secretory burst for each cytokine studied (IL-10, IL-1β, IL-6, IL-8, and TNF-α) and for every subject (black bars for healthy, subjects 1–9; gray bars for FM, subjects 10–16). Lower right panel aggregates the data for each cytokine for healthy and FM subjects. The number of subjects for each cytokine in healthy controls and FM patients are gray-level coded [0 (white) to all (black)].

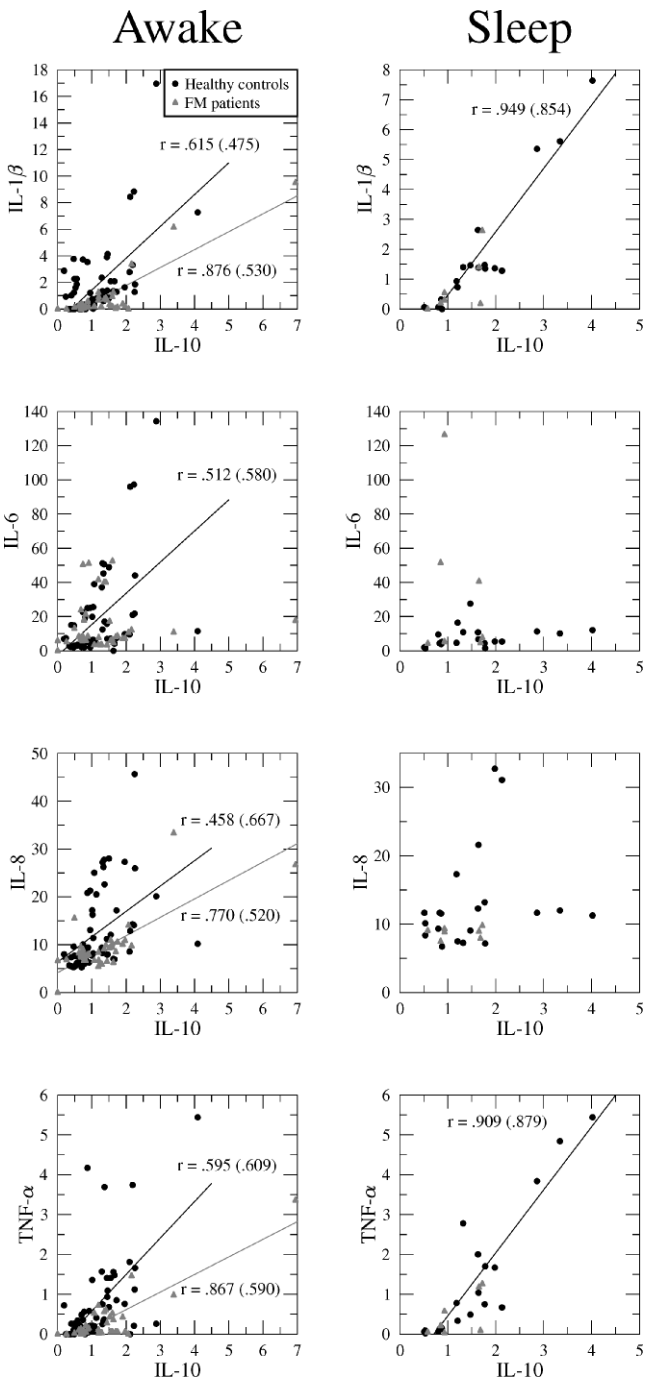

Figure 4. The relation between the statistically significant bursts of secretion of the anti-inflammatory cytokine IL-10 to those of the pro-inflammatory cytokines, IL-1β, IL-6, IL-8, and TNF-α for healthy controls (circle) and FM patients (triangle). Data from all subjects in each group are shown. Pearson (Spearman) correlation coefficients are presented if $P < 0.05$.

Additional evidence supporting a disconnection between the two classes of cytokines in FM was the fact that healthy controls but not FM patients had statistically significant and positive correlations between IL-10 with IL-1β and with TNF-α during sleep.

Although not every subject had high CWT moduli attaining statistical significance for IL-10 during wake and sleep states, we were able to do statistical comparisons of mean phase differences between IL-10 and pro-inflammatory cytokines when present (see Table 5). Phase differences were all positive and quite small; with the variability among subjects, they approached zero indicating bursts of secretion of the four cytokines occurred at approximately the same times. There were no significant differences in mean phases of significant secretory bursts of any of the cytokines between healthy controls and FM patients either during wake or sleep states (Table 5).

Discussion

Our understanding of hormonal regulation has been greatly altered by results of studies using high frequency sampling. For example, cortisol is secreted in periodic bursts (18), and these bursts cannot be seen unless sampling is done rapidly enough to capture the basic structure of the burst. In contrast to the wealth of knowledge on hormonal dynamics, there is little information available on cytokine dynamics, and this was a major reason for our doing this study. As a result, we now report that statistical evaluation of cytokine levels over time reveals patterns that are relatively quiescent and then interrupted by relatively large increases, consistent with episodic secretion. In contrast to hormones, however, secretion was not periodic except in a local sense. That is, when a burst began, there was a high probability that a second burst would follow shortly thereafter. Interestingly, the duration between bursts in doublets averaged approximately 110 mins (Table 3), and this is close to the basic period known to occur in ultradian rhythms across modalities and species (19). Thus, although quite preliminary, the data suggest an element of entrainment to an underlying ultradian rhythm. What is unknown, however, is the reason these bursts only occurred during 13% of the 24-hr study period rather than throughout the entire time.

A second intriguing finding is the apparent synchronization of these bursts of cytokine secretion. These tended to occur early in sampling and then late in the night. The most striking of these synchronized bouts occurred in the patient group for IL-8 coincident with onset of sampling. While these initial episodes of synchronized cytokine secretion probably reflected some social entrainment related to venous cannulation and initiation of sampling, we do not have an explanation for the late night apparent synchronization.

Collecting repeated samples over time and using an assay method that allows determination of levels of multiple cytokines with one small sample allows us to determine whether pro-inflammatory cytokines are secreted in or out of phase with the anti-inflammatory cytokine IL-10. We found the former result. This result argues against the idea that separate or independently regulated cell types exist for secretion of pro-inflammatory and anti-inflammatory cytokines but instead suggests that cell communication between

Table 5. Mean Phase Difference Between Anti-Inflammatory (IL-10) and Pro-Inflammatory (IL-1β, IL-6, IL-8, and TNF-α) Cytokines at Regions Selected as Having High Moduli of CWT in a Statistically Significant ($P < 0.05$) Manner for IL-10 for Healthy Controls and FM Patients[a]

	Healthy		FM	
Phase difference (min) between IL-10 and:	Awake ($n = 7$)	Sleep ($n = 4$)	Awake ($n = 3$)	Sleep ($n = 3$)
IL-1β	2.5 ± 1.7	1.8 ± 1.0	6.0 ± 6.8	0.5 ± 0.2
IL-6	7.9 ± 8.4	10.1 ± 6.8	2.1 ± 1.0	6.3 ± 8.5
IL-8	10.8 ± 10.5	15.1 ± 10.0	2.9 ± 2.5	4.3 ± 3.4
TNF-α	2.7 ± 1.0	3.6 ± 1.5	7.1 ± 8.5	1.3 ± 1.5

[a] Number of subjects with moduli of both cytokines that were statistically significant in parentheses. Values are means ± SD.

systems must be operating (20). However, across these cytokine systems, in the unstimulated human immune system of healthy people, levels are usually low but are characterized by bursts of secretion that tend to occur across cytokine class. However, at least for healthy subjects, the ratio of secretion of these two classes of cytokines occurs in favor of pro-inflammatory ones.

The cytokine findings in patients with FM differed from those of healthy controls—primarily in nighttime, a time that has not been previously studied in this illness group. Healthy controls but not patients showed nocturnal correlations between IL-10 and IL-1β and TNF-α. In the same vein, healthy subjects showed daytime correlations between IL-10 and IL-6 which were not seen in FM. These correlations suggest an interaction among these cytokines, perhaps in regulating normal sleep; the fact that these correlations did not extend to the nighttime for IL-6 may reflect the fact that it is more pro-inflammatory than soporific (21).

The magnitude of these regression slopes, when significant, was higher in controls than in patients. We understand this to mean that for every molecule of pro-inflammatory cytokines secreted in controls, significantly more molecules of IL-10 were secreted in FM patients. This suggests a tilt away from the normal balance of these two systems in controls to one favoring anti-inflammatory cytokines in FM. In addition, the patients showed a shift from pro-inflammatory toward anti-inflammatory cytokines by having significantly higher levels of IL-10 during the nighttime than the controls. This result is different from an earlier report of increases in IL-8, a pro-inflammatory chemokine, relative to controls with no change in other pro-inflammatory cytokines (22, 23). We cannot explain this discrepancy except to note our use of a substantially larger sample size due to repeated measures over time within subjects.

Interestingly nonetheless, this increase in nocturnal IL-10 suggests dysregulated circadian patterns which may explain in part the patient complaint of unrefreshing sleep. Vgontzas has reported that insomniacs show a shift of pro-inflammatory cytokine secretion from nighttime to daytime (11). Although anti-inflammatory cytokines were not measured in that study, the shift reported would be consistent with the same effect noted here—namely a shift in nocturnal cytokine secretion in favor of anti-inflammatory cytokines. Pro-inflammatory cytokines are known to have sleep promoting effects while anti-inflammatory cytokines have sleep disrupting effects. Healthy people have compensatory increases in pro-inflammatory cytokines when bursts of secretion of the anti-inflammatory cytokine IL-10 occur; this balance permits normal, restful sleep. In contrast to what occurs in healthy people, FM patients have three problems: first, they have frank increases in IL-10 in night-time; second, they do not show bursting of pro-inflammatory cytokines to compensate for bursting in the anti-inflammatory cytokine IL-10 in night-time; and third, even during day-time, the ratio of pro- to anti-inflammatory cytokines is reduced relative to controls. The first two differences between FM and healthy controls may explain the complaint of disturbed sleep in FM because of an imbalance in favor of sleep disturbing anti-inflammatory cytokines during sleep. The third difference may reflect an underlying dysregulation in cytokine dynamics in FM. Further research will be needed to explore what may be a fundamental problem in cytokine organization in FM.

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