

Circadian characteristics of circulating interleukin-6 in men

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Background: The cytokine interleukin-6 (IL-6) is a multifunctional small-peptide molecule that is produced by various types of lymphoid and nonlymphoid cells. It plays a central role in hematopoiesis, host defense mechanisms, and acute-phase reactions, including regulation of inflammatory and immune responses.

Methods: Because a circadian time structure has been shown to characterize nearly every biologic function tested in human beings, including some cytokines, we sought to investigate the 24-hour pattern of circulating IL-6 in a group of 11 clinically symptom-free men (median age, 50 years; range, 46 to 72 years). Blood samples were obtained every 3 hours for 24 hours (eight samples per subject), and serum was frozen until analysis for IL-6 with a solid-phase ELISA.

Results: Average IL-6 values ranged from 1.66 to 5.38 pg/ml, with lowest to highest values within 24 hours ranging from 1.20 to 7.58 pg/ml (120% to 531%) between subjects. On average, values were greater than the mean throughout the night, with a peak at 01:00 hours and less than the mean throughout the day, with a nadir at 10:00 hours. A significant time effect was found by analysis of variance; and a high-amplitude circadian rhythm was described by the least-squares fit of a 24-hour cosine ($p < 0.001$; amplitude, $41\% \pm 5\%$; acrophase, $02:16 \pm 00:28$ hours). In addition, a positive correlation between mean IL-6 levels and age was found ($r = 0.63$, $p = 0.037$).

Conclusions: Because monitoring of endogenous cytokine levels is suggested for assessing immune function and pathologic condition, clinical decisions and immunotherapies may be significantly influenced by the large and predictable day-night variations in endogenous cytokine production and bioactivity. (*J ALLERGY CLIN IMMUNOL* 1995;95:1029-35.)

Key words: Blood, circadian, cytokine, interleukin-6, rhythm

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Abbreviations used

IFN: Interferon
IL: Interleukin
ROC: Range of change

As biologic response modifiers, cytokines are peptide mediators used by immune and inflammatory cells for intercellular communication. The soluble nature of cytokines provides intercellular signaling ranging from a local cell-cell level to a widespread systemic response, and in one form or another cytokines act on every cell in the body as regulators of growth, differentiation, and mature function. Interleukin (IL)-6 is a multifunctional protein produced by lymphoid and nonlymphoid cells and by normal and transformed cells, includ-

TABLE I. Circadian data and characteristics for serum IL-6 levels measured every 3 hours for 24 hours in healthy men

Subject No.	Age (yr)	Time (clock hr of beginning of sampling interval)							
		19:00	22:00	01:00	04:00	07:00	10:00	13:00	16:00
1	46	1.22	1.38	2.64	2.83*	0.70	0.96	2.41*	1.14
2	47	2.23	4.49*	3.63	4.75*	1.63	1.39	1.90	1.96
3	48	0.81	1.64	2.57*	2.43	2.22	1.74	1.75	0.97
4	50	0.50	1.71	2.65	2.89*	3.18	1.20	2.32	1.10
5†	50	1.00	1.82	2.11*	1.92	1.88	1.12	2.20*	1.35
6	50	0.85	2.72	3.11*	2.64	1.16	1.02	1.26	0.69
7	50	1.71	2.93	5.65*	1.80	3.07	2.45	1.47	1.89
8	58	3.21	10.06	10.18*	4.63	5.01	2.60	3.00	4.38
9	65	2.32	3.18	4.47*	3.36	2.45	1.20	1.39	1.65
10	71	3.74	4.35	5.67	6.83*	4.98	2.08	2.11	2.44
11	72	4.00	3.63	4.40	3.66	5.09*	1.76	2.96	4.24
Mean ± SE (pg/ml)		1.96 ± 0.37	3.45 ± 0.74	4.28* ± 0.70	3.43 ± 0.45	2.85 ± 0.47	1.59 ± 0.17	2.07 ± 0.18	1.98 ± 0.53
Mean ± SE (% of mean)		68.6 ± 7.2	120.8 ± 10.5	157.0* ± 9.3	132.6 ± 10.9	103.7 ± 10.8	62.2 ± 5.4	85.3 ± 10.1	69.7 ± 5.7

Mean of two duplicate determinations per sample.

*Highest value.

†Subject had chronic asthma but was asymptomatic during study.

ing T and B cells, monocytes and macrophages, fibroblasts, hepatocytes, vascular endothelial cells, cardiac myxomas, bladder cell carcinomas, myelomas, astroglomas, and glioblastomas.^{1,2} On the basis of its various activities, IL-6 has also been called interferon (IFN)- β , 26 kd protein, B-cell stimulatory factor-2, hybridoma-plasmacytoma growth factor, hepatocyte stimulating factor, cytotoxic T-cell differentiation factor, and macrophage-granulocyte inducing factor 2A.

The various activities attributed to IL-6 on different cells are numerous and varied, because IL-6 is both a mediator of specific immune function (by promoting an antigen-specific response to a previously encountered antigen) and a mediator of natural immunity and of amplification of the inflammatory response (by promoting primary responses to newly encountered antigens). This suggests that IL-6 has a major role in the mediation of the inflammatory and immune response initiated by infection or injury.^{3,4} Although the exact functions of IL-6 in vivo are not known, elevated IL-6 levels have been reported to be associated with a variety of diseases, including autoimmune diseases,⁵ such as rheumatoid arthritis,⁶ and chronic allograft rejection,⁷ and malignancies such as myelomas,^{2,8} plasmacytomas,⁸ lymphomas and leukemias,⁹ and ovarian cancers.¹⁰

A great deal of work is currently in progress to understand better the role of IL-6 in the modula-

tion of normal and pathologic processes.^{1,2} Temporal considerations, however, are generally overlooked. Genetically determined, environmentally synchronized circadian rhythms have been described for most endogenous biodynamic processes,¹¹ including variables involving hematopoiesis¹² and the hypothalamic-pituitary-adrenal axis.¹³ Less is known, however, about circadian fluctuations in the immune system, including functions of immune effector cell populations and changes in the multifunctional growth factors and cytokines. The objective of this study was to establish normal baseline circadian characteristics of circulating IL-6 in clinically symptom-free adult male subjects tested every 3 hours for 24 hours to understand better the biologic time structure of this cytokine in health to serve as a more complete reference for disease.

METHODS

Subjects

As part of an ongoing study of biologic rhythms and aging in a group of former army reservists,¹⁴ 11 clinically healthy men, 46 to 72 years old, were used as subjects in this study, conducted May 14 and 15, 1993, at a clinical research unit at the Edward Hines, Jr., Veterans Affairs Hospital in Hines, Ill. Each subject gave a medical and social history and received a complete physical. The medical history of one subject (No. 5) included mild chronic asthma. None had any clinically overt disease (subject 5 was asymptomatic) or was undergoing any

ROC		Mean $\pm$ SE (pg/ml)	Acrophase $\pm$ SE (hr/min)
pg/ml	%		
2.13	303	1.66 $\pm$ 0.29	02:30 $\pm$ 00:32
3.37	243	2.75 $\pm$ 0.47	00:45 $\pm$ 01:04
1.77	219	1.76 $\pm$ 0.23	04:49 $\pm$ 00:44
2.67	531	1.94 $\pm$ 0.34	05:36 $\pm$ 01:12
1.20	120	1.67 $\pm$ 0.16	04:20 $\pm$ 03:12
2.42	354	1.68 $\pm$ 0.34	01:45 $\pm$ 00:56
4.18	284	2.62 $\pm$ 0.48	02:10 $\pm$ 02:04
7.58	292	5.38 $\pm$ 1.08	00:36 $\pm$ 01:12
3.27	273	2.50 $\pm$ 0.40	01:17 $\pm$ 00:32
4.75	229	4.02 $\pm$ 0.62	02:22 $\pm$ 02:24
3.33	189	3.72 $\pm$ 0.36	00:12 $\pm$ 03:12
3.33 $\pm$ 0.53	276 $\pm$ 32	2.70 $\pm$ 0.36	

ROC is from lowest to highest value within 24 hours. Acrophase (objectively determined time of peak) = time of peak (hr/min) of best-fitting 24-hour cosine.

immunosuppressive therapeutic intervention. However, two subjects (Nos. 3 and 4) had used aspirin within the previous week. All subjects were found to have a satisfactory nutritional status with a mean weight of 189 pounds (range, 144 to 250 pounds) and average height of 70 inches (range, 66 to 74 inches). Meals during the study consisted of a general hospital diet totaling 2500 Kcal and were served at 16:30, 07:30, and 13:30 hours. The subjects were not restricted in their water intake, except for the half hour before sampling, but were required to abstain from other liquids and food between meals. All subjects had followed their regular diurnal activity schedule with sleep at night for at least 2 weeks before the study. During the study participants were active by day, with bed rest between 23:00 and 07:00 hours; they were briefly awakened for the 01:00 and 04:00 hour blood samplings. Four subject coinvestigators (Nos. 1, 9, 10, and 11) slept fewer hours and more intermittently because of procedural activities related to performing the study.

Informed consent was obtained from each of the volunteers after the nature and possible consequences of the study were fully explained. This study was approved by the institutional review board of the Edward Hines Veterans Affairs Hospital.

Blood collection

Blood samples (eight samples per subject) were collected at 3-hour intervals beginning at 19:00 hours with subsequent sampling beginning at 22:00, 01:00, 04:00, 07:00, 10:00, 13:00, and 16:00 hours. Blood was immediately centrifuged and the serum separated, aliquoted, and frozen at -25°C until analysis.

Measurement of IL-6

A quantitative sandwich enzyme immunoassay technique was used to determine IL-6 (Quantikine Human IL-6 Immunoassay Kit, R & D Systems, Minneapolis, Minn.) This solid-phase ELISA contains recombinant human IL-6 and antibodies against recombinant human IL-6 and accurately measures the recombinant factor. Because the measurement of IL-6 is insensitive to the addition of the recombinant form of the IL-6-soluble receptor, it is probable that sample measurements reflect the total amount of IL-6 present, that is, the total amount of free IL-6 plus the amount of IL-6 initially bound to soluble receptors (manufacturer's developmental observation). All determinations were performed in duplicate, and each assay was repeated twice, with the average value used for statistical analysis. Values less than the lower standard sensitivity (1.0 pg/ml) were determined by extrapolation. The intraassay coefficients of variation for the IL-6 assays were 4.4% and 3.6% for interassay precision.

Statistical analysis

Each individual time series was displayed in a chronogram (x-y plot) to inspect the data for patterns, trends, and outliers. No values were excluded from data analysis (88 total values). All data were analyzed for time effect by comparing the eight time point means by one-way analysis of variance, with values in original units (picograms per milliliter) and as a percentage of individual means. In addition, the data were analyzed individually and as a group for circadian rhythm by a computerized inferential statistical method (single cosinor) involving the fit of a 24-hour cosine curve by the method of least

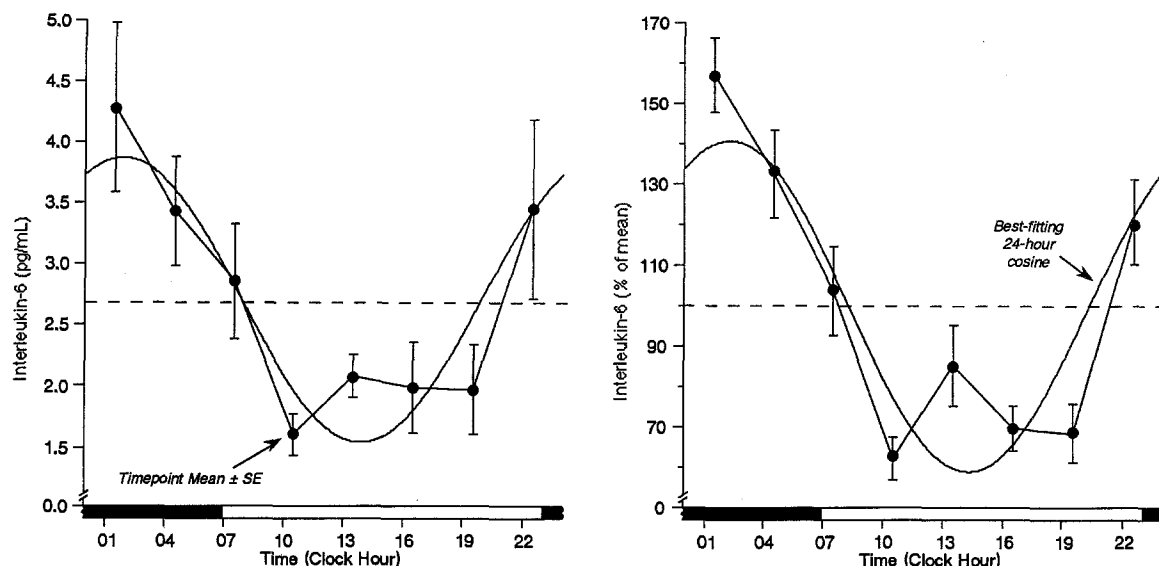


FIG. 1. Circadian rhythm in serum IL-6 in 11 men, 46 to 72 years old. Samples were taken every 3 hours for 24 hours, beginning at 19:00 May 14, 1993. IL-6 was measured in duplicate, and mean values were determined. Data were analyzed in original units (picograms per milliliter; *left panel*) and in normalized units (percentage of individual mean; *right panel*) for group analysis. Analysis for time effect was significant at $p < 0.001$ by both analysis of variance and least-squares fit of 24-hour cosine. *Dashed line*, 24-hour mean; *dark bar*, sleep and/or rest span.

squares,¹⁵ which has been adapted to personal computer.¹⁶ A p value for the rejection of the zero amplitude assumption was determined, with rhythm detection considered statistically significant if p is 0.05 or less. Rhythm characteristics that can be determined from the best-fitting cosine include the MESOR (the middle of the cosine representing an adjusted 24-hour average), the amplitude (half the peak-trough difference of the cosine), and the acrophase (the timing of the peak of the fitted cosine). To test for any effect of age, mean IL-6 levels were correlated to age by simple linear regression.

RESULTS

Individual data for each subject, the range of change (ROC) between the lowest and highest values throughout the 24-hour observation span, the mean serum IL-6 levels, and the time of the peak as estimated by the best-fitting 24-hour cosine are listed in Table I. Average 24-hour IL-6 levels ranged 224% between subjects, from 1.66 ± 0.29 to 5.37 ± 1.08 pg/ml (mean $\pm$ SE). Considerable within-day variation from lowest to highest IL-6 values was observed in each subject, ranging from 1.20 pg/ml to more than 7 pg/ml. As percentages, the average within-day ROC between lowest and highest values was $276\% \pm 32\%$ (range, 120% to 531%). The average peak IL-6 level was 4.62 pg/ml (range, 2.20 to 10.18 pg/ml), whereas the average lowest value was 1.29 pg/ml (range, 0.50 to 2.60

pg/ml). Acrophases objectively approximating times of individual peak values all occurred during nighttime hours after midnight (from 00:12 to 05:36 hours), including acrophases for the four subject study coordinators with intermittent sleep (subjects 1, 9, 10, and 11) (Table I).

Overall timepoint means $\pm$ SE are listed in original units (picograms per milliliter) and as a percentage of the mean at the bottom of Table I and are shown graphically with the best-fitting 24-hour cosines in Fig. 1. Results of statistical evaluation for circadian time effects are presented in Table II. Average IL-6 levels were greater than the daily mean at 22:00, 01:00, and 04:00 hours, with the overall time point average being maximal at 01:00 hours. Average IL-6 levels were near the daily mean at 07:00 hours and less than the daily mean at 10:00, 13:00, 16:00, and 19:00 hours, with the overall minimum at 10:00 hours. A significant time effect was found by analysis of variance, when original units were used ($F = 4.1$, $p < 0.001$) or when data were normalized to a percentage of the mean ($F = 15.0$, $p < 0.001$). A high-amplitude circadian rhythm was also detected at a p level less than 0.001 when a 24-hour cosine was fit to the data with both original units and the percentage of the mean (Table II). With data expressed as a percentage of the mean, the

TABLE II. Statistical evaluation for circadian time effect and rhythm for circulating IL-6 in healthy men

Units	For time effect: ANOVA		Least-squares fit of 24-hr cosine			Acrophase (95% confidence limits)
	F	p	p	MESOR $\pm$ SE	Amplitude $\pm$ SE	
pg/ml	4.1	<0.001	<0.001	2.70 $\pm$ 0.17	1.17 $\pm$ 0.24	01:52 (00:24-03:24)
% of mean	15.0	<0.001	<0.001	100.0 $\pm$ 3.5	40.9 $\pm$ 4.9	02:16 (01:24-03:12)

MESOR is the rhythm-adjusted mean; acrophase expressed in hours/minutes (local midnight, 00:00 hours). ANOVA, Analysis of variance across eight time points at 3-hour intervals.

peak of the best-fitting cosine shown in Fig. 1 was located at 02:16 hours with 95% confidence limits from 01:24 to 03:12 hours and the amplitude was $41\% \pm 5\%$.

In addition, overall IL-6 levels showed a positive correlation with age ($r = 0.63$, $p = 0.037$) within the 27-year age range available (46 to 72 years).

DISCUSSION

Recent advances in peptide research have provided an abundance of information about the roles of cytokines in crossregulation of various immune effector functions. The regulation of expression of cytokines has also received considerable attention with reference to mechanisms of cell activation or inhibition of cellular activity. The various immune effector cell types produce a select profile of cytokines in response to stimuli, and thus the levels of these cytokines can be reflective of the immune functional status. Despite a clear association between the neuroendocrine and immune systems and the established circadian rhythmicity of the neuroendocrine system, the possibility that the immune system also fluctuates rhythmically has not been thoroughly studied.¹⁷ Circadian rhythms in circulating T-lymphocyte subtypes have been described, some with a single 24-hour peak (total cells, CD3, CD4, and CD4/CD8 ratio) and some with two peaks (CD8).¹⁸ Also, a circadian rhythm in human bone marrow total proliferating cells (DNA synthesis) and in myeloid progenitor cells (cells having the capacity to give rise to colonies composed of granulocytes and macrophages in soft agar) has been reported,¹² with highest values during daytime hours between 08:00 and 16:00 hours, and lowest values at midnight. Thus the circadian rhythmicity in hematopoiesis may also be expected to influence circadian observations in circulating cytokines. In addition, endogenous serum corticosteroids exhibit a predictable circadian pattern, generally with highest levels near awakening. Corticosteroids are known to influence lymphocyte distribution, modulate immune cell functional activity, influence the production of some cytokines, and alter the expres-

sion of cytokine receptors.¹⁹ Some cytokines, such as IL-1, have been shown to modulate adrenocorticoid levels, thereby creating an immunoregulatory feedback system. Circadian variations in serum cortisol levels may therefore be one mechanism by which circadian-dependent variations in the immune network are coordinated.

We report that circulating IL-6, an indicator of immune effector and regulatory cells, demonstrates a high-amplitude circadian rhythm in healthy diurnally active persons, with highest values during sleep. On an individual basis, the ROC from lowest to highest values during the 24-hours of study ranged from 120% to 531%. On average, values were lowest during the day and 276% higher during sleep, indicating that time-specified reference intervals,²⁰ so-called chronodesms, need to be constructed for the interpretation of single IL-6 samples. In conventional terms the same value may be too high (false positive), normal, or too low (false negative), depending on the circadian stage of sampling a rhythmic variable, especially if the circadian amplitude is high, as is the case for IL-6.

As part of a separate report,²¹ we have also reported in these same persons the presence of circadian patterns in four additional cytokines: IL-2, IL-10, tumor necrosis factor- α and granulocyte-macrophage colony-stimulating factor. Dramatic temporal differences were observed, with daytime peaks in these cytokines as compared with the nighttime peak for IL-6. IL-2 showed a single peak near midday (12:48 hours), whereas two daily peaks were observed in the remaining cytokines, with major peaks (acrophases) at 08:28 hours for IL-10, 14:00 hours for tumor necrosis factor- α , and at 19:28 hours for granulocyte-macrophage colony-stimulating factor. Other studies have reported circadian variations in cytokines, some with nighttime peaks. Normal sleep was associated with physiologic nocturnal rises in IL-1 β ^{22, 23} and IL-6²³ secretion, whereas the capability of cultured blood cells to produce IL-1 β and IFN- γ on endotoxin challenge was reported to be maximal around the time sleep began or during the

first hours of sleep, and minimal in the morning.²⁴ Erythropoietin has also been observed to peak near midnight in normal subjects¹⁹ and in patients with chronic lung disease²⁵ or with obstructive sleep apnea syndrome.²⁶ Another study found pulsatile patterns of IL-1 α and IL-2 levels with no clear daily pattern but concluded that further study was warranted because a single-point or integrated analysis for 1 hour would not allow for an accurate assessment of cytokine levels.²⁷

Studies of cytokine receptors can provide important clues to the overall functional relationships between particular cytokines involved in the immune response. Cytokine receptors, however, may also display circadian characteristics, as has been shown for soluble IL-2 receptor in serum of healthy adults^{28,29} and of patients with active rheumatoid arthritis.³⁰ Highest IL-2 receptor concentrations were found in the early afternoon and lowest values around 04:00 hours in both health and disease, paralleling the circadian pattern we observed in circulating IL-2 levels in our normal subjects.²⁰

The prominent fluctuations observed during 24 hours in circulating cytokine levels may reflect underlying endogenous circadian fluctuations in various immune effector cells and as such may be of importance both experimentally and clinically. If the circadian pattern varies systematically between different cytokines, with some peaking by night and others throughout the day, this may imply a temporal sequencing of function indicating a systematic regulation of different immune effector cell activities throughout the day. Such variations can clearly contribute to heterogeneity in clinical studies and suggest that such variability might be reduced if studies were performed at specified circadian stages. Proper timing of single samples may also improve the usefulness of circulating cytokines as clinically useful markers, as has been suggested for IL-6.³¹ The results presented here are meant to serve as chronobiologically defined reference values for the circadian rhythm in serum IL-6 in diurnally active, clinically healthy, men without atopic disease. Such reference values need to be established, under similar investigative protocols for all age groups, not only of men but also of women (where consideration of stage of menstrual cycle is critical) and possibly in different seasons, to make informed comparisons with circadian variations in disease (e.g., asthma, arthritis, cancer, and human immunodeficiency virus). For example, even though asymptomatic, our subject with a history of mild asthma (No. 5) had apparently normal circadian characteristics for mean level (1.67 ± 0.16 pg/dl) and acrophase (04:20 hours), but showed the smallest

ROC between lowest and highest IL-6 values for 24 hours of any subject (1.20 pg/dl [120%]).

Of clinical significance is the question of whether the observed large day-night variations in endogenous cytokine production and bioactivity of IL-6 (and other cytokines) might influence clinical decisions and the effectiveness of immunotherapies such as those being evaluated for the treatment of patients with cancer,³²⁻³⁴ transplant recipients,³⁵ and persons with rheumatoid arthritis,⁶ among others. In preclinical and clinical studies proper timing (chronotherapy) of anticancer medication(s) has been shown to increase the maximum tolerated dose up to 300%, increase tumor control and/or cure, and decrease undesired toxicity.^{36,37} Similar studies recognizing that immune cytokines exhibit circadian periodicity are needed to consider the possible significance, both experimentally and clinically, of use of immune rhythmometry to assist in achieving a reliable therapeutic effect. Because of their small size, low effective concentration, and short half-life (minutes to hours), cytokine activities are difficult to evaluate *in vivo*. Because most cytokines act locally rather than systemically and influence multiple cellular functions, systemic administration of cytokines as therapy often leads to undesirable side effects. Circadian stage (time of day) of exogenous cytokine administration may play a role in treatment outcome. Indeed, preliminary studies of circadian-patterned immunotherapy have yielded significant effects on the magnitude of hematopoietic, immunologic, antitumor, and toxic responses.¹⁹ For example, in a phase I trial, IFN- α was administered to patients with cancer as a 21-day continuous venous infusion, with a daily peak dose between 18:00 and 22:00 hours.³⁸ This circadian schedule of infusion allowed a large increment in total daily dose and dose intensity, and increased stabilization of previously progressive disease. It is thus imperative to develop a more complete understanding of the very complex cytokine temporal sequencing and the circadian-dependent dichotomous effects (stimulation or inhibition) of some of these peptides, possibly to increase diagnostic capabilities and to design optimally timed therapeutic regimens with lower toxicity and higher efficacy in patients, thereby maximizing the true clinical utility of each of the various cytokines.

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