Cyclic Changes in the Concentrations of Peripheral Blood Immune Cells During the Normal Menstrual Cycle (43795)

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Abstract. **The optimal availability of immune cells in the peripheral blood streams of women may play a critical role in their response to disease and therapeutic interventions. This study was designed to examine concentrations of circulating white blood cells (WBC) including lymphocyte subsets, during the 24-hr daily and 28-day menstrual cycles. Venous blood (20 ml) from five healthy young women was obtained at 0, 6, 12, and 18 hr on the 6th and 22nd day of the normal menstrual cycle. Cortisol, progesterone (P4), estradiol (E2), total WBC, granulocyte, monocyte, and total lymphocyte levels were measured. Using fluorescent activated cell scanning, levels of T, B, Helper (H), Suppressor (S) and Natural Killer (NK) cells were also determined. Significant differences in the diurnal and Day 6 and 22 means were identified using analysis of variance and the Student's *t* test.**

Mean WBC counts differed significantly between individuals and ranged from 3.63 ± 0.33 to 8.60 ± 1.00 on Day 6 and 3.75 ± 0.56 to 9.45 ± 0.98 on Day 22 ($P < 0.05$). Fluctuations in the concentrations of peripheral blood immune cells followed a similar pattern for the time points selected. They were lowest in the morning at 6 hr and reached peak concentrations in the evening at 17 hr or at midnight at 24:00 hr. Total WBC and granulocyte levels were consistently highest in the evening at 18 hr while lymphocyte levels either peaked in the evening or at midnight. Between midnight and early morning, levels of WBC, lymphocytes, T, B, H, and S cells all decreased significantly ($P < 0.01$) and subsequently increased significantly, between 6 hr and noon or noon and 18 hr ($P < 0.01$). When compared every 12 hr, the levels of WBC, granulocytes, lymphocytes, T, B, H, and S cells showed a significant day time rise between 6 and 18 hr ($P < 0.02$). NK cells revealed no significant fluctuations for any of the diurnal time point comparisons examined in this study.

The WBC means for all subjects on Day 6 was 6.15 ± 1.96 and on Day 22 was 6.39 ± 2.14, evidence that the total number of white blood cells was not significantly altered between the 2 days. However when comparing specific time points during the day for the follicular (Day 6) and luteal (Day 22) phases of the menstrual cycle, significant differences were found. Most striking were the monocyte patterns, which revealed a nadir at 12:00 noon on Day 6 and a peak at the same time on Day 22. WBC, and granulocyte levels were significantly higher at 12:00 noon on Day 22 ($P < 0.05$) and NK cells significantly lower on Day 6 at 18 hr ($P < 0.01$). Thus, within this population of normal menstruating women, quantitative and qualitative differences in the circadian and circalunar levels of peripheral blood immune cells do exist.

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Growing evidence identifies both cyclic and rhythmic variation in the concentrations and functions of immune cells (1–5). Circadian rhythms have been demonstrated for total circulating white blood cells, lymphocytes (T and B cells), and thymosin in humans and mice (1, 2, 6). Immune functions such as those involved in kidney allograft rejection (7, 8) inflammation (9, 10) and antibody responses to sheep erythrocytes in mice (11) as well as sympa-

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thetic nerve activity (12) have demonstrated characteristic periodicities.

Periodic changes in the levels of circulating immune cells have also been linked to the daily and monthly patterns of endogenous hormones (13–17). The Abo and Kawate murine studies demonstrated an inverse relationship between the daily fluctuations in levels of cortisol and the numbers of lymphocytes (2). Murine lymphocyte levels peaked when cortisol concentrations dropped during the hours of light or the murine sleep cycle. A corresponding shift in cell counts was observed when artificial light was used to reverse the normal light and dark cycles (2). Peripheral blood lymphocyte levels in humans are reported to be opposite those of the murine levels with peak levels occurring during the dark hours, corresponding to periods of low plasma cortisol and sleep (17).

Both clinical and experimental evidence support the hypothesis that gonadal steroids regulate immune function (18–22). Receptors for steroid hormones, as demonstrated by cytosolic and nuclear assays, have been found in circulating lymphocytes (23–25) and steroid hormones are known to induce immunological events such as altering immunoglobulin (IgM) secretion and depression of suppressor T-cell activity (13). However, few studies to date have examined the daily and monthly concentrations and patterns of immune cells in circulation, including specific lymphocytes subsets, within the normal menstrual cycle (26).

Interaction between the reproductive and immune systems plays an important and immunoregulatory role. Recent clinical studies for example show that survival rates for women with operable breast cancer varied based on the time of the menstrual cycle at operation (21, 22). Overall and recurrence-free survival was greatly reduced in women whose surgery took place 3–12 days after menses onset as compared in those operated on from Day 0–2 or day 13–32.

Other studies have identified the importance of circadian rhythms in the administration of cancer chemotherapy (27–29). Hrushesky and von Roemeling tested if circadian timing of the drug floxuridine (FUDR) was important for its toxicity and antitumor activity in female rats. Circadian differences in both drug tolerance and tumor growth reduction were found. The best tolerated and most effective infusion time occurred during the circadian stage of late activity-early rest or between 9:00 PM and 3:00 AM (28) for therapeutic dose levels. Lethal toxicity also differed significantly depending upon the circadian stage of maximum drug delivery.

The present study was designed to examine both the circadian and circalunar levels of white blood cells with specific lymphocyte subsets, during the follicular and luteal phases of the 28-day menstrual cycle in normal women. Also explored were the variations in white blood cell numbers in relationship to plasma estradiol and progesterone levels.

Materials and Methods

Subjects. Five female subjects aged 24 to 38 years in good health and not taking medications volunteered for this study. Each had a history of regular and normal menstrual cycles and documented the time of ovulation by charting basal body temperatures 1 month prior to, and during the month of, sampling. Each followed her normal daily activities during the course of the study and gave informed, witnessed consent.

Sample Acquisition and Handling. Blood (20 ml) was drawn every 6 hr starting at 0, 6, 12, and 18 hr on Day 6 and 22 of the menstrual cycle. Day 6 was 6 days after the beginning of menses and Day 22 was luteal as determined by the rise in basal body temperature. Serum was separated within 1 hr of sampling time and frozen at −80°C until analysis for hormone levels.

Hormone Analysis. Levels of cortisol, progesterone and estradiol were quantified by radioimmunoassay Coat-A-Count (Diagnostic Products Co., Los Angeles, CA). Hormonal analyses were performed as a single run on all specimens.

Hemogram. The total WBC count (cells per cubic millimeter of blood) was determined by automatic analyses (Coulter, Hialeah, FL) within 1 hr of sampling time. The total number of circulating granulocytes, monocytes, and lymphocytes per cubic millimeter was also calculated at the same time using the automatic analyzer.

Lymphocyte Subset Surface Marker Determination. Within 1 hr of collection of each sample, blood was prepared for fluorescent activated cell scanning (FACScan; Becton Dickinson, Mountain View, CA). Fifty microliters of whole blood was lysed using FACS Lysing Reagent (Becton Dickinson, Mountain View, CA) then centrifuged to remove red blood cells. The remaining blood cells (WBC) were bound to the appropriate fluorochrome conjugated antibodies (Ab) and fixed in a 1% solution of glutaraldehyde until sorted. Isotype controls were used for each surface marker in order to insure a negative control for each subset. Table I lists the specific lymphocyte subsets examined, with the corresponding antigen clusters, antibodies, fluorochrome markers, and isotype controls.

Statistical Analysis. Diurnal data and menstrual phase data were analyzed for significant differences using the analysis of variance (ANOVA) for repeated measures and the Student's *t* test for paired observations. Cosinor analysis was not used in this study due to the minimum number of diurnal data points avail-

Table I. Lymphocytes Examined with Fluorochrome Markers

Immune cells	Antigen cluster	Antibody to antigens	Fluorochrome markers	Isotype controls
Pan T[a]	CD 2	L 5	RD 1[b]	IgG1-RD 1
Pan B[a]	CD 19	L 16, L 12	FITC[c]	IgG1-FITC
Helper[a]	CD ± 4	CD ± 4	RD 1	IgG1-RD 1
Suppressor[a]	CD ± 8	CD ± 8	FITC	IgG1-FITC
Natural Killer	NKH-1		FITC	IgG1-FITC

[a] Dual straining procedures used.
[b] RD 1 = Phycoerythrin.
[c] FITC = Fluorescein.

able. Results of these tests are given with their 95% confidence levels with ANOVA values significant at $F > 2.3$ and the Student's t test at $t \geq 2.776$ and $P < 0.05$.

Results

Table II documents, by subject, the Day 6 and Day 22 means and standard deviations (SD) for every cell type. Significant interindividual differences were found with mean levels of total WBC ranging from 3.63 ± 0.33 to 8.60 ± 1.00 on Day 6 and 3.75 ± 0.56 to 9.45 ± 0.98 on Day 22 ($P < 0.05$). The group means of WBC, monocytes, granulocytes, lymphocytes, T cells, B cells, helper cells, suppressor cells, the H/S ratio, and NK cells were not significantly different between the 2 days.

The mean basal body temperatures and hormone profiles for P4 and E2 for all subjects were significantly different on Day 6 and Day 22. Estrogen levels were 52.79 ± 21.72 and 135.38 ± 53.99 ($P < 0.001$) during the follicular and luteal phases respectively. Progesterone values on Day 6 and 22 were 0.36 ± 0.22 and 12.57 ± 6.89 ($P < 0.001$) and basal body temperatures 97.79 ± 0.68 and 98.65 ± 0.64 ($P < 0.001$). The

Table II. Means ± SD[a] of WBC ($\times 10^3$ Cells/μl) by Subject on Day 6 and 22 of the Menstrual Cycle

Subjects:	1	2	3	4	5	Group[b]
WBC						
Day 6	620 ± 1.08	8.60 ± 1.00	3.63 ± 0.33	4.68 ± 0.56	6.30 ± 1.04	6.15 ± 1.96
22	6.80 ± 0.67	9.45 ± 0.98	3.75 ± 0.56	5.40 ± 0.32	7.10 ± 0.73	6.39 ± 2.14
Granulocytes						
Day 6	3.06 ± 0.62	5.20 ± 0.93	2.10 ± 0.18	2.96 ± 0.58	2.98 ± 0.58	3.29 ± 1.57
22	3.63 ± 0.56	5.78 ± 0.59	2.33 ± 0.46	3.93 ± 0.85	3.93 ± 0.85	3.83 ± 1.24
Monocytes						
Day 6	0.28 ± 0.05	0.43 ± 0.05	0.18 ± 0.05	0.20 ± 0.08	0.30 ± 0.16	0.28 ± 0.12
22	0.23 ± 0.10	0.60 ± 0.08	0.20 ± 0.00	0.25 ± 0.06	0.28 ± 0.05	0.33 ± 0.16
Lymphocytes						
Day 6	2.86 ± 0.66	2.98 ± 0.57	1.35 ± 0.17	2.35 ± 0.39	3.05 ± 0.52	2.58 ± 0.85
22	2.98 ± 0.54	3.13 ± 0.46	1.25 ± 0.25	2.45 ± 0.31	2.90 ± 0.50	2.63 ± 0.83
T-Cells						
Day 6	2.48 ± 0.56	2.27 ± 0.40	1.06 ± 0.12	1.70 ± 0.13	2.35 ± 0.16	2.13 ± 0.76
22	2.51 ± 0.48	2.45 ± 0.32	1.10 ± 0.19	1.73 ± 0.35	2.46 ± 0.39	2.13 ± 0.72
B-Cells						
Day 6	0.26 ± 0.08	0.57 ± 0.15	0.23 ± 0.10	0.21 ± 0.08	0.25 ± 0.06	0.29 ± 0.15
22	0.27 ± 0.05	0.58 ± 0.19	0.21 ± 0.06	0.23 ± 0.05	0.28 ± 0.05	0.30 ± 0.16
H-Cells						
Day 6	1.38 ± 0.36	1.00 ± 0.25	0.47 ± 0.06	0.85 ± 0.10	1.23 ± 0.09	1.07 ± 0.44
22	1.34 ± 0.37	1.20 ± 0.17	0.42 ± 0.08	0.86 ± 0.24	1.30 ± 0.26	1.07 ± 0.42
S-Cells						
Day 6	0.68 ± 0.14	0.58 ± 0.09	0.24 ± 0.05	0.31 ± 0.06	0.55 ± 0.05	0.54 ± 0.23
22	0.66 ± 0.14	0.60 ± 0.05	0.23 ± 0.05	0.40 ± 0.06	0.56 ± 0.12	0.55 ± 0.23
NK Cells						
Day 6	0.24 ± 0.04	0.29 ± 0.01	nd[c]	0.16 ± 0.03	0.31 ± 0.04	0.24 ± 0.11
22	0.21 ± 0.09	0.22 ± 0.03	nd[c]	0.18 ± 0.02	0.26 ± 0.03	0.22 ± 0.09[c]

[a] SD: Standard Deviation from the daily means of four blood draws at 6, 12, 18 and 0 hours on day 6 and day 22.
[b] Group means for all subjects.
[c] nd: no data available.

biochemical data confirm the achievement of the targeted sampling days during the follicular and luteal phases of the normal ovulatory menstrual cycle. Mean cortisol levels did not change significantly when Day 6 and Day 22 levels were compared ($P = 0.97$). The daily mean cortisol levels for all subjects on Day 6 was 9.02 ± 6.27 and 9.16 ± 2.72 on Day 22.

Diurnal variations were found for a number of hormones and cell types. As expected, cortisol levels showed significant elevations at 6 hr and significant declines by 12 hr on both Day 6 and 22. A further significant decline at 18 hr on Day 22 was also revealed. Progesterone levels did not change significantly on Day 6 but showed a significant rise in levels on Day 22 from 0 to 6 hr. Estradiol levels also remained unchanged during the 24-hr time period on Day 6, but showed a significant decline in levels between 12 hr to 18 hr on Day 22. Table III documents the *P* and *F* values where levels of white blood cells were found to vary significantly on pooled data. Both tests yielded consistent results, with the greatest number of cell types differing between midnight and morning and between morning and evening. Significant variations were apparent for all variables except NK cells and monocytes, these showed no significant differences for the time points selected in this study.

As seen in Figure 1, WBC, granulocytes, lymphocytes, T cells, B cells, helper cells, and suppressor cells all reveal a nadir at 6 hr. WBC count levels significantly increase between 6 hr and 12 hr and peak at 18 hr then decline overnight to a nadir at 6 hr. Total lymphocytes differ significantly between 12 and 18 hr, peak at 24:00 hr then decline to the nadir at 6 hr. The observed patterns for the four time points for WBC, granulocyte, and total lymphocytes, as well as for lymphocyte subsets, did not appear different between Day 6 and Day 22 of the menstrual cycle and are remarkably similar (Fig. 1). However, monocyte patterns for the 2 days, at 0, 6, 12, and 18 hr, do show different periodicities (Fig. 2). The nadir for monocyte levels is at 12:00 noon on Day 6 and at 24:00 hr, or midnight, on Day 22. At each time point, when Day 6 and 22 levels are compared, the direction of change is opposite, with minimum levels occurring at 12 hr on Day 6 and maximum levels occurring at 12 hr on Day 22.

Quantitative differences were found in cell counts at specific time points during the normal menstrual cycle (Fig. 3). WBC were significantly higher on Day

Table III. Significant Diurnal Differences in the Levels of WBC and Subsets[a]

	ANOVA TEST: *F* Values[b]					
Comparisons:	Every 6 hours			Every 12 hours		
	0 vs. 6 hr	6 vs. 12 hr	12 vs. 18 hr	18 vs. 0 hr	0 vs. 12 hr	6 vs. 18 hr
Variables:						
Total WBC	3.89	3.20	—	—	—	10.74
Granulocytes	—	3.37	—	—	3.50	5.16
Monocytes	—	—	—	—	—	—
Lymphocytes	2.26	—	4.47	—	6.95	8.87
T-Cells	11.00	6.43	—	—	—	10.13
B-Cells	5.82	—	—	—	2.23	3.56
H-Cells	12.03	—	4.52	—	7.01	8.69
S-Cells	10.36	—	8.88	—	1.92	7.54
NK Cells	—	—	—	—	—	—
	Student's *t* test: *p* values[b]					
Comparisons:	Every 6 hours			Every 12 hours		
	0 vs. 6 hr	6 vs. 12 hr	12 vs. 18 hr	18 vs. 0 hr	0 vs. 12 hr	6 vs. 18 hr
Variables:						
Total WBC	0.006	0.009	0.014	—	—	0.000
Granulocytes	—	0.012	—	0.010	0.039	0.001
Monocytes	—	—	—	—	—	—
Lymphocytes	0.000	—	0.000	—	0.002	0.001
T-Cells	0.000	0.000	—	—	—	0.001
B-Cells	0.000	—	—	—	0.037	0.019
H-Cells	0.000	—	0.005	—	0.000	0.003
S-Cells	0.000	—	0.000	—	0.001	0.001
NK Cells	—	—	—	—	—	—

[a] Levels were compared every 8 and 12 hr.
[b] Only *p* and *F* values which demonstrate significant differences between time points are indicated.
ANOVA ($F > 2.30$) and Student's *t*-tests (*t* values ≥ 2.776, $p < 0.05$).
Nonsignificant *p* and *F* values are indicated by a dotted line.

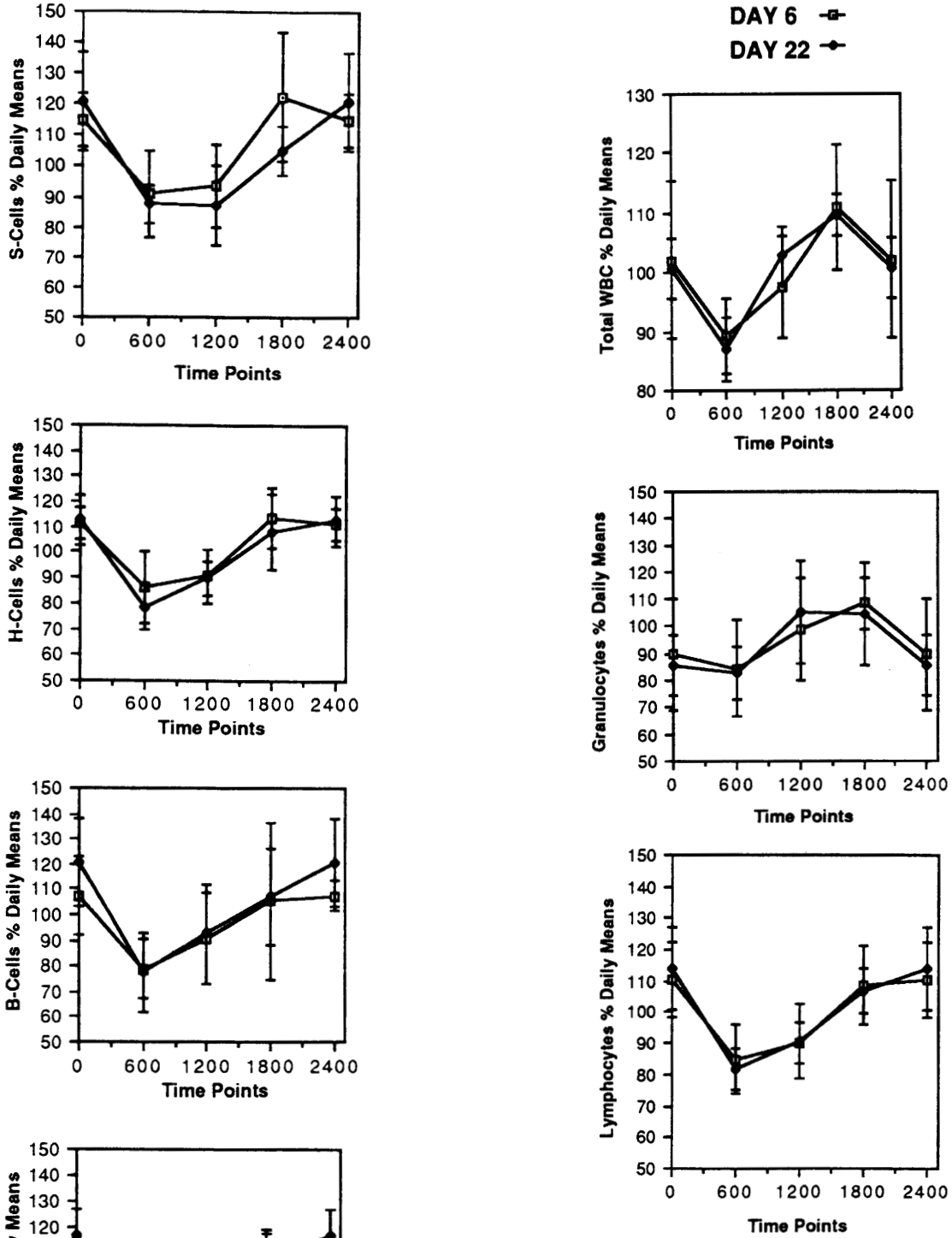

Figure 1. Diurnal variations for total WBC, granulocytes, lymphocytes, T and B cells, Helper and Suppressor cells, and NK cells on Day 6 (open symbols) and 22 (closed symbols) of the normal menstrual cycle at 0, 6, 12, and 18 hr. The percent of daily means for all five subjects is represented at each time point. Significant differences between each time point are indicated on Table III. The error bars represent standard deviations.

22 at 12 hr ($P < 0.01$). B-cell counts were significantly higher on Day 22 at 24:00 hr ($P = 0.01$). NK cell counts were unique in being significantly higher on Day 6 of the menstrual cycle at 18 hr ($P = 0.01$).

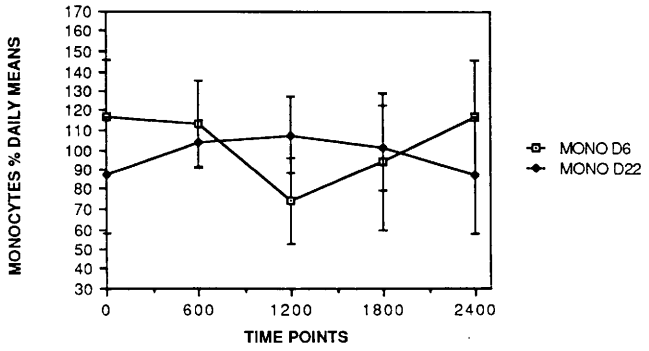

Figure 2. Periodicities for circulating monocytes on Day 6 (open squares) and Day 22 (closed circles) at 0, 6, 12, and 18 hr of the normal menstrual cycle. Error bars indicate standard deviation.

Discussion

Our study was conducted to examine circulating white blood cell profiles in normal menstruating women. The design and methods begin to address the need for analyzing the temporal dimensions of immune cell variation in women. With a limited number of subjects and sampling frequencies, significant differences in the daily concentrations and patterns of white blood cells were found. The times selected (0, 6, 12, and 18 hr) were chosen because of the known hormonal changes occurring during the light/dark cycle. The days selected (6 and 22) reflect significant shifts in the hormone profiles for the 28-day monthly cycle. Within these daily and monthly time frames, white blood cell counts were found to significantly differ.

In general, fluctuations in the daily concentrations of white blood cells followed a similar pattern. They were lowest in the morning at 6 hr and then peaked at 18 hr or 24:00 hr. Total WBC counts peaked at 18 hr on both days while lymphocyte subsets peaked at 18 hr on Day 6 and at 0 hr on Day 22. Granulocyte levels peaked at 12:00 noon or 18 hr, and then declined overnight to a nadir at 6 hr. Total WBC, granulocytes, total lymphocytes, T, B, H, and S cell levels all declined between 0 and 6 hr, but differed in their patterns of increase, as determined by the four time points. To define the precise variations and to examine their overall significance, further studies with additional time points will be needed.

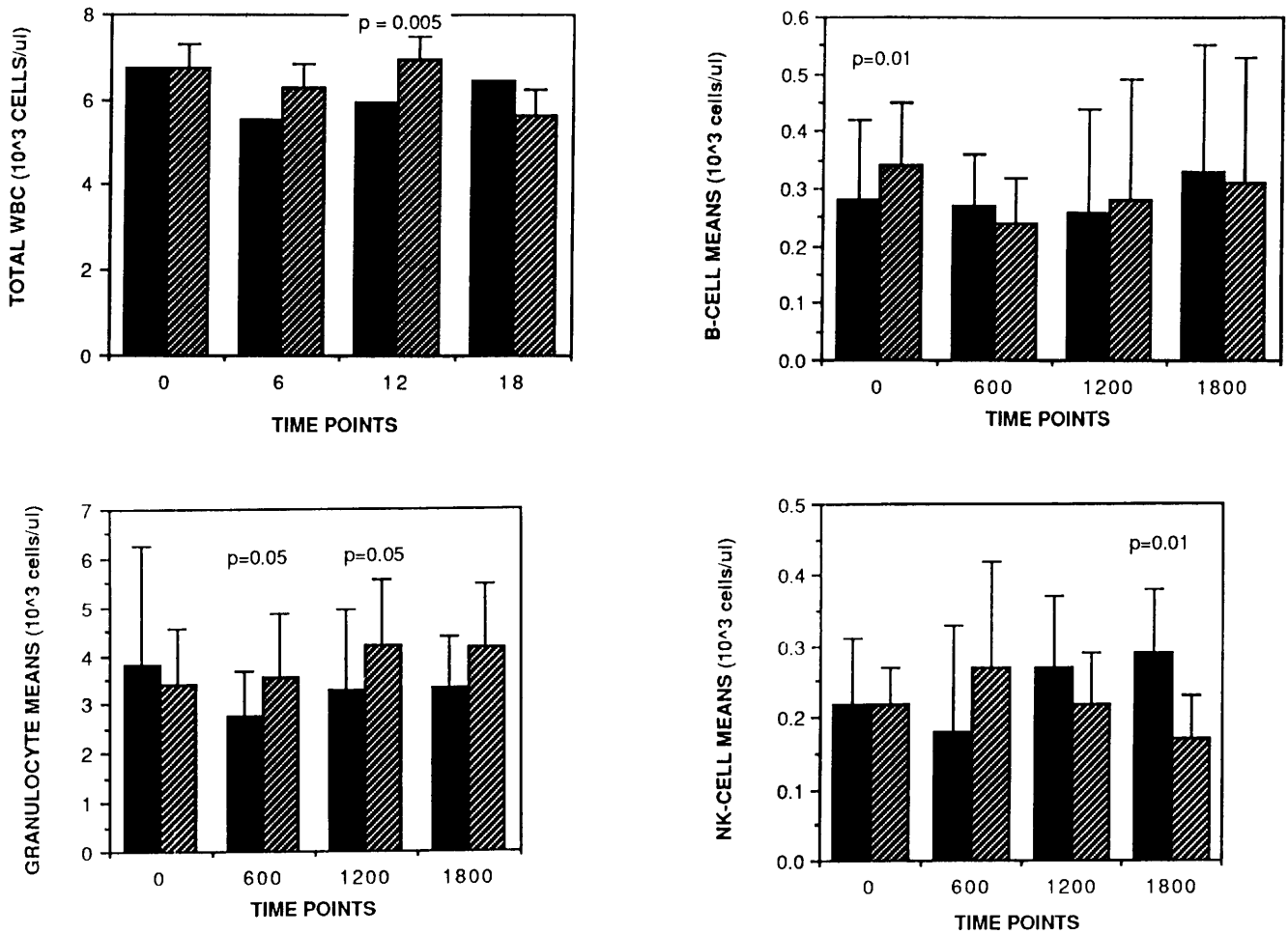

Figure 3. Total WBC, granulocyte, B-cell, and NK-cell counts at each time point studied on Day 6 (closed bars) and Day 22 (hatched bars) of the menstrual cycle. Significant differences in Day 6 and Day 22 concentrations at diurnal time points are indicated by *P* values. Error bars indicate standard deviation.

Changes in the levels of steroid hormones are characteristic of the normal monthly menstrual cycle. During the follicular phase, before ovulation, estrogen peaks subsequent to a surge of luteinizing hormone (LH) and in the absence of progesterone (28). A second estrogen surge occurs later in the cycle during the luteal phase coinciding with the secretion of progesterone (28). Previous results have demonstrated that significant differences in the levels of circulating lymphocytes were found between Day 6 and Day 14 when estradiol levels surge in response to LH (20).

In this study, we examined Day 22 of the normal menstrual cycle, when the progesterone-estrogen level is elevated, and found no significant difference in the daily mean white blood cell levels when Day 6 and Day 22 were compared. While the daily mean concentrations of circulating immune cells did not differ, we did find qualitative and quantitative differences between Day 6 and Day 22. Most striking were the differences observed in monocyte patterns. The diurnal patterns for other cell types on Day 6 and 22 were similar, however monocyte patterns differed. At noon, there is a minimum on Day 6 and a maximum on Day 22. The nadir during the follicular phase is at 12:00 noon and the nadir during the luteal phase is at midnight, consistent with different periodicities. Other significant differences reveal that levels of WBC, granulocytes, and B lymphocytes were higher at specific time points on Day 22. Granulocyte levels differed at two time points, while the other variables differed at a single time point. Whether the differences observed are influenced by fluctuations in the diurnal and monthly hormone levels needs further examination.

These results identify significant fluctuations in the numbers and patterns of circulating immune cells in women during the normal menstrual cycle. Such evidence points to the need for determining the role that variations in immune cell levels may play in women's immune response. Whether the elevated levels of progesterone-estradiol, WBC, granulocytes, and B cells constitute a "window of opportunity" for cancer or for immunotherapy remains to be determined. The potential value of discovering optimal monthly and daily times for medical interventions is compelling.

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