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Lymphocyte subpopulations and catecholamines; daytime variations and relationships

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The diurnal variations and their possible relations with endogenous catecholamines have been studied in 16 apparently healthy subjects (8 women and 8 men, between 22 - 35 years of age). A butterfly cannula was inserted into the flexure vein of the elbow at 8 h and at 20 h, a blood sample being taken after three 15-minute intervals. Leukocytes, lymphocytes, monocytes, and granulocytes were quantified in a cell counter while lymphocyte subpopulations were determined with flow cytometry in cells labelled with specific monoclonal antibodies. Plasma catecholamine concentrations were measured by high-pressure liquid chromatography. Significant differences for leukocyte circulating levels were found, as well as for all subpopulations measured at different times of day, save NK cells and monocytes. A statistically significant correlation was also found both between leukocytes, all subpopulations and epinephrine save B lymphocytes and NK cells, and between total lymphocytes, T lymphocytes, CD4, CD4/45RA$^+$ subtypes and norepinephrine. In conclusion, diurnal rhythms were proved to exist in leukocyte and lymphocyte subpopulations; the significant correlation between these cells and catecholamine plasma levels suggests the presence of a possible mechanism that connects the cellular immunity with determined hormones.

Key words: Norepinephrine, Epinephrine, Leukocytes, Lymphocyte subpopulations, Circadian rhythms.

The growing knowledge of the immune system regulation has demonstrated that there is a close relationship between this and the neuroendocrine axis. Lymphocytes are able to express the proopiomelanocortina gene and to secrete peptides derived from this structure (8). Receptors for catecholamines, CRF, ACTH and

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β-endorphine in immunocytes have been described (2).

In the last years numerous studies on the circadian variations of the circulation rate of leucocytes have been carried out, Although their existence was known for some time (4). The monoclonal antibody development has made possible the determination of the circadian rhythms in the different lymphocytarian subpopulations (1, 9, 16).

Since these rhythms are the contrary of the corticosteroids and other axis hormones, these have been considered for years as the sole modular effect mediators of the neuroendocrine axis on the glucocorticoids immune system. However, as this modulation has been proved to occur in adrenalectomized rats, other factors must also be involved (7). Thus, the lymphoid organs are extensively innervated by the autonomous noradrenergic nerves, the activation of which has an important influence on the migration of the blood lymphocytes to the tissues (3, 5).

The discrepancies of different authors related to the diurnal variations of the lymphocytal subpopulations and the modulation which plasma catecholamine levels could have on these, led to the present study, the aim of which is to establish the daytime variations and relations between the different leukocyte subtypes and the mentioned hormones.

Materials and Methods

Subjects in study.– Eight 8 women and 8 men, ages 22 to 35, were selected. None of them presented any apparent disease or was undergoing treatment, their jobs or social situations not presenting a high anxiety level. Each one of them was given a previous medical examination which included a physical examination, biochemical parameter quantification in blood and anxiety level determination through the questionnaire State-Trait Anxiety Inventory (18). In no case is acute or chronic pathology observed, their anxiety levels being within normal values for the Spanish population. The subjects were previously familiarized with the place where the test was carried out to minimize the possible anxiety caused by it. With the women, the test was carried out during the first phase of the menstrual cycle. Morning samples were taken after the overnight fast of 12-hours and after 6 hours fasting for evening samples.

To consider the possible interference caused by hormone ultradian fluctuation, three samples of venous blood were obtained by using a forearm catheter at 15-minute intervals from 9 a.m. and from 8 p.m. A butterfly cannula was inserted into the flexure vein of the elbow of the seated subject; the cannula was connected to a drip system (physiological saline solution Grifols Inc.), ensuring a permeable pathway. After a 15-minute interval, allowing subjects to overcome the injection stress, a first blood sample was taken. Two further samples were taken at the subsequent 15-minute intervals. The first 5 ml of blood were rejected from each sample.

For each sample, 12 ml blood were collected in standard EDTA-containing tubes (Terumo Europe, Leuven, Belgium) and were processed for an immediate haemogram determination and subsequent hormonal and lymphocyte subpopulation analysis.

Hormonal determination.– Plasma concentrations of catecholamines, norepinephrine (NE) and epinephrine (E), were measured by high-pressure liquid chromatography. A methanol and phosphate mixture tampon (ESA Co., Inc., Bedford, M, USA) has been employed as mobile phase. A NE and E mixture, was used as

external standard, while dihydroxibenzilamine (DHBA) was the internal standard (ESA Plasma Catecholamine Analysis System).

Hemogram and lymphocyte subpopulations.– Leukocytes and the three subtypes neutrophils, monocytes and total lymphocytes, have been quantified by an automatic cell counter Sysmex K-1000 (TOA Medical Electronics Co., Kobe, Japan). Lymphocyte subpopulations were determined with flow cytometer Facsort from Becton Dickinson, in cells labelled with specific monoclonal antibodies. The defined cells in which these antigens are expressed are shown in table I.

Quantification on mononuclear cells isolated in Hypaque-Ficoll has been accomplished. After incubating 50 µl cells suspension with 5 µl antibody during 30 minutes at 4 °C, the cells were washed twice in 0.15 M, pH 7.2 phosphate tampon and fixed in 1 % formaldehyde. A minimum of 5000 cells. have been measured per sample.

Statistical analyses.– Dates were statistically analysed through the application of the statistic program, Statistical Graphics System (STATGRAF), version 6.0, by Statistical Graphics Corporations. For all the statistical tests, the level of significance was set at 95 % ($p > 0.05$). Normality and randomness of data were checked by using D'Agostino's test and Hart's test, respectively. Sample sizes were selected with an α error = 0.05 (95 % interval of confidence), and a β error = 0.1 (90% of the population). Differences between mean experimental values of the variables were subjected to analysis of variance (ANOVA). Analysis of variable dependency has been accomplished through the calculation of Pearson correlation coefficient (13).

Table I. *Leukocyte and lymphocyte surface antigens and respective cells.*

CD	CELL
$CD45^+$	Leukocytes
$CD45^+/CD14^-$	Total lymphocytes
CD3	T lymphocytes
CD19	B lymphocytes
CD3/CD4	T4 lymphocytes
CD3/CD8	T8 lymphocytes
$CD16/CD56/CD3^-$	NK cells
$CD4/CD45RA^+$	T4 Naive lymphocytes
$CD4/CD45RA^-$	T8 Memory lymphocytes

Results

Circulating leukocyte levels and plasma concentrations of NE and E have been quantified in 16 apparent healthy subjects.

Results of the circulating leukocyte values subpopulations and plasmatic rates of catecholamines are given as the mean value of the three determinations taken in the two periods of the day. No significant differences have been observed when comparing the concentrations obtained in the three samples. In this way a possible secretion peak in some of the hormones, which might alter the results, was discarded.

The results corresponding to leukocytes and their subpopulations (table II) show that there exists a diurnal rhythm in their circulating rates in most of them. Thus, significant differences ($p < 0.001$, $p < 0.01$) between morning and evening levels of total leukocytes and subtypes have been found. Both monocyte and NK cell circulating levels are the only ones similar in the two periods of the day.

A significant difference for NE concentrations has been found ($p < 0.05$) when comparing the mean values at 8 h (236.8 ± 21.0 pg/ml) and 20 h (175.6 ± 17.4 pg/ml), while E levels do not present these differences (196.7 ± 23.8 pg/ml versus 165.1 ± 20.8 pg/ml).

Table II. *Circulating leukocyte and subpopulations levels.*
Mean values ± SEM at 8 h and at 20 h together with the statistical significance of the morning-evening differences (*** P<0.001, ** P<0.01).

CELLS	8 h	20 h
Leukocytes	5158.7 ± 166.1	6596.3 ± 176.6***
Neutrophils	955.8 ± 121.3	3793.1 ± 135.0***
Monocytes	408.6 ± 36.3	427.9 ± 24.3
Total lymphocytes	1801.1 ± 56.8	2374.0 ± 68.1***
B	180.0 ± 10.7	308.7 ± 45.6**
T	1438.1 ± 44.6	1886.2 ± 54.0***
CD4	902.6 ± 34.2	1220.0 ± 290.8***
CD4/45RA$^+$	413.8 ± 26.3	541.5 ± 41.6**
CD4/45RA$^-$	500.6 ± 182.0	665.8 ± 228.7**
CD8	525.8 ± 25.4	669.2 ± 28.8**
NK cells	121.2 ± 11.0	123.2 ± 7.6

When analyzing the correlations between catecholamine plasma levels and the number of circulating cells, a significant one is proven ($p < 0.01$) between total leukocytes, granulocytes, total lymphocytes, T lymphocytes, CD4 and CD4/45 RA$^-$ lymphocytes and E; a significant correlation also exists ($p < 0.05$) between this hormone and monocytes, CD4/45RA$^+$ lymphocytes and CD8 lymphocytes. NE plasma levels shown a correlation with total lymphocytes, T lymphocytes ($p < 0.05$) and CD4 lymphocytes ($p < 0.01$). Significant correlations have not been found either between B lymphocytes or NK cell circulating levels or the two catecholamines.

Discussion

A daily rhythm has been demonstrated in leukocytes and in most subpopulations studied by finding significant differences between the 8.00 h and the 20.00 h recounts . The existence of significant lineal correlations between the in plasma catecholamine amount and part of the circulating subtype lymphocytes has been established.

The present work relations are in agreement with the important relations found in the last years. The lymphoid organs are extensively enervated by autonomous noradrenergic nerves and their activation bears on the lymphocyte migration from the blood into the tissues (3, 5), in such a way that the vascular perfusion of the lymphoid tissue is sensitive to α and β-adrenergical signals. When the sympathetic activity alters, so does the blood flow in the postcapillary venules, thus modulating the lymphocyte probability of colliding with the vascular endothelium (14).

The existence of β-adrenergic lymphocyte receptors has been proven, as well as the E inducing effect in the different subpopulation distribution through the receptors (6, 11). On the other hand, physical exercise is known to produce a lymphocyte redistribution (12).

The significant correlation between CD4 lymphocyte and NE rates, and the lack of relation between this hormone concentration and the CD8 lymphocytes could explain the proportion modifications of CD4/CD8 lymphocytes, produced on the autonomous nervous system activation (6). In these situations no

changes are kown to exist in the B lymphocyte percentage, which would explain the lack of dependence found between this subpopulation and the two catecholamines.

The above plus the significant correlations between plasma NE and E levels and the total of T lymphocyte subpopulations, leads to the localization of the possible immunocyte modulation by the hormones in the thymic vias of the T lymphocyte activation. It should be pointed out that in a previous study no significant lineal relation was found between the circulating B lymphocytes number and the cortisol and ACTH plasma concentrations (17).

As to the diurnal leucocytal variations, the present results are in agreement with those of POWNALL *et al.* (15) and LEVI *et al.* (9), who described lymphocytic subpopulation modifications along the entire day, with higher circulatory levels in the evening. On the other hand, the lack of daily rhythm in the NK cells has equally been found by RITCHIE *et al.* (16).

The circadian NE rhythm, with higher levels in the morning, has already been established in previous studies (19). Catecholamine secretion pulses superposed on the normal rhythm have equally been described (10). These pulses or the possible secretion peaks, due to the stress from the test, which could alter the results, seem to have been obviated, as there were no significant differences when the three sample concentrations were compared at each period of the day.

In conclusion, diurnal rhythms have been found both for leukocytes and subpopulations and for NE and E. The significant correlations between the aforesaid cells and the catecholamine plasma levels suggest the existence of a possible regulating mechanism of the circulating immune cells by certain hormones.

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J. R. INFANTE, F. PERÁN, M. MARTÍNEZ, R. POYATOS, A. ROLDÁN, C. RUIZ y F. GARRIDO. *Subpoblaciones linfocitarias y catecolaminas; variaciones diurnas y relaciones.* J. Physiol. Biochem. (Rev. esp. Fisiol.), 52 (3), 143-148, 1996.

Se estudian los ritmos circadianos en subpoblaciones de linfocitos circulantes y sus posibles relaciones con las catecolaminas endógenas. El estudio se realiza en 16 indivíduos aparentemente sanos (8 mujeres y 8 hombres de 22-35 años de edad). A las 8 h y a las 20 h se inserta en la vena de la flexura del codo una cánula tipo "butterfly" y tras un descanso de 15 minutos se extraen tres muestras de sangre a intervalos de 15 minutos. Se cuantifican leucocitos, linfocitos, monocitos y granulocitos en un contador automático. Las subpoblaciones linfocitarias se miden en un citómetro de flujo en células marcadas con anticuerpos monoclonales específicos. Las concentraciones plasmáticas de catecolaminas se determinan mediante cromatografía líquida de alta presión. En los niveles circulantes de leucocitos y todas las subpoblaciones medidas a diferentes horas del día, excepto para las células NK y los monocitos, se encuentran diferencias significativas. Hay correlaciones estadísticamente significativas entre los leucocitos y sus subpoblaciones y la epinefrina, excepto para los linfocitos B y las células NK; y entre los linfocitos totales, linfocitos T, subpoblaciones CD4 y CD4/CD45RA$^+$ y la norepinefrina. Se concluye que existen ritmos diurnos en leucocitos y subpoblaciones linfocitarias y que la existencia de correlaciones significativas entre estas células y los niveles de catecolaminas plasmáticas sugiere un posible mecanismo de conexión entre el sistema inmune y determinadas hormonas.

Palabras clave: Norepinefrina, Epinefrina, Leucocitos, Subpoblaciones linfocitarias, Ritmos circadianos.

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