EXPERIMENTAL BIOLOGY

Diurnal Dynamics of Cytokine Spectrum Produced by Immunocompetent Cells of Intact Mice

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The production of cytokines by immunocompetent cells of intact mice was studied in various phases of the diurnal cycle. The ratio of produced cytokines was shown to differ in various phases of the diurnal cycle. The correlation between spontaneous production of various cytokines disappeared at 20:00, which reflects the development of physiological immunosuppression due to evening increase in physical activity of mice and corresponding changes in neuroendocrine status. Our results illustrate the existence of diurnal variations in intercellular interactions in the immune system. These data should be taken into account in the study of the mechanisms for cytokine immunoregulation and development of new schemes for cytokine immunocorrection.

Key Words: *cytokines; lymphocytes; diurnal variations*

Functions of the immune system are determined not only by several parameters that reflect the state of cells, but also by the relationship between these parameters. This principle was postulated in the conception of "optimal morphofunctional ratio" between components of the immune system [4]. The ratio is characterized by diurnal variations, which provides different functional states of the system in various phases of the circadian rhythm [1]. It may be suggested that diurnal variations in cell subpopulations of immune organs contribute to differences in cytokine production [5,9,11]. Previous studies revealed diurnal variations in the relationship between several regulatory factors (glucocorticoids and melatonin) and immune parameters [1,3]. These specific features probably determine different effects of corticosterone and thymic hormones in mice during various phases of the diurnal cycle [1]. Chronoeffectiveness is also typical of several cytokines, including interferon-γ (IFN-γ) and interleukin-2 (IL-2) [2,8]. It can be hypothesized that a diurnal biorhythm of intrasystemic cytokine immunoregulation also exists, but this assumption was not yet confirmed.

Here we studied the interrelation between cytokine production by immunocompetent cells (ICC) of intact mice in various phases of the diurnal cycle.

MATERIALS AND METHODS

Experiments were performed with cells of the thymus and spleen from male $(CBA \times C57Bl)F_1$ mice aging 3-4 months and obtained from the nursery of laboratory animals of the Siberian Division of the Russian Academy of Medical Sciences (Nizhnyaya El'tsovka, Novosibirsk). The animals were maintained in a vivarium of the Institute of Clinical Immu-

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nology. They were housed in plastic cages (Animark) under natural light/dark conditions and had free access to water and food. The communities of constant composition were presynchronized for at least 14 days.

The animals were killed by cervical dislocation under light ether anesthesia (10:00, 15:00, and 20:00). The thymus and spleen were removed under sterile conditions. The cells (10^6 cells/ml) were cultured in RPMI-1640 medium containing 2 mM L-glutamine, 10% fetal bovine serum, 100 mg/liter ampicillin, and 50 mg/liter gentamicin in the presence or absence of T cell mitogen concanavalin A for 48 h. The concentrations of cytokines IFN-γ, IL-2, IL-10, and granulocyte-macrophage colony-stimulating factor (GM-CSF) in the supernatant were measured by the electrochemiluminescence method on an ORIGEN Analiser device. We used specific anticytokine antibodies and recombinant proteins (R&D Systems) [10].

Intergroup differences were evaluated by Kruskal—Wallis test (one-factor analysis of variance, ANOVA). Correlation analysis involved Spearman rank correlation coefficient.

RESULTS

Intact mice exhibited significant diurnal variations in the production of IL-2 in the culture of stimulated and unstimulated thymocytes (Table 1). IL-2 production was minimum in the morning and maximum in the evening. This dynamics probably reflects a correlation between IL-2-producing function of ICC and diurnal rhythm of melatonin [6]. The acrophase of melatonin production corresponds to nighttime.

Correlation analysis revealed a relationship between cytokine parameters of conditioned media from cultured ICC that were isolated at various time points (total value, Table 2). A positive correlation was found between GM-CSF production by stimulated thymocytes and spontaneous IL-2 secretion in these cells. Moreover, a positive correlation was revealed between the production of GM-CSF and IFN-γ by stimulated splenocytes. Spontaneous production of IL-2 by thymocytes positively correlated with stimulated production of IL-2 by thymocytes and spontaneous production of IL-2 by splenocytes.

Our results suggest that thymocytes spontaneously producing considerable amount of IL-2 are characterized by increased secretion of GM-CSF and IL-2 under conditions of stimulation (positive correlations, Table 2). The diurnal cycle includes synchronous variations in the ability of splenocytes to respond to stimulation by enhanced production of GM-CSF and IFN-γ (positive correlation, Table 2). It concerns the same cells or various populations of cells whose number and/or activity exhibit synchronous diurnal variations. Synchronous diurnal

TABLE 1. Diurnal Variations in the Concentration of Cytokines in Plasma and Supernatants from Unstimulated and Stimulated Splenocytes of Intact Mice (pg/ml, $M \pm m$)

Medium to measure cytokine concentration	10:00 (n=6)	15:00 (n=6)	20:00 (n=6)
IFN-γ			
CM from unstimulated splenocytes	301.46±212.76	145.46±83.65	270.93±145.9700
CM from stimulated splenocytes	13 651.44±2125.33	15 420.24±2853.5	29 337.630±9546.18
IL-2			
CM from unstimulated splenocytes	21.78±12.62	145.29±92.89	239.03±117.15
CM from stimulated splenocytes	616.53±196.84	348.43±79.19	795.42±333.39
CM from unstimulated thymocytes	0.00±0.00	74.18±35.13*	1191.58±573.20*
CM from stimulated thymocytes	0.00±0.00	126.27±56.66*	4365±2902.44*
GM-CSF			
CM from unstimulated splenocytes	20.74±11.39	166.61±119.87	130.49±69.19
CM from stimulated splenocytes	2057.43±333.69	2174.29±296.69	1764.78±653.48
CM from unstimulated thymocytes	547.42±336.93	122.35±71.50	74.62±42.41
CM from stimulated thymocytes	65.31±22.15	294.08±172.79	318.82±148.91
IL-10			
CM from unstimulated splenocytes	659.05±406.60	966.07±478.25	1201.37±615.85
CM from stimulated splenocytes	868.50±228.48	1258.19±263.38	1443.31±463.15

Note. CM: conditioned medium; *p<0.05 compared to 10:00.

TABLE 2. Correlation Analysis of Cytokine Production by Cells of the Thymus and Spleen in Intact Mice (Data at All Time Points)

Pairs of parameters	N	Spearman correlation coefficient (*r*)	Significance level (*p*)
GM-CSF (T_{ST})—IL-2 (T_{SP})	18	0.48	0.04
GM-CSF (S_{ST})—IFN-γ (S_{ST})	18	0.72	0.0007
IL-2 (T_{SP})—IL-2 (T_{ST})	18	0.84	0.0001
IL-2 (T_{SP})—IL-2 (S_{SP})	18	0.67	0.002

Note. Here and in Table 3: T_{SP}, spontaneous cytokine production by thymocytes; T_{ST}, stimulated cytokine production by thymocytes; S_{SP}, spontaneous cytokine production by splenocytes; and S_{ST}, stimulated cytokine production by splenocytes.

TABLE 3. Correlation Analysis of Cytokine Concentration in Supernatants of ICC and Blood Plasma at Individual Time Points

	Pairs of parameters	Number of measurements	Spearman correlation coefficient (*r*)	Significance level (*p*)
10:00				
	GM-CSF (T_{ST})—IL-2 (S_{ST})	6	0.83	0.04
	GM-CSF (S_{SP})—IL-2 (S_{SP})	6	-0.88	0.02
	GM-CSF (S_{ST})—IFN-γ (S_{ST})	6	0.94	0.005
15:00				
	GM-CSF (T_{SP})—GM-CSF (S_{SP})	6	-0.81	0.04
	GM-CSF (S_{SP})—IL-10 (S_{SP})	6	0.93	0.008
	GM-CSF (S_{SP})—IL-2 (T_{ST})	6	-0.93	0.008
	GM-CSF (S_{ST})—IFN-γ (S_{ST})	6	0.88	0.02
	IL-10 (S_{SP})—IL-2 (T_{ST})	6	-0.94	0.004
20:00				
	GM-CSF (S_{ST})—IFN-γ (S_{ST})	6	0.94	0.005
	IFN-γ (S_{ST})—IL-2 (S_{ST})	6	0.83	0.04

variations were revealed in spontaneous production of IL-2 by thymocytes and splenocytes (positive correlation, Table 2).

Positive correlations were found between the parameters for secretion of type 1 cytokines by ICC at the specified time interval. Our results are consistent with published data that these cytokines induce mutual potentiating and stimulatory effects and are produced by the same cells [7]. IL-2 production by thymocytes positively correlated with other cytokine parameters, including GM-CSF production by stimulated thymocytes and spontaneous IL-2 production by splenocytes (Table 2). IL-2 production in the thymus increased in the morning and evening. These data suggest that the ability of immune cells to produce proinflammatory cytokines in the evening is higher than in the morning. These specific features probably reflect circadian variations in baseline immune reactions of the intact organism to constantly present autoantigens and heteroantigens.

We studied the correlations between cytokine production by ICC at individual time points.

The relationship between cytokine production by ICC differed in various phases of the diurnal cycle (Table 3). These data attest to specific diurnal variations in the regulatory relationship between cells of the immune system. A positive correlation between production of GM-CSF and IFN-γ by stimulated splenocytes was found at all time points. Hence, splenocytes respond to mitogenic stimulation by changes in the production of these cytokines. No correlations were found between spontaneous production of various cytokines at 20:00. It is most likely that they characterize regulatory relationships in the intact immune system. These data are consistent with the results of our previous experiments. We showed that immunization of mice in the evening is followed by a decrease in the immune response [1], which probably results from physiological immunosuppression due to evening increase in physical activity of animals and corresponding changes in neuroendocrine status.

Our results attest to the existence of diurnal variations in intercellular interactions in the immune system. These data should be taken into account in the study of the mechanisms for cytokine immunoregulation and development of new schemes for cytokine immunocorrection.

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