Journal of Neuroendocrinology, 2000, Vol. 12, 758–765

Effect of Melatonin Treatment on 24-h Variations in Responses to Mitogens and Lymphocyte Subset Populations in Rat Submaxillary Lymph Nodes

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Key words: melatonin, submaxillary lymph nodes, lymphocyte subsets, circadian rhythms, Freund's adjuvant arthritis.

Abstract

Wistar male rats were injected s.c. with melatonin (30 μg) or vehicle, 1 h before lights off, for 11 days. Ten days after beginning melatonin treatment, rats received Freund's complete adjuvant or its vehicle s.c., and after 2 days, they were sacrificed at six different time intervals throughout a 24-h cycle. The mitogenic effect of lipopolysaccharide (LPS) and concanavalin A (Con A), the activity of ornithine decarboxylase (ODC) and the relative size of lymphocyte subset populations were measured in submaxillary lymph nodes. In control rats, the mitogenic effects of LPS and Con A and ODC activity peaked during the afternoon. Injection of Freund's adjuvant induced a 10-h shift in the diurnal rhythm of the mitogenic effect of LPS to attain maximal values at night. Melatonin pretreatment blunted the daily variations in the mitogenic activity of Con A or LPS and, when given to Freund's adjuvant-injected rats, augmented mesor and amplitude of diurnal rhythm in ODC activity. Maxima in B cell number occurred at night whereas those of T and B-T cell number occurred during the afternoon. During the early phase of immunization tested, the number of B cells augmented and the amplitude of its diurnal rhythmicity increased both after immunization and following melatonin pretreatment. Maxima of 24-h rhythms in CD4+ and CD4+/CD8+ cell populations occurred during the afternoon while those of CD8+ cells occurred at late night. Melatonin significantly augmented CD4+ cell number and decreased CD8+ cell number; it therefore augmented the CD4+ : CD8+ ratio. The results suggest that pretreatment with a pharmacological dose of melatonin exerts immunomodulating effects at an early, preclinical, phase of Freund's adjuvant-induced arthritis in rats.

Many biological functions wax and wane in cycles that repeat every 24 h. The rationale for this circadian organization is the predictive value of the mechanisms that enable an organism to maintain equilibrium in response to anticipated variations of the environment (1). One essential circadian rhythm of adaptive responses is that of the immune reaction. Circadian changes in the circulation of T, B, or natural killer (NK) lymphocyte subsets in peripheral blood and in the density of epitope molecules at their surface have been reported (2), which may be related to cell reactivity to antigen exposure. Changes in lymphocyte subset populations can depend on time-of-day associated changes in cell proliferation in immunocompetent organs and/or on diurnal modifications in lymphocyte release and traffic among lymphoid organs.

During recent years, we have examined the regulation of circadian rhythmicity of lymph cell proliferation taking the rat submaxillary lymph nodes as a model. The bilateral anatomical location of submaxillary lymph nodes and their easily manipulable autonomic innervation allowed us to carry out the analysis of the humoral and neural mechanisms regulating the lymphoid organs. Both in immunized and in nonimmunized rats, a significant diurnal variation of submaxillary lymph node ornithine decarboxylase activity (ODC), an index of cell proliferation in a number of organs, including immunocompetent organs (3) and endocrine glands (4), was reported, displaying maximal activity in early afternoon (5). Such a maximum coincided with peak mitotic responses to lipopolysaccharide (LPS) and concanavalin A (Con A) in

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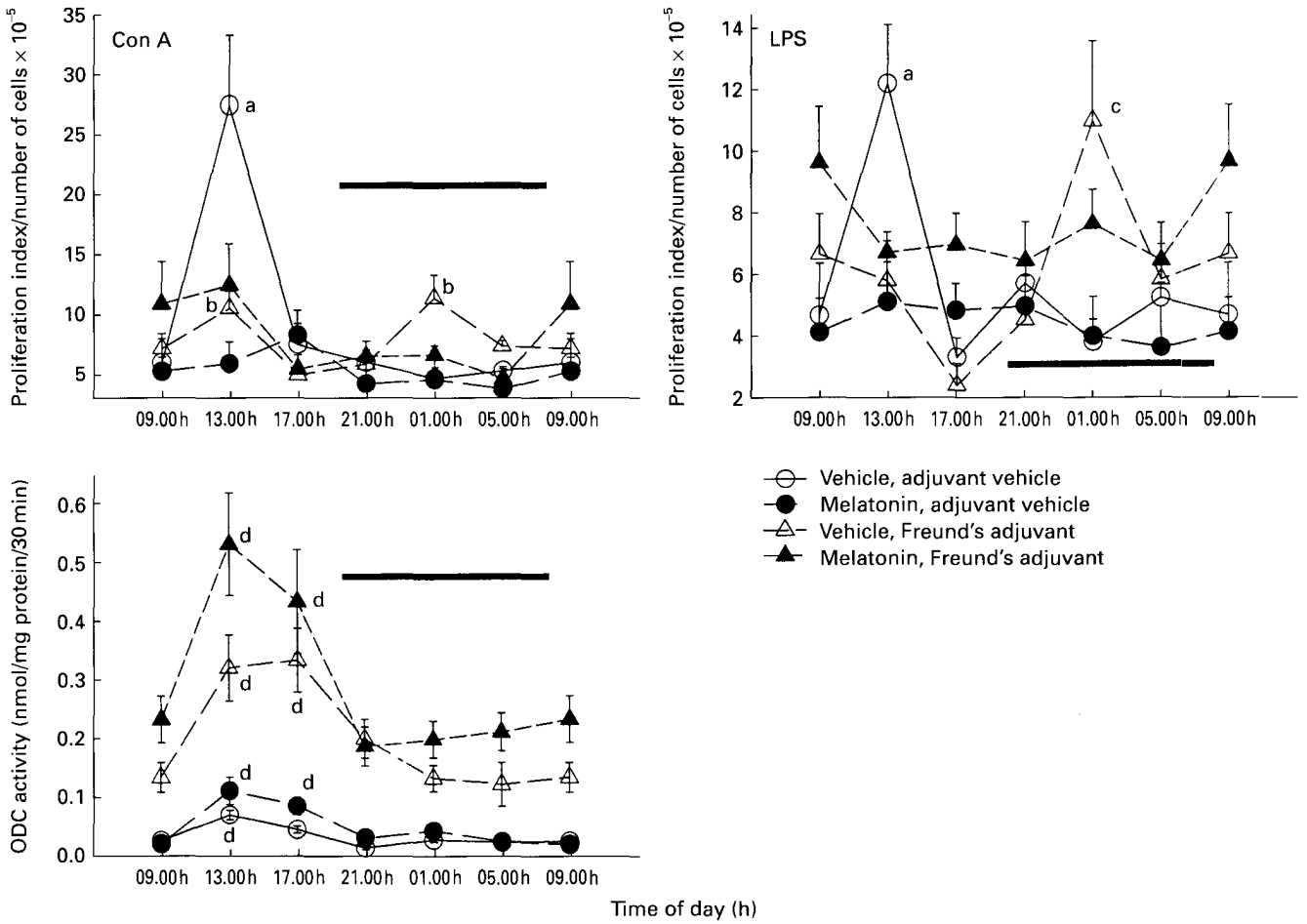

FIG. 1. Twenty-four hour changes in mitogenic activity of concanavalin A (Con A) and lipopolysaccharide (LPS), and in ornithine decarboxylase (ODC) activity, in submaxillary lymph nodes of rats injected with Freund's complete adjuvant or adjuvant's vehicle and treated with melatonin or vehicle (control). Groups of 4–7 rats were killed by decapitation at six different time intervals throughout a 24-h cycle, 2 days after injection of Freund's complete adjuvant or its vehicle. Shown are the means ± SEM. Letters indicate significant differences among time intervals within every experimental group (one-way ANOVA followed by a Student–Newman–Keuls test). $^{a}P<0.01$ compared to the remaining intervals; $^{b}P<0.05$ compared to 17.00 h and 21.00 h; $^{c}P<0.05$ compared to the remaining time intervals; $^{d}P<0.05$ compared to 01.00 h, 05.00 h, 09.00 h and 21.00 h. Further analysis employing a two-factorial ANOVA and a Cosinor analysis are described in the text and Table 1.

incubated lymph node cells (6). A purely neural pathway was identified (7), including, as a motor leg, the autonomic nervous system innervating the lymph nodes. In addition, a hormonal pathway involving the circadian secretion of melatonin may also play a role in inducing rhythmicity (2, 8). Indeed, several *in vivo* and *in vitro* data now support the existence of potent immunomodulatory effects of melatonin in both humans and experimental animals (9–11).

As a continuation of those studies on melatonin's role in regulation of circadian immune activity, we report the effect of pretreating rats with pharmacological amounts of melatonin on 24-h changes in the *in vitro* mitogenic effect of LPS and Con A and in the relative size of lymphocyte subset populations in submaxillary lymph nodes. Rats received a s.c. injection of Freund's complete adjuvant or its vehicle 2 days before sacrifice. As a control, ODC activity in submaxillary lymph nodes was measured.

Materials and methods

Chemicals

Thymidine [methyl-^{3}H] (specific activity 20 Ci/mmol) and L-[1-^{14}C] ornithine hydrochloride (specific activity 58 Ci/mol) were purchased from NEN Research Products (Boston, MA, USA). Freund's complete adjuvant was purchased from Difco (Detroit, MI, USA). All other drugs and reagents employed were obtained from Sigma Chemical Co (St Louis, MO, USA).

Animals and experimental design

Experiments were carried out in adult male Wistar rats (180–220 g), kept under light between 08.00 h and 20.00 h daily. Rats had access to food and water *ad libitum*. The studies were conducted in accordance with the principles and procedures outlined in the NIH guide for the Care and Use of the Laboratory Animals.

The rats received s.c. injections of melatonin (30 μg) in 0.1 ml of vehicle (10% ethanol in saline), or its vehicle, 1 h before lights off for 11 days. At these doses of melatonin (i.e. 10–100 μg), significant effects on 24-h rhythms of preand postsynaptic activity in lymphoid organs of young and old rats have

TABLE 1. Cosinor Analysis of the Effect of Melatonin on 24-h Changes of Concanavalin A (Con A) and Lipopolysaccharide (LPS) Mitogenic Activity, and of Ornithine Decarboxylase (ODC) Activity in Rat Submaxillary Lymph Nodes.

	Mesor	Amplitude	Acrophase (h, min)	Percent of rhythm
Control, adjuvant's vehicle				
Con A	9.5±1.2	8.0±0.9	13.18±02.04	48.5±5 6
LPS	5.8±0.7	2.3±0.5	12.35±03.00	30.5±3 4
ODC	0.04±0.01	0.02±0.004	14.04±01.23	67.2±11.3
Melatonin, adjuvant's vehicle				
Con A	NS	NS	NS	NS
LPS	NS	NS	NS	NS
ODC	0.05±0.01	0.05±0.009	15.10±01.56	61.1±9.8
Control, Freund's adjuvant				
Con A	–	–	–	–
LPS	6.0±0.7	2.5±0.5	03.38±01.45	46.8±6.5
ODC	0.21±0.05*	0.12±0.03*	15.54±02.31	88.9±10.0
Melatonin, Freund's adjuvant				
Con A	NS	NS	NS	NS
LPS	NS	NS	NS	NS
ODC	0.30±0.06*	0.16±0.04*	14.18±02.22	75.4±7.7

Shown are the means±SEM (n=4–7/group). Mesor and amplitude values are expressed as proliferation index/number of cells $\times 10^{-5}$ (ConA, LPS mitogenic activity) or nmol/mg protein/30 min (ODC activity). Asterisks designate significant differences compared to the control, adjuvant's vehicle or melatonin, adjuvant's vehicle-treated groups in a one-way ANOVA followed by a Student–Newman–Keuls test, $P<0.05$. NS, no significant daily changes in a one-way ANOVA; (–): no significant changes in Cosinor.

been described (2, 12). On the tenth day of treatment, the rats were s.c. injected with Freund's complete adjuvant (0.5 mg heat-killed Mycobacterium butyricum/rat) or its vehicle (0.5 ml paraffin oil containing 15% mannide monooleate) at 11.00 h. Therefore, our study comprised four experimental groups: (i) melatonin's vehicle-treated, adjuvant's vehicle injected rats; (ii) melatonin-treated, adjuvant's vehicle injected rats; (iii) melatonin's vehicle-treated, Freund's adjuvant-injected rats; and (iv) melatonin-treated, Freund's adjuvant-injected rats.

On the second day after Freund's adjuvant or adjuvant's vehicle injection, groups of 4–7 rats from each experimental group were sacrificed by decapitation at six different time intervals throughout a 24-h cycle. Their submaxillary lymph nodes were removed aseptically, weighed and placed in Petri disks. Aliquots of tissue were frozen to −70 °C for ODC measurement (performed within 2 weeks). The remaining tissue was put on balanced salt solution and the cells gently teased apart. After removing the clumps by centrifugation, the cells were suspended in sterile supplemented medium (RPMI 1640), containing 10% heat-inactivated, foetal bovine serum, 20 mM L-glutamine, 0.02 mM 2-mercaptoethanol and gentamicin (50 mg/ml) and were counted.

Mitogen assays

Mitogen assays were performed as described in detail elsewhere (6). Submaxillary lymph node cells were obtained as described above and used at a final number of cells/well (0.1 ml) of 5×10^5. Control and experimental cultures were run in triplicate. Mitogens were added to the cultures at final supramaximal concentrations of 5 µg/ml. The cultures were incubated in a humidified 37 °C incubator in an atmosphere of 5% CO_2. After 48 h incubation, ^{3}H-thymidine (0.2 µCi) was added to each well in a volume of 0.02 ml. Cells were harvested 5 h later using a automated sample harvester, and the filters were counted in a liquid scintillation spectrometer. The proliferation index was estimated as the ratio between stimulation in the presence of mitogens/controls. Results were expressed as proliferation index/number of cells.

Lymphocyte subsets

The relative size distributions of lymph cells in submaxillary lymph nodes of rats were determined by fluorescence-activated cell sorter analysis, as previously described (13, 14). For these studies, we used the following monoclonal antibodies: Anti-rat LCA (OX-33) for B lymphocytes (Serotec, Oxford, UK), anti-rat TCR alpha/beta (R7.3) for T lymphocytes (Serotec, Oxford, UK), anti-rat CD4 (OX-35) which recognizes a rat T helper cell differentiation antigen (Pharmingen, San Diego, CA, USA), and anti-rat CD8a (OX-8) which recognize the reactive antigen expressed on rat T cytotoxic/suppressor cells (Pharmingen). Lymphocytes from submaxillary lymph nodes isolated as indicated above, were washed in cold phosphate buffered saline (PBS) with 0.02% sodium azide and then incubated (3×10^5 cells/tube) with appropriate primary antibodies for 30 min at 4 °C. Following two washes, the cells were incubated with 1 ml of PBS-BSA 1%, during 5 min at 4 °C, washed three times, resuspended in 1% paraformaldehyde in PBS. Fluorescence intensity was analysed by fluorescence activated cell sorting (FACStar Beckton Dickinson, Mountain View, CA, USA). Dead cells were excluded by gating with propidium iodide.

ODC activity

ODC activity was measured as described previously (5). Submaxillary lymph nodes were homogenized in chilled phosphate buffer containing 5 mM NaF, 0.1 mM pyridoxal-PO_4, 0.1 mM EDTA-Na and 2 mM dithiothreitol. Homogenates were centrifuged and aliquots of supernatants were incubated in glass tubes fitted with rubber stopper and centre wells containing a filter paper disk spotted with hyamine hydroxide. L-[1-^{14}C]ornithine hydrochloride (1 µCi/tube) was then added, together with unlabeled L-ornithine to adjust the assay concentration to 0.25 mM. After 30 min of incubation at 37 °C, the enzymatic reaction was stopped with citric acid and the $^{14}CO_2$ liberated from the enzymatic reaction was collected on the filter papers. The radioactivity was counted in a Triton X100-toluene phosphor solution. Results were expressed as nmol of $^{14}CO_2$ released/mg supernatant protein/30 min. Validation of the procedure is provided elsewhere (5).

Statistical analysis

Statistical analysis of results was performed by employing a two-way factorial analysis of variance (ANOVA), a one-way ANOVA, followed by a Student–Newman–Keuls test, and a Cosinor analysis. The latter was used to analyse general rhythmic parameters, i.e. acrophase (the maximum of the sinus function fit by the experimental data), mesor (the statistical estimate of the mean) and amplitude (half the difference between calculated maximal and minimal values). $P<0.05$ was considered statistically significant.

Results

Figure 1 shows the effect of melatonin pretreatment on 24-h changes in mitogenic effects of LPS and Con A, and in ODC activity, in submaxillary lymph nodes of rats injected with Freund's complete adjuvant or adjuvant's vehicle two days earlier. Significant time-of-day dependent changes were

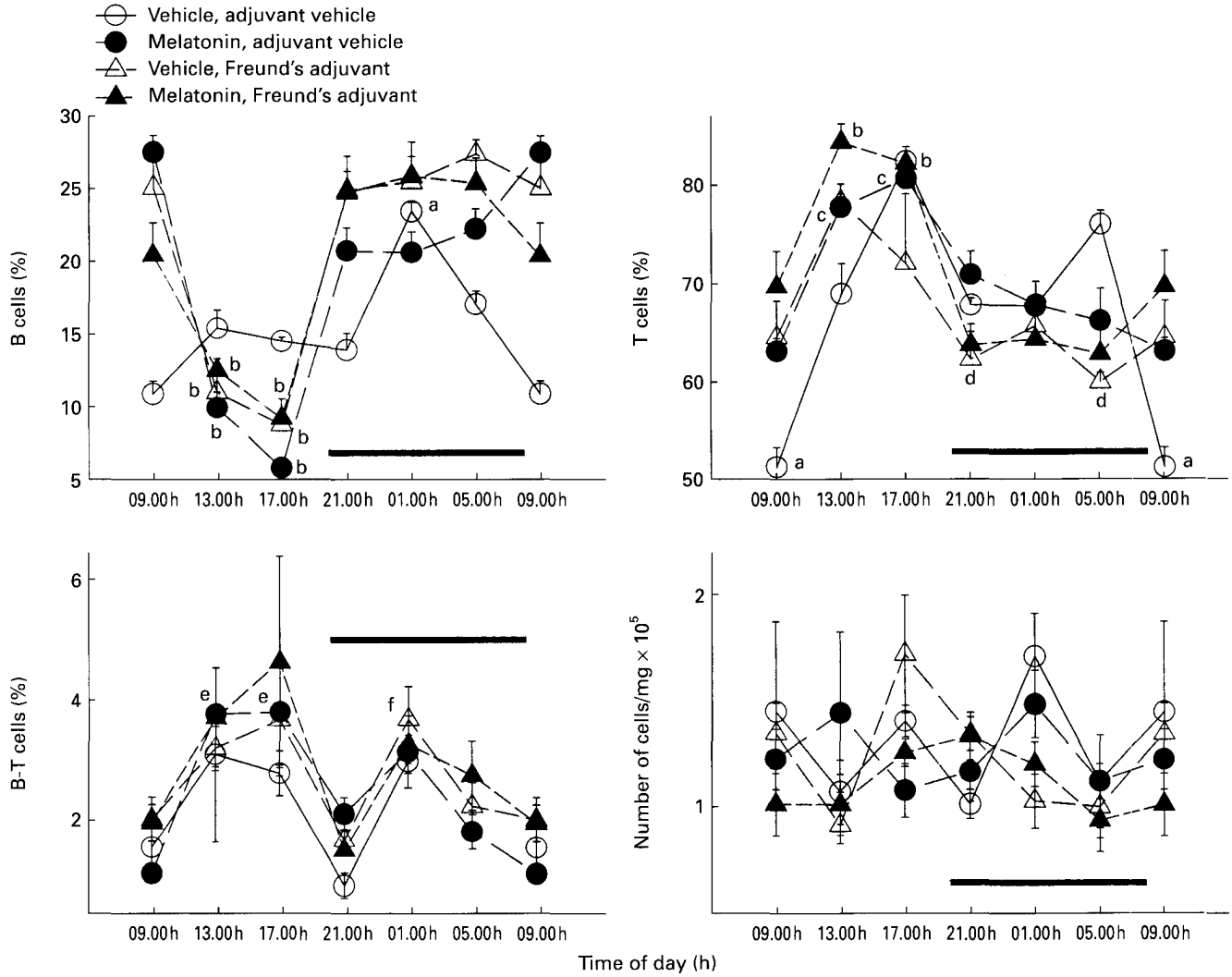

FIG. 2. Twenty-four hour changes in B, T and B-T cell populations and cellularity in submaxillary lymph nodes of rats injected with Freund's complete adjuvant or adjuvant's vehicle and treated with melatonin or vehicle (control). Cell populations were evaluated by fluorescence-activated cell sorter analysis. Shown are the means ± SEM. Superscript letters indicate significant differences among time intervals within every experimental group (one-way ANOVA followed by a Student–Newman–Keuls test). [a]$P<0.01$ compared to the remaining intervals; [b]$P<0.01$ compared to 09.00 h, 21.00 h, 01.00 h and 05.00 h; [c]$P<0.05$ compared to 09.00 h, 21.00 h, 01.00 h and 05.00 h; [d]$P<0.05$ compared to 13.00 h; [e]$P<0.05$ compared to 05.00 h, 09.00 h and 21.00 h; [f]$P<0.05$ compared to 21.00 h. Further analysis employing a two-factorial ANOVA and a Cosinor analysis are described in the text and Table 2.

observed for the Freund's adjuvant-or adjuvant's vehicle-treated animals injected with melatonin vehicle (Con A and LPS mitogenic effect) or in the four experimental groups (ODC activity) ($P<0.01$). When a two-way, factorial ANOVA was employed for the analysis of main factors, significant stimulatory effects of immunization in the case of both Con A and LPS mitogenic effect were revealed. Grand mean values (proliferation index/number of cell $\times 10^{-5}$) were: (i) Con A, Freund's adjuvant, 8.45; adjuvant's vehicle, 6.29; $F=5.37$, $P=0.021$. (ii) LPS, Freund's adjuvant, 8.11; adjuvant's vehicle, 5.35; $F=20.5$, $P<0.00001$). Time-of-day changes in the factorial ANOVA were significant for Con A only ($F=4.59$, $P=0.0006$). In the case of ODC activity, a significant stimulation after immunization or melatonin injection was observed. Grand mean value (nmol/mg protein/30 min): (i) Freund's adjuvant, 0.31; adjuvant's vehicle, 0.08; $F=28.6$, $P<0.00001$. (ii) Melatonin, 0.38; vehicle, 0.17; $F=24.5$, $P<0.00001$) was observed. Time-of-day changes in ODC activity were significant in the factorial ANOVA ($F=25.5$, $P<0.00001$).

In adjuvant's vehicle-treated rats administered with saline, the three parameters examined peaked at afternoon hours, acrophases being 13.18 h (Con A), 12.35 h (LPS) and 14.04 h (ODC). Administration of Freund's adjuvant caused a 10-h-shift in acrophase with respect to LPS activity (03.38 h), while acrophases of ODC activity did not change significantly (15.54 h). In Freund's adjuvant-injected rats, time-of-day changes in Con A activity did not fit a cosinoid function (Table 1). Melatonin administration blunted the daily variations in mitogenic activity of Con A or LPS (Fig. 1 and

TABLE 2. Cosinor Analysis of the Effect of Melatonin on 24-h Changes in B, T and B-T Cells, As Well as Cellularity in Rat Submaxillary Lymph Nodes.

	Mesor	Amplitude	Acrophase (h, min)	Percent of rhythm
Control, adjuvant's vehicle				
B cells	15.9±3.8	3.6±0.5	00.52±02.01	43.9±6.5
T cells	69.0±8.7	6.6±1.2	19.44±03.48	24.5±4.4
B-T cells	–	–	–	–
Cellularity	NS	NS	NS	NS
Melatonin, adjuvant's vehicle				
B cells	17.9±3.5	8.5±1.2**	04.28±01.19	64.5±8.8
T cells	71.2±7.8	7.8±0.9	16.42±00.56	78.2±9.8
B-T cells	2.6±0.7	0.9±1.2	17.24±01.47	43.6±7.6
Cellularity	NS	NS	NS	NS
Control, Freund's adjuvant				
B cells	20.6±3.1	9.6±1.2**	03.18±00.56	79.9±8.7
T cells	67.3±7.8	7.2±0.8	14.34±01.11	66.5±7.6
B-T cells	–	–	–	–
Cellularity	NS	NS	NS	NS
Melatonin, Freund's adjuvant				
B cells	19.7±2.6	8.6±1.1*	02.34±00.42	85.3±9.8
T cells	71.2±9.8	11.6±3.4	14.18± 02.32	85.7± 12.3
B-T cells	NS	NS	NS	NS
Cellularity	NS	NS	NS	NS

Shown are the means±SEM (n=4–7/group). Mesor and amplitude values are expressed as percentage (B, T and B-T cells) or number of cells/mg tissue × 10^5 (cellularity). Asterisks designate significant differences compared to the control, adjuvant's vehicle group in a one-way ANOVA followed by a Student–Newman–Keuls test (*) $P<0.05$ (**) $P<0.01$. NS, no significant daily changes in a one-way ANOVA; (–): no significant changes in Cosinor.

Table 1). In rats pretreated, or not, with melatonin and injected with Freund's adjuvant 2 days earlier, mesor and amplitude of ODC activity rhythm were augmented significantly (Table 1).

Figure 2 depicts melatonin and Freund's adjuvant effects on 24-h rhythms in B, T and B-T cells, and in cell number, in submaxillary lymph nodes of the same group of rats. Significant time-of-day dependent changes were observed in the four experimental groups for B and T cells and for Freund's adjuvant injected rats treated with vehicle, or adjuvant's injected rats treated with vehicle or melatonin in the case of B-T cells ($P<0.01$). Cell number did not vary significantly as a function of time-of-day or treatment employed. The analysis of main factors in a factorial ANOVA for B cells indicated stimulatory effects of immunization (grand mean values: Freund's adjuvant, 20.7%; adjuvant's vehicle, 17.2%; $F=20.2$, $P<0.00001$) and occurrence of time-of-day changes ($F=35.2$, $P<0.00001$). In the case of T cells, main factor analysis indicated a significant stimulatory effect of melatonin treatment (grand mean values: melatonin, 70.8%; vehicle, 67.8%; $F=6.67$, $P=0.011$) and time-of-day changes ($F=27.3$, $P<0.00001$). In the case of B-T cells, main factor analysis in a factorial ANOVA indicated significant time of day-related changes only ($F=9.52$, $P<0.00001$).

The significant time of day changes for B and T cells were generally described in the different groups by a cosinoid function, with acrophases occurring at night (00.52 h to 04.28 h) for B cells and during the afternoon (14.18 h to 19.44 h) for T cells (Table 2). In the case of B cells, Cosinor analysis indicated an increase in the amplitude of rhythm both after immunization or after melatonin pretreatment, while for B-T cell changes were significant in rats pretreated with melatonin and injected with adjuvant's vehicle only (acrophase at 17.24 h) (Table 2).

The effect of melatonin pretreatment and of immunization on T lymphocyte subsets in submaxillary lymph nodes is depicted in Fig. 3. Individual one-way ANOVA indicated existence of significant time of day changes for each experimental group of rats in every parameter tested. A factorial ANOVA of changes in CD4+ cells indicated a significant stimulatory effect of melatonin treatment (grand mean values: melatonin, 49.4%; vehicle, 44.2%; $F=30.5$, $P<0.00001$) and occurrence of time of day changes ($F=34.1$, $P<0.00001$). In the case of CD4+/CD8+ cells, main factor analysis in a factorial ANOVA was significant only for time of day changes ($F=78.4$, $P<0.00001$) while for CD8+ cells main factor analysis indicated a significant decrease after melatonin treatment (grand mean values: melatonin, 14.2%; vehicle, 15.6%; $F=9.16$, $P=0.032$) as well as significant daily changes ($F=10.4$, $P<0.00001$). With respect to CD4+ : CD8+ ratio, main factor analysis in a factorial ANOVA indicated a significant stimulatory effect of melatonin treatment (grand mean values: melatonin, 3.91; vehicle, 3.22; $F=9.17$, $P=0.032$) as well as time of day-related changes ($F=13.2$, $P<0.0001$).

As shown in Table 3, such time-of-day changes could be described in terms of a cosinoid function, except for CD4+ and CD4+/CD8+ cells in rats injected with melatonin's and adjuvant's vehicle. Cosinor analysis indicated acrophases of 15.10 h to 15.53 h (CD4+ cells), 04.14 h to 07.16 h (CD8+ cells), 15.06 h to 15.17 h (CD4+/CD8+ cells) and 17.01 h to 20.38 h (CD4+ : CD8+ ratio). Melatonin augmented CD4+

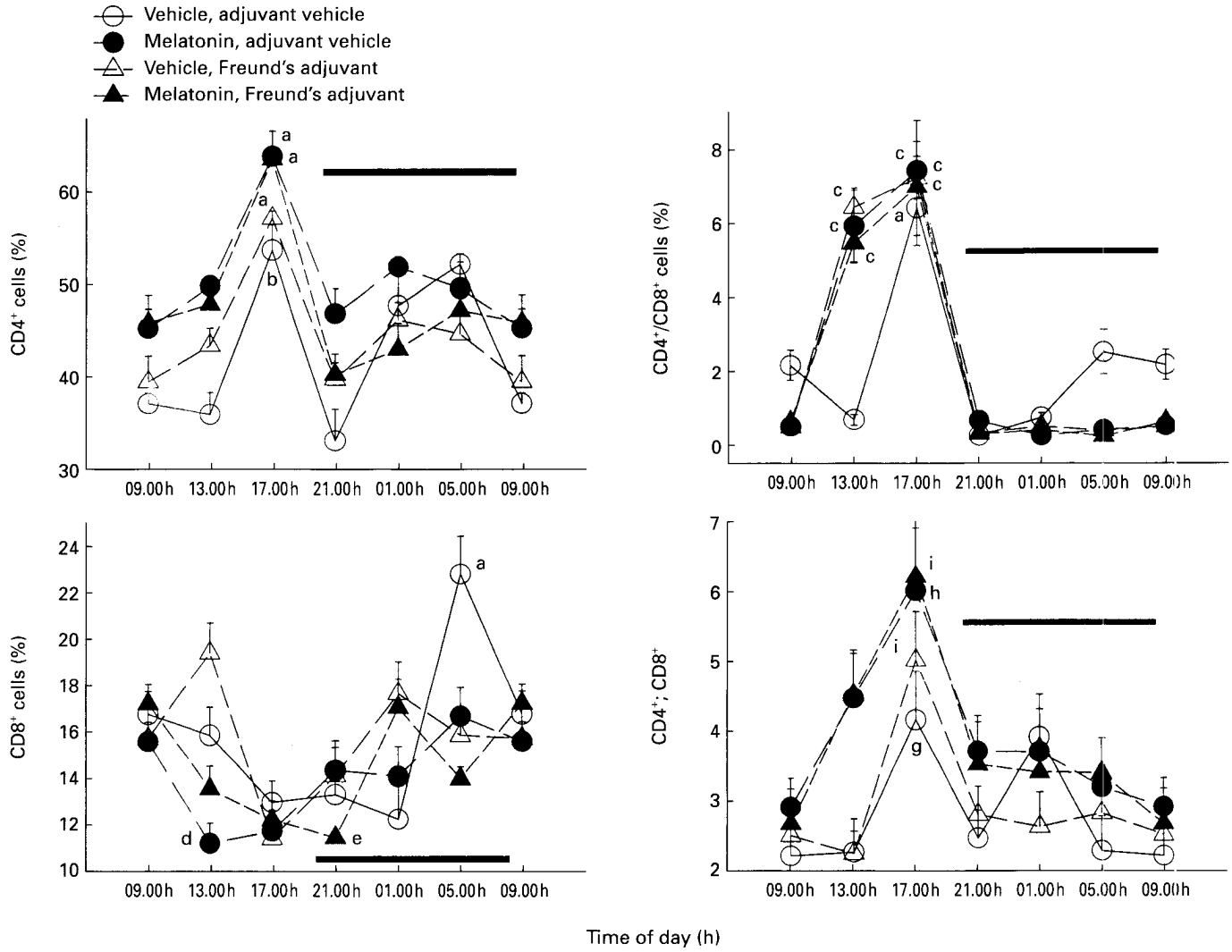

FIG. 3. Twenty-four hour changes in CD4+, CD4+/CD8+ and CD8+ cell populations, as well as CD4+ : CD8+ ratio, in submaxillary lymph nodes of rats injected with Freund's complete adjuvant or adjuvant's vehicle and treated with melatonin or vehicle (control). Cell populations were evaluated by fluorescence-activated cell sorter analysis. Shown are the means ± SEM. Superscript letters indicate significant differences among time intervals withon every experimental group (one-way ANOVA followed by a Student–Newman–Keuls test). $^{a}P<0.01$ compared to the remaining intervals; $^{b}P<0.01$ compared to 09.00 h, 13.00 h and 21.00 h; $^{c}P<0.01$ compared to 01.00 h, 05.00 h, 09.00 h and 2100 h; $^{d}P<0.05$ compared to 05.00 h and 21.00 h; $^{e}P<0.05$ compared to 01.00 h and 09.00 h; $^{f}P<0.05$ compared to 01.00 h and 1300 h; $^{g}P<0.05$ compared to 09.00 h; $^{h}P<0.05$ compared to 05.00 h, 09.00 h and 21.00 h; $^{i}P<0.05$ compared to the remaining time intervals. Further analysis employing a two-factorial ANOVA and a Cosinor analysis are described in the text and Table 3.

cells and decreased CD8+ cells, thus increasing significantly CD4+ : CD8+ ratio as well as mesor and amplitude of its 24-h rhythm in a Cosinor analysis (Fig. 3 and Table 3).

Discussion

Pineal ablation, or any other experimental procedure that inhibits melatonin synthesis and secretion, induces a state of immunodepression, which is partly counteracted by melatonin in several species (9–11). Melatonin treatment increases T cell proliferation (15, 16), enhances antigen presentation by macrophages to T cells by increasing the expression of MHC class II molecules (15), activates lymph node and bone marrow cells (17), stimulates antibody-dependent cellular citotoxicity (18) and augments natural and acquired immunity (19). Melatonin also stimulates NK cell activity (20), activates monocytes (21), increases the number of Th2 lymphocytes (22), restores impaired Th cell activity in immuno-depressed mice (23) and augments antibody responses *in vivo* (9, 15, 19).

The present results indicate that pretreatment for 11 days with a pharmacological dose of melatonin affected some aspects of the early phase of the immune response elicited by Freund's adjuvant injection. The stimulation by a pharmacological dose of melatonin of B and T (CD4+ cells) observed in lymph nodes are compatible with a promoting role of immune response by melatonin. Indeed, melatonin, in physiological concentrations, stimulates activated CD4+ T cells *in*

TABLE 3. Cosinor Analysis of the Effect of Melatonin on 24-h Changes In CD4+, CD4+/CD8+ and CD8+ Lymphocyte Subpopulations, and on CD4+ : CD8+ Ratio, in Rat Submaxillary Lymph Nodes.

	Mesor	Amplitude	Acrophase (h, min)	Percent of rhythm
Control, adjuvant's vehicle				
CD4+ cells	–	–	–	–
CD4+/CD8+ cells	–	–	–	–
CD8+ cells	15.6±3.2	3.8±0.7	07.02±01.48	57.0±8.7
CD4+ : CD8+ ratio	2.9±0.2	0.7±0.1	20.38±05.43	33.7±6.5
Melatonin, adjuvant's vehicle				
CD4+ cells	51.4±0.7	4.8±0.7	17.50±02.22	31.4±6.5
CD4+/CD8+ cells	2.5±0.8	3.7±0.7	15.17±01.05	76.7±8.9
CD8+ cells	13.9±3.0	2.4±0.5	04.14±00.52	74.6±7.6
CD4+ : CD8+ ratio	4.0±0.2*	1.2±0.1*	17.01±04.32	68.6±8.1
Control, Freund's adjuvant				
CD4+ cells	45.1±7.4	3.8±1.1	17.53±03.32	20.8±5.6
CD4+/CD8+ cells	2.6±0.6	3.7±0.9	15.06±01.23	74.1±9.8
CD8+ cells	15.7±4.3	1.7±0.4	07.16±01.03	23.6±5.0
CD4+ : CD8+ ratio	3.0±0.2	0.7±0.2	18.02±03.45	32.5±5.4
Melatonin, Freund's adjuvant				
CD4+ cells	48.6±5.9	6.1±0.9	15.10±02.20	33.4±4.5
CD4+/CD8+ cells	2.3±0.7	3.4±0.8	15.11±00.47	75.1±9.2
CD8+ cells	14.8±2.9	2.2±0.9	06.10±01.53	51.3±6.7
CD4+ : CD8+ ratio	3.9±0.3*	1.3±0.2*	16.45±02.03	61.2±9.8

Shown are the means±SEM (n=4–7/group). Mesor and amplitude values are expressed as percentage. Asterisks designate significant differences compared to the control, adjuvant's vehicle group in a one-way ANOVA followed by a Student–Newman–Keuls test, $P<0.05$. NS, no significant daily changes in a one-way ANOVA; (–): no significant changes in Cosinor.

vitro to release opioid agonist(s) with immunoenhancing and antistress properties (24). Melatonin also activates the production of interleukin-1, 2 and 6, ′-interferon, tumour necrosis factor, reactive oxygen intermediates and nitric oxide in lymphocytes (21, 25).

It could be argued that, 2 days after Freund's adjuvant challenge, only the intensity of the inflammatory reaction which precedes the real immune response is assessed. Relevant to this point is a study (26) that examined the course of adjuvant-induced arthritis in rats over an 11-week postinoculation period. The preclinical stage (first week) was characterized by very few, if any, signs of inflammation with discrete radiological lesions of the forepaws. Inflammatory events were marked in the acute stage of the disease (weeks 2–4), with signs of hyperalgesia and increases in hindpaw and forepaw joint diameters. Similarly, in a previous study, we examined inflammation of hind paws by a plethysmographic procedure in rats on days 6 and 16 after injection of Freund's adjuvant or adjuvant vehicle (2). In young rats, significant changes in hind paw volume, an indicator of inflammation, were revealed in Freund's adjuvant-injected rats on day 16 but not on day 6 of treatment. Daily administration of 100 μg melatonin starting on the day of Freund's adjuvant injection did not affect hind paw volume on day 6 and increased it significantly on day 16 after challenge. Therefore, it does not seem feasible that the activity of melatonin reported here was due to an effect on the inflammatory reaction.

Our present results also indicate that melatonin treatment suppressed the subsequent *in vitro* stimulation by the mitogenic agents LPS (which stimulates B cells) and Con A (which stimulates T cells). Several explanations are possible for this inhibiting effect of melatonin. It is possible that the combination of two immune stimulating agents in supramaximal amounts could obliterate each other. In addition, an inhibitory influence of melatonin on parameters of the immune function has also been demonstrated in human lymphocytes *in vitro*. Natural killer cell activity, DNA, α-interferon and tumour necrosis factor-γ synthesis, as well as the proliferation of T lymphocytes and lymphoblastoid cell lines were depressed by melatonin (27–30). In the present study, melatonin treatment exerted an inhibitory influence on cytolytic, CD8+ cells.

The nature of the immunomodulatory activity of melatonin remains to be determined. There is evidence of high-affinity binding sites for melatonin, mainly in human circulating CD4+ T helper lymphocytes, with few in CD8+ T cytolytic cells and none in B lymphocytes (25). Such a predominant effect on CD4+, T helper cells is supported by the present observations on melatonin efficacy to augment CD4+ cells in submaxillary lymph nodes.

However, expression of Mel_{1a}-melatonin receptor was found in rat thymus and spleen, melatonin receptor mRNA being expressed in all the thymic lymphocyte subpopulations (CD4+, CD8+, doubled positive, doubled negative, and B cells), indicating possible effects of melatonin on all these cells (31). Nuclear melatonin receptors may also mediate immunomodulation, since drugs that bind to retinoid Z receptor/retinoid acid receptor-related orphan receptor receptors are active in experimental models of autoimmune diseases (32). Melatonin is also a potent antioxidant, acting by itself rather than through specific binding sites (33). In addition, melatonin could affect centrally hypothalamic hormone release (34) as well as the activity of autonomic pathways to the lymphoid organs (12). Whether the melatonin rhythm is an important physiological efferent pathway for the suprachiasmatic nuclei to export synchronicity to the immune system

awaits further experiments employing physiological rather than pharmacological conditions (i.e. melatonin suppression and replacement in physiological amounts).

Acknowledgements

This work was supported by grants from the DGES (PB 97–0257), Spain, University of Buenos Aires (TM 07), Consejo Nacional de Investigaciones Científicas y Tēcnicas, Argentina (PIP 4156) and Agencia Nacional de Promoción Científica y Tecnológica, Argentina (PICT 2350 and A97B01).

Accepted 15 February 2000

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