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Aging-induced changes in 24-h rhythms of mitogenic responses, lymphocyte subset populations and neurotransmitter and amino acid content in rat submaxillary lymph nodes during Freund's adjuvant arthritis

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Abstract

In young (two months) and aged (18 months) male rats injected s.c. with Freund's adjuvant or adjuvant's vehicle 18 days earlier, 24-h variations in mitogenic responses, lymphocyte subsets and monoamine and amino acid content were examined in submaxillary lymph nodes. Mitogenic responses to concanavalin A (Con A) and lipopolysaccharide (LPS) were higher during the light phase of daily photoperiod. Old rats exhibited a suppressed or impaired mitogenic response to Con A but not to LPS. Acrophases of 24-h rhythm in lymphocyte subset populations in submaxillary lymph nodes were: 18:37–19:44 h (B cells), 09:00–10:08 h (T and $CD4^+$ cells) and 12:19–15:58 h ($CD8^+$ cells). Aging augmented B cells and decreased T, $CD4^+$ and $CD8^+$ cells. Significant correlations were found between Con A activity and T cells, between lymph node 5HT content and B, T and $CD8^+$ lymphocytes, and between lymph node 5HT and taurine and GABA content. Aging increased lymph node 5HT content but did not modify NE content. Lymph node concentration of aspartate, glutamate and taurine was higher at night while that of GABA attained peak values at late afternoon. Old rats injected with Freund's adjuvant showed a higher mean value (glutamate) and smaller amplitude (glutamate, taurine) than their respective young controls. The results further document the effects of aging on the chronobiology of the immune system.

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1. Introduction

Aging is characterized by changes in circadian rhythms, the most marked modification being the attenuation of amplitude. An advance of phase, a shortening of period and a desynchronization of rhythms were also reported during the aging process (Copinschi and Van Cauter, 1995; Myers and Badia, 1995; Ferrari et al., 1996; Touitou et al., 1997). An aspect of the circadian organization in aged subjects less often addressed is the modification in amplitude or phase of circadian rhythms during an immune challenge. Both humoral and delayed (cellular) immunity show regularly rhythmic variations in various frequencies (remarkable the circadian one) (Haus and Smolensky, 1999) and thus the response of the immune system to introduction of an antigen varies in extent in this frequency range (Nelson and Demas, 1996).

Since aging is associated with declines in multiple areas of immune function it seems feasible that differences in circadian response to an immune challenge occur with age (Born et al., 1995; LeMaoult et al., 1997; Pawelec et al., 1999). Among the immune challenges employed in animal studies, one widely used is the injection of Freund's complete adjuvant. Its administration to rats leads to an inflammatory disease of the joints that shows four stages in its time-course: preclinical (first week), acute (weeks 2–4), post-acute (weeks 5–8) and recovery (weeks 9–11) (Pearson and Wood, 1959; Calvino et al., 1987). In Freund's adjuvant-injected rats, the 24-h organization of the biologic responses is altered (Neidhart and Fluckiger, 1992; Sarlis et al., 1992; Holmes et al., 1995; Cardinali et al., 1998a; Duvilanski et al., 1998).

The objective of the present study was to examine the 24-h changes in mitogenic responses and in lymphocyte subset groups in submaxillary lymph nodes of young (two months) and aged (18 months) rats injected with Freund's adjuvant or adjuvant's vehicle 18 days earlier. The changes in immune parameters were correlated with lymph node content of norepinephrine (NE) and serotonin (5HT), and with lymph node amino acid content.

2. Materials and Methods

2.1. Chemicals

Thymidine [methyl-^{3}H] (specific activity 20 Ci/mmol) was purchased from NEN Research Products, Boston, MA, USA. *O*-Phthalaldehyde (OPA), 2-mercaptoethanol and amino acid standards were purchased from Sigma Chemical Co., St. Louis, MO, USA. Freund's complete adjuvant was obtained from Difco, Detroit, IL, USA. Double-distilled deionized water was used for preparation of solutions and buffers.

2.2. Animals and experimental design

Experiments were carried out in adult male Wistar rats (180–220 g), kept under light between 08:00 and 20:00 hours daily. Rats had access to food and water ad libitum.

Adequate measures were taken to minimize pain or discomfort, in accordance with the principles and procedures outlined in European Communities Council Directives (86/609/EEC).

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 hours. Assessment of arthritis development was made clinically (Pearson and Wood, 1959; Calvino et al., 1987). Although arthritis is induced most easily in inbred Lewis rats, it is also produced, to a milder extent, in Wistar rats (Pearson, 1956; Holoshitz et al., 1984; Knight et al., 1992; Stenzel Poore et al., 1993; Tanaka et al., 1996). The course of adjuvant-induced arthritis was followed by behavioral observations including those of spontaneous behavior-mobility, exploring, rearing and scratching. Eighteen days after Freund's adjuvant injection a lack of mobility and exploring behavior, an increase in scratching behavior and signs of hyperalgesia were clearly established as compared with the adjuvant's vehicle-injected group. At this time, groups of 5–7 rats from each experimental group were killed by decapitation at six different time intervals throughout a 24-h cycle and their submaxillary lymph nodes were removed aseptically, weighed and placed in Petri dish containing balanced salt solution, the cells being gently teased apart. After removing the clumps by centrifugation, the cells were suspended in sterile supplemented medium (RPMI 1640), containing 10% heat-inactivated, fetal bovine serum, 20 mM L-glutamine, 0.02 mM 2-mercaptoethanol and gentamicin (50 mg/ml), and were counted.

2.3. Mitogen assays

Mitogen assays were performed as described elsewhere (Esquifino et al., 1996). Submaxillary lymph node cells were 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.

2.4. Lymphocyte subsets

The relative size distributions of lymph cells in submaxillary lymph nodes of rats were determined by FACS analysis, as previously described (Castrillón et al., 2000; Esquifino et al., 2000). 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 recognize 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, San Diego, CA, USA). Lymphocytes from submaxillary lymph nodes isolated as indicated above, were washed in cold 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 analyzed by fluorescence activated cell sorting (FACStar^{plus}; Beckton Dickinson, Mountain View, CA). Dead cells were excluded by gating with propidium iodide.

2.5. High pressure liquid chromatography

To measure monoamine transmitter content, aliquots of lymph nodes were weighed and homogenized in chilled (0–1°C) 2 M acetic acid. After centrifugation (at 15 000*g* for 30 min, at 5°C), the samples were analyzed by HPLC, using electrochemical detection (Coulochem, 5100A, ESA; USA). A C-18 reverse-phase column eluted with a mobile phase (pH 4, 0.1 M sodium acetate, 0.1 M citric acid, 0.7 mM sodium octylsulphate and 0.57 mM EDTA containing 10% methanol, v/v), was employed. Flow rate was 1 ml/min, at a pressure of 2200 psi. Fixed potentials against H_2/H^+ reference electrode were: conditioning electrode: −0.4 V; preoxidation electrode: +0.10 V; working electrode: +0.35 V. NE and 5HT concentration were calculated from the chromatographic peak heights by using external standards. The linearity of the detector response was tested within the concentration ranges found in supernatants.

Amino acid concentration was measured by HPLC using fluorescence detection after pre-column derivatization with OPA as described previously (Duvilanski et al., 1998). An aliquot of tissue supernatant containing homoserine as an internal standard was neutralized with NaOH (4 M) and was reacted with OPA reagent (4 mM OPA, 10% methanol, 2.56 mM 2-mercaptoethanol, in 1.6 M potassium borate buffer, pH 9.5) for 1 min at room temperature. At the end of this period, the reaction was stopped by adding acetic acid (0.5% v/v). Samples were immediately loaded through a Rheodyne (Model 7125) injector system (50 μl loop) to reach a C-18 reverse-phase column (4.6 mm ID × 150 mm, Nucleosil 5, 100A). Elution was achieved by means of a mobile phase consisting of 0.1 M sodium acetate buffer (pH 6.5) containing 35% methanol, at a flow rate of 1 ml/min and a pressure of 140 bars. The column was subsequently washed with the same buffer containing 70% methanol and re-equilibrated with the elution buffer before reuse. The filter fluorometer was set at the following wavelengths: excitation: 340 nm, emission: 455 nm. The procedure allowed a distinct separation and resolution of the amino acids measured. Amino acid content was calculated from the chromatographic peak heights by using standard curves and the internal standard. The linearity of the detector response for aspartate, glutamate, taurine and GABA tested within the concentration ranges found in lymph node extracts.

2.6. Statistical analysis

Statistical analysis of results was performed by employing a one-way analysis of

variance (ANOVA), a two-way factorial ANOVA, regression analysis or a Cosinor analysis. The latter was used to analyze general rhythmic parameters, i.e. acrophase (the maximum of the sinusoidal 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 values lower than 0.05 were considered significant.

3. Results

Fig. 1 shows the 24-h changes in mitogenic responses to Con A and LPS in cells derived from submaxillary lymph nodes of young and old rats 18 days after receiving Freund's adjuvant or its vehicle. As revealed by individual one-way ANOVA, time-of-day changes were significant for young rats injected with Freund's adjuvant or its vehicle (Con A and LPS) and for old rats injected with Freund's adjuvant (Con A). Mitogenic responses were highest during the light phase of daily photoperiod, with acrophases varying from 11:47 to 14:44 hours, as shown by Cosinor analysis (Table 1). As compared to young rats injected with Freund's adjuvant, lymph node cells derived from old rats receiving the mycobacterial adjuvant showed significantly lower mean values (mesor) and amplitude values of 24-h rhythm in Con A activity. Analyzed as a main factor in a two-way ANOVA, aging decreased Con A activity ($F = 7.04$, $p = 0.009$) but was devoid of effect on that of LPS. The differences between rats receiving Freund's adjuvant and its vehicle did not attain significance (two-way factorial ANOVA).

Fig. 2 depicts the 24-h changes in B, T, $CD4^+$ and $CD8^+$ lymphocytes in submaxillary lymph nodes in young and old rats. Time-of-day changes, as detected by one-way ANOVA, were significant for all experimental groups examined, maxima in B cell group occurring at late afternoon–early night. Acrophases of daily rhythm in B cells, as studied by Cosinor analysis, were 18:37–19:44 hours (Table 2). Maxima in B cells tended to correlate with minima observed in T, $CD4^+$ and $CD8^+$ cell groups. Acrophases found were: 09:00–10:08 hours (T cells, $CD4^+$ cells) and 12:19–15:58 hours ($CD8^+$ cells) (Table 2). Factor analysis in a two-way factorial ANOVA indicated that aging augmented significantly B cells ($F = 24.6$, $p = 0.00001$) while it decreased T ($F = 28.1$, $p = 0.00001$), $CD4^+$ ($F = 9.16$, $p = 0.0032$) and $CD8^+$ cells ($F = 33.2$, $p = 0.00001$). Cosinor analysis indicated significantly higher mesor values for 24-h rhythm in B cells in old rats injected with adjuvant's vehicle ($p < 0.05$), a decrease in mesor and amplitude of daily rhythm of $CD8^+$ cells in old rats injected with adjuvant's vehicle ($p < 0.05$), and a decrease in mesor of daily rhythm of $CD8^+$ cells in old rats injected with Freund's adjuvant ($p < 0.03$) (Table 2). Con A activity correlated significantly with T cells ($p < 0.001$; Fig. 3).

The 24-h variations in NE and 5HT content of submaxillary lymph nodes in the same groups of young and old rats are depicted in Fig. 4. Time-related changes in NE or 5HT content were not significant. Aging increased significantly submaxillary lymph node 5HT content, when analyzed as a main factor in a factorial ANOVA ($F = 35.6$, $p = 0.00001$). Lymph node 5HT content correlated significantly with B, T and $CD8^+$ lymphocyte subsets, and with lymph node taurine and GABA content (Fig. 5).

The 24-h changes in lymph node concentration of aspartate, glutamate, taurine and

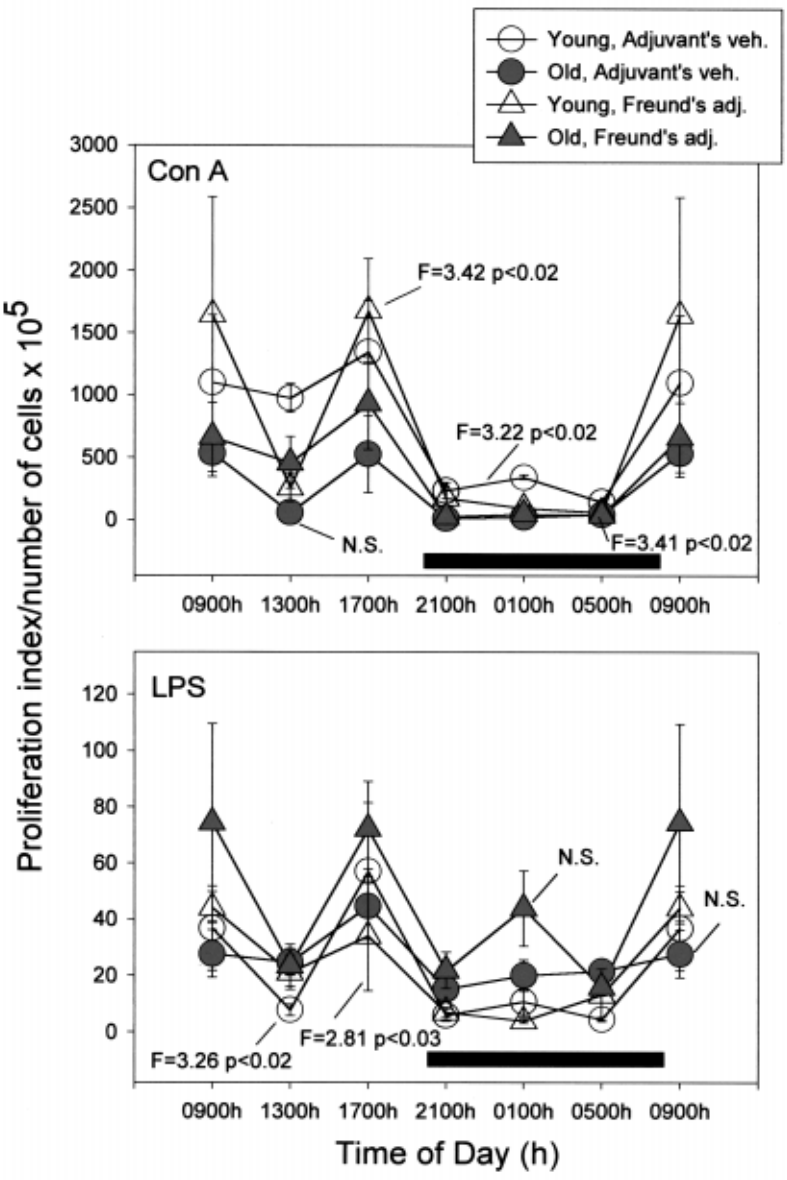


Fig. 1. Twenty-four hour variations in mitogenic responses to Con A and LPS in cells derived from submaxillary lymph nodes of young and old rats receiving Freund's adjuvant or its vehicle. Groups of 5–7 rats were killed by decapitation at six different time intervals throughout a 24-h cycle, as described in Methods. Shown are the means ± SEM. *F* and *p* values in a one-way ANOVA are depicted for those experimental groups in which time-of-day-related differences among means were significant; NS: not significant. For further statistical analysis, see text.

Table 1
Cosinor analysis of 24-h variations in mitogenic responses to Con A and LPS in cells derived from submaxillary lymph nodes of young and old rats receiving Freund's adjuvant or its vehicle. For details see legend to Fig. 1 (Shown are the means ± SEM ($n = 5$–7/group). Mesor (the statistical estimate of the mean in cosinor analysis) and amplitude values are expressed as proliferation index/number of cells × 10^5. Labels designate significant differences as compared to young rats injected with Freund's adjuvant in a Student's t-test: * $p < 0.02$; **, $p <$ 0.05; and NS, not significant daily changes in a one-way ANOVA)

	Mesor	Amplitude	Acrophase (h, min)	Percent of rhythm
Con A				
Young, adjuvant's vehicle	690 ± 81	564 ± 75	13:40 ± 02:32	73.1 ± 11.2
Old, adjuvant's vehicle	NS	NS	NS	NS
Young, Freund's adjuvant	651 ± 78	570 ± 62	13:17 ± 03:32	32.2 ± 9.4
Old, Freund's adjuvant	360 ± 55*	398 ± 44**	13:43 ± 02:02	64.0 ± 11.1
LPS				
Young, adjuvant's vehicle	19.1 ± 2.6	14.1 ± 3.7	14:44 ± 01:24	27.6 ± 4.5
Old, adjuvant's vehicle	NS	NS	NS	NS
Young, Freund's adjuvant	20.0 ± 3.4	16.3 ± 5.4	11:47 ± 02:34	64.5 ± 7.8
Old, Freund's adjuvant	NS	NS	NS	NS

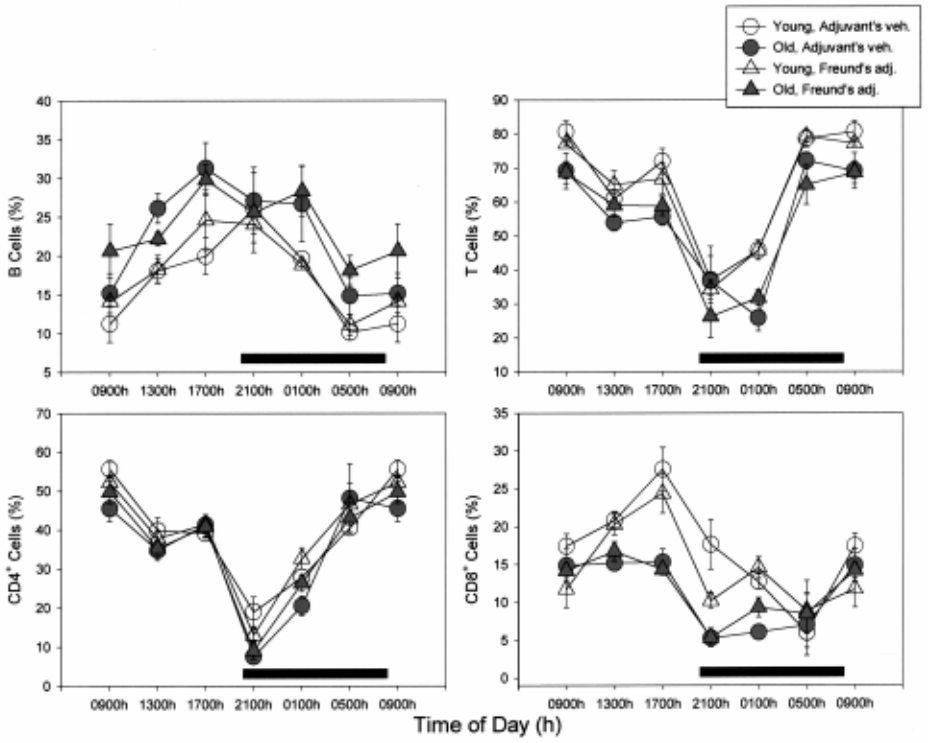


Fig. 2. Twenty-four hour changes in B, T, $CD4^+$ and $CD8^+$ lymphocytes in submaxillary lymph nodes of young and old rats receiving Freund's adjuvant or its vehicle. Cell groups were evaluated by FACS. Shown are the means ± SEM. Time-of-day changes were significant, as detected in a one-way ANOVA, for all experimental groups examined. For further statistical analysis, see text.

Table 2
Cosinor analysis of 24-h variations in B, T, $CD4^+$ and $CD8^+$ lymphocytes in submaxillary lymph nodes of young and old rats receiving Freund's adjuvant or its vehicle. For details see legend to Fig. 2(Shown are the means ± SEM ($n = 5$–7/group). Mesor (the statistical estimate of the mean in cosinor analysis) and amplitude values are expressed as %. Labels designate significant differences as compared to a similar treatment in young animals, *, $p < 0.03$, **, $p < 0.05$, Student's t test, and NS, not significant daily changes in a one-way ANOVA)

	Mesor	Amplitude	Acrophase (h, min)	Percent of rhythm
B Cells				
Young, adjuvant's vehicle	16.0 ± 2.4	7.2 ± 1.6	19:44 ± 03:32	85.4 ± 12.2
Old, adjuvant's vehicle	23.1 ± 1.8*	8.4 ± 2.2	18:37 ± 01:54	87.3 ± 9.8
Young, Freund's adjuvant	18.1 ± 2.5	6.6 ± 1.7	18:43 ± 03:32	97.0 ± 11.3
Old, Freund's adjuvant	23.6 ± 2.5	4.7 ± 0.8	19:49 ± 02:22	68.8 ± 11.0
T Cells				
Young, adjuvant's vehicle	62.1 ± 7.8	18.2 ± 3.5	09:36 ± 01:56	62.5 ± 4.9
Old, adjuvant's vehicle	51.6 ± 6.8	18.7 ± 4.7	09:39 ± 02:09	62.8 ± 7.6
Young, Freund's adjuvant	61.3 ± 6.9	19.9 ± 3.9	09:23 ± 01:19	74.2 ± 11.6
Old, Freund's adjuvant	51.1 ± 7.7	20.7 ± 3.5	10:08 ± 02:32	79.5 ± 12.7
$CD4^+$ Cells				
Young, adjuvant's vehicle	36.5 ± 4.6	14.5 ± 2.0	09:51 ± 02:05	82.8 ± 10.9
Old, adjuvant's vehicle	32.5 ± 4.5	16.2 ± 3.7	09:28 ± 03:35	62.3 ± 9.5
Young, Freund's adjuvant	36.8 ± 6.7	14.6 ± 2.0	09:00 ± 01:43	69.9 ± 10.2
Old, Freund's adjuvant	36.6 ± 7.2	15.4 ± 3.4	09:26 ± 02:09	69.3 ± 11.1
$CD8^+$ Cells				
Young, adjuvant's vehicle	16.5 ± 2.1	8.6 ± 1.1	15:58 ± 01:56	88.4 ± 11.8
Old, adjuvant's vehicle	10.3 ± 1.4*	5.5 ± 0.8**	12:49 ± 02:45	88.7 ± 13.0
Young, Freund's adjuvant	15.5 ± 1.6	6.5 ± 0.9	15:47 ± 03:23	64.6 ± 11.0
Old, Freund's adjuvant	11.0 ± 1.2**	4.9 ± 1.7	12:19 ± 01:15	78.6 ± 9.7

GABA are depicted in Fig. 6. Generally, the concentrations of aspartate, glutamate and taurine were higher at night, acrophases varying from 20:58 to 04:08 hours (Table 3). In the case of GABA, the acrophase of 24-h rhythm in old rats receiving adjuvant's vehicle was 18:25 hours. Analyzed a main factor in a two-way ANOVA, aging augmented lymph node levels of glutamate ($F = 7.39$, $p = 0.007$) and taurine ($F = 20.2$, $p = 0.00001$), but not those of aspartate or GABA. In a Cosinor analysis, old rats injected with Freund's adjuvant showed a higher mean value (glutamate) and a smaller amplitude (glutamate, taurine) than their respective group of young rats ($p < 0.05$) (Table 3). The differences between rats receiving Freund's adjuvant and its vehicle did not attain significance in a factorial ANOVA for any of the amino acids studied.

4. Discussion

As shown by the foregoing results, an age-dependent, significant effect of immune-mediated inflammatory response to Freund's adjuvant on 24-h rhythms in immune parameters occurred in submaxillary lymph node of Wistar rats. Cells derived from lymph nodes of old rats injected with Freund's adjuvant or its vehicle exhibited a suppressed or impaired 24-h rhythmicity in mitogenic response to Con A. Aging decreased the activity

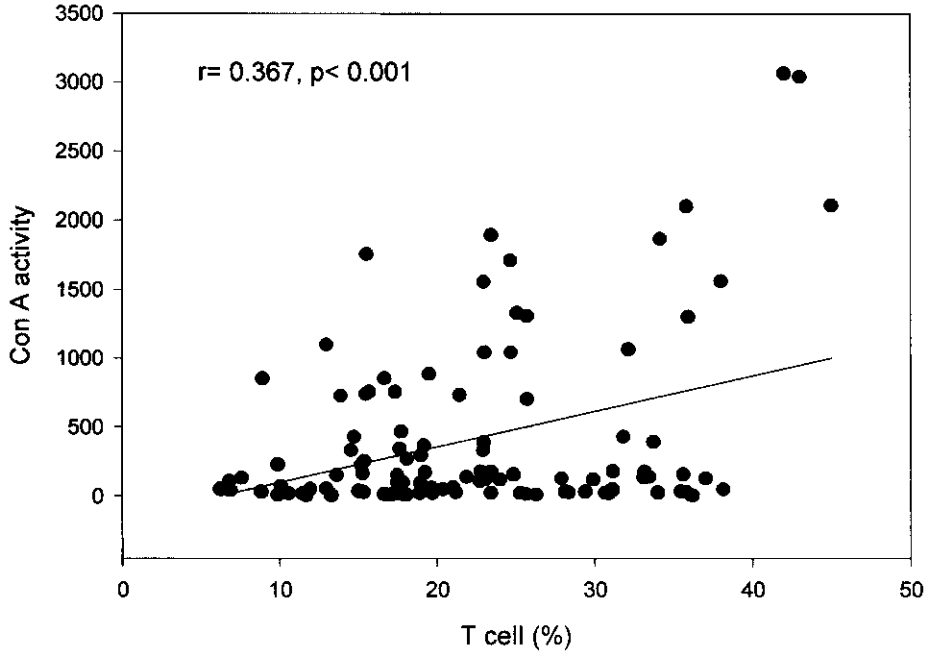


Fig. 3. Correlation between lymph node T cells and Con A mitogenic effect in the whole group of rats studied. Shown are *r* value and its statistical significance.

of Con A, but not that of LPS, in lymph nodes, and decreased T, $CD4^+$ and $CD8^+$ cell subsets. The findings keep in pace with the widely held hypothesis that the continuous activation, clonal expansion and elimination of T cells with age eventually lead to a decreased T cell clonal expansion, changes in T cell repertoire and reduced efficiency of T cell effector functions (LeMaoult et al., 1997). As far as the increase in B cell number observed in aged rats, it agreed with the observation that in immunological aging, B cells or antigen-presenting cells remain relatively unimpaired (Pawelec et al., 1999). Indeed, the activity of LPS (a T cell-independent phenomenon) in submaxillary lymph node cells reported herein remained unaffected.

The subject of age-related changes in 24-h rhythms of immune parameters during adjuvant's arthritis was addressed in a previous study by measuring ornithine decarboxylase as an index of lymph cell proliferation in submaxillary lymph nodes and spleen of Sprague Dawley rats (Cardinali et al., 1998a). After Freund's adjuvant injection, mean value and amplitude of 24-h rhythm in lymph node and splenic ornithine decarboxylase were lowest in old rats. In the same study, an increase in hindpaw and forepaw joint diameters as compared to adjuvant's vehicle-treated controls was found on day 16 after Freund's adjuvant injection. Old rats receiving the mycobacterial adjuvant showed a milder inflammatory response of the ankle joints than the young animals (Cardinali et al., 1998a). Collectively, the previous and present results allow the conclusion that aging decreases the amplitude of 24-h rhythm in immune responsiveness in rats.

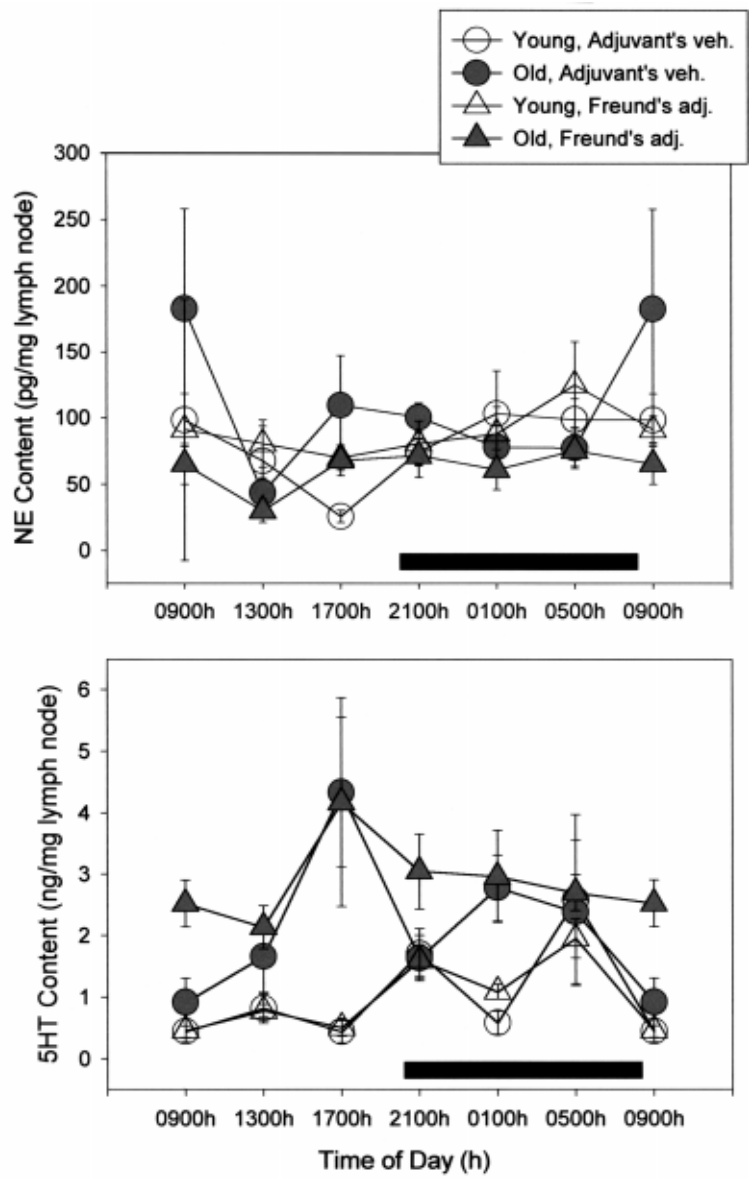


Fig. 4. Twenty-four hour variations in NE and 5HT content in submaxillary lymph nodes of young and old rats receiving Freund's adjuvant or its vehicle. Groups of 5–7 rats were killed by decapitation at six different time intervals throughout a 24 h cycle, as described in Methods. Shown are the means ± SEM. Individual one-way ANOVAs performed for 24-h-related changes in each experimental group indicated absence of significant differences among means. For further statistical analysis, see text.

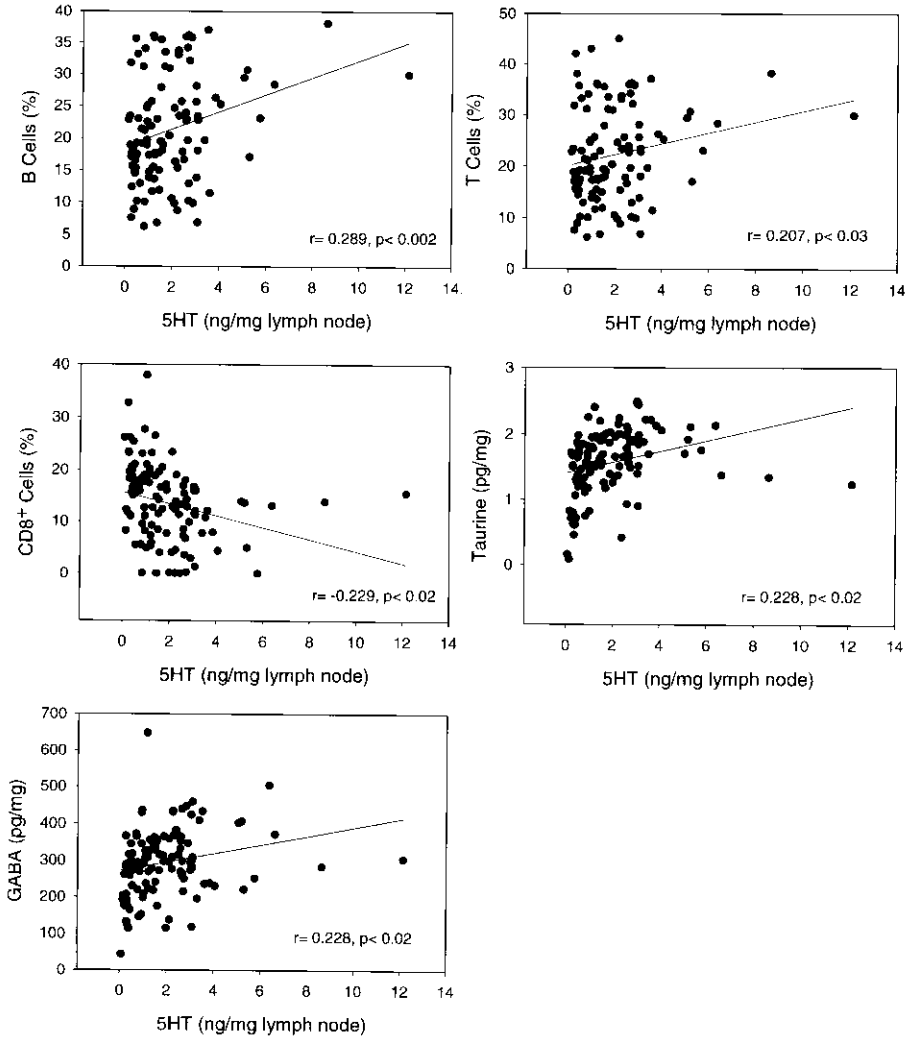
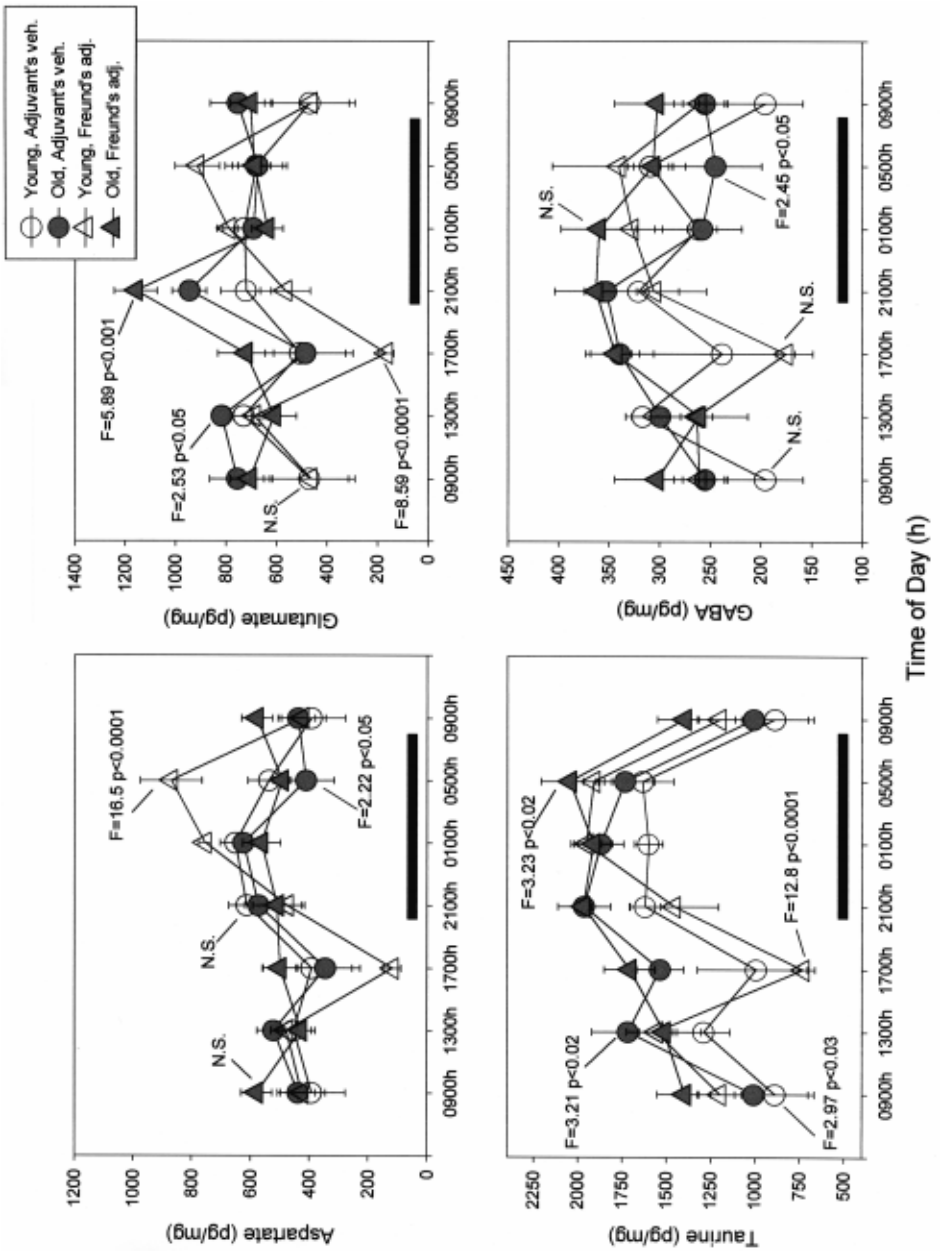


Fig. 5. Correlation between lymph node 5HT content and B, T and $CD8^+$ lymphocyte subsets, and taurine and GABA content, in the whole group of rats studied. Shown are *r* values and their statistical significance.

Among brain areas whose activity decline with age, the impaired circadian activity of the suprachiasmatic nuclei, needed for the generation of circadian rhythms and for synchronization of rhythms with lightdark cycles, could account for the blunting of circadian rhythms (Benloucif et al., 1997; Cai et al., 1997; Li and Satinoff, 1998). The circadian output of the suprachiasmatic nuclei is transmitted to the rest of the body through neuronal and endocrine pathways, the principal neuronal pathway being circuitry of the autonomic nervous system. In previous studies we demonstrated that the activity of the central oscillator, driven to the organs in part via the autonomic nervous system and


Young, Adjuvant's veh.
Old, Adjuvant's veh.
Young, Freund's adj.
Old, Freund's adj.
Glutamate (pg/mg)
F=5.89 p<0.001
F=2.53 p<0.05
N.S.
F=8.59 p<0.0001
GABA (pg/mg)
N.S.
N.S.
N.S.
F=2.45 p<0.05
Aspartate (pg/mg)
F=16.5 p<0.0001
N.S.
N.S.
F=2.22 p<0.05
Taurine (pg/mg)
F=3.23 p<0.02
F=3.21 p<0.02
F=12.8 p<0.0001
F=2.97 p<0.03
0900h 1300h 1700h 2100h 0100h 0500h 0900h
Time of Day (h)

by the melatonin signal, deteriorates significantly with age (Brusco et al., 1998; Cardinali et al., 1998b). Since the suprachiasmatic nuclei themselves are sensitive to immune-derived signals, the chronic stress condition given by mycobacterial adjuvant injection is probably instrumental to inhibit a number of circadian rhythms at different phases of the disease.

It must be noted that the view of a general impairment of immune functions associated with aging was challenged by clinical studies evaluating various cytokines and lymphocyte subsets (Born et al., 1995). Advanced age was associated with a decreased number of T cells and decreases in the major T cell subsets ($CD4^+$, $CD8^+$ cells). However, the production of interferon-gamma was enhanced, and numbers of activated T cells, natural killer cells and T cells expressing interleukin-2 receptors increased in the aged subjects (Born et al., 1995). In the present study, a significant positive correlation was found between Con A activity and lymph node T cell number, suggesting that the decrease in number of T cells in aging was accompanied by parallel decreases in T cell-mediated effects, like the response to Con A. Thus, the present results do not support the existence of an enhanced responsiveness of the T cell compartment in aged rats that may compensate for the decrease in T cells.

In the present study, aging increased lymph node 5HT content, but did not modify that of NE. The results agree with the lack of diurnal differences found in splenic NE content in mice (Kelley et al., 1996). In a previous study on the effect of aging on tyrosine hydroxylase activity in submaxillary lymph nodes, we reported a smaller amplitude and mean value of enzyme activity rhythm in old rats up to 18 days after Freund's adjuvant injection (Cardinali et al., 1998a). Therefore, as in other situations, the determination of NE concentration is considerably less sensitive as a parameter than to measure the activity of the rate-limiting enzyme in NE synthesis.

As shown above, submaxillary lymph node 5HT content correlated significantly with B, T and $CD8^+$ lymphocyte groups. 5HT in lymph nodes is associated with the presence of mast cells, as well as with interfollicular macrophages in the parenchyma of the lymphoid organs and may modulate immune responsiveness in rodents, as shown by the depression of T cell-dependent primary immune responses after repeated injection of 5HT to mice (Bliznakov, 1980).

A last aspect of the present study deserves comment. The foregoing results indicate that aging augmented lymph node levels of glutamate and taurine. The data agreed with the existence of an increased production of glutamate in the mesenteric lymph nodes of aged rats (Almeida et al., 1996). Therefore, amino acid metabolism in lymph nodes appears to be significantly affected by the aging process. It must be noted that glucose is not the

Fig. 6. Twenty-four hour variations in aspartate, glutamate, taurine and GABA content in submaxillary lymph nodes of young and old rats receiving Freund's adjuvant or its vehicle. Groups of 5–7 rats were killed by decapitation at six different time intervals throughout a 24-h cycle, as described in Methods. Shown are the means ± SEM. F and p values in a one-way ANOVA are depicted for those experimental groups in which time-of-day-related differences among means were significant; NS: not significant. For further statistical analysis, see text.

Table 3
Cosinor analysis of 24-h variations in aspartate, glutamate, taurine and GABA content in submaxillary lymph nodes of young and old rats receiving Freund's adjuvant or its vehicle. For details see legend to Fig. 6(Shown are the means ± SEM ($n = 5$–7/group). Mesor (the statistical estimate of the mean in cosinor analysis) and amplitude values are expressed as pg/mg lymph node. * Significantly different as compared to young rats injected with Freund's adjuvant, $p < 0.05$, Student's t-test, NS, not significant daily changes in a one-way ANOVA; (–): not significant changes in Cosinor)

	Mesor	Amplitude	Acrophase (h, min)	Percent of rhythm
Aspartate				
Young, adjuvant's vehicle	NS	NS	NS	NS
Old, adjuvant's vehicle	485 ± 68	71 ± 11	23:53 ± 03:22	27.1 ± 5.4
Young, Freund's adjuvant	520 ± 79	302 ± 53	03:47 ± 01:14	78.5 ± 11.7
Old, Freund's adjuvant	NS	NS	NS	NS
Glutamate				
Young, adjuvant's vehicle	NS	NS	NS	NS
Old, adjuvant's vehicle	–	–	–	–
Young, Freund's adjuvant	598 ± 62	256 ± 35	04:08 ± 02:00	56.1 ± 7.7
Old, Freund's adjuvant	785 ± 69*	161 ± 22*	20:58 ± 02:22	37.9 ± 5.5
Taurine				
Young, adjuvant's vehicle	1339 ± 222	332 ± 45	00:42 ± 01:23	59.6 ± 7.8
Old, adjuvant's vehicle	1639 ± 156	337 ± 36	22:12 ± 02:09	55.6 ± 6.6
Young, Freund's adjuvant	1469 ± 150	445 ± 54	03:23 ± 01:08	59.4 ± 7.1
Old, Freund's adjuvant	1760 ± 180	292 ± 41*	00:07 ± 01:17	74.8 ± 9.9
GABA				
Young, adjuvant's vehicle	NS	NS	NS	NS
Old, adjuvant's vehicle	291 ± 43	57 ± 11	18:05 ± 02:09	90.7 ± 11.2
Young, Freund's adjuvant	NS	NS	NS	NS
Old, Freund's adjuvant	NS	NS	NS	NS

only quantitatively significant energy substrate in lymph nodes, since glutamine at near-physiological concentration can be readily utilized by these cells (Wu et al., 1991).

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