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Diurnal changes in cyclosporine effect on ornithine decarboxylase and noradrenergic and cholinergic activities in submaxillary lymph nodes

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Abstract

Diurnal changes in cyclosporine efficacy to affect ornithine decarboxylase activity, [^{3}H]norepinephrine uptake and [^{3}H]choline conversion into [^{3}H]acetylcholine were examined in rat submaxillary lymph nodes. Cyclosporine (5 or 20 mg/kg) caused a dose-dependent decrease in lymph node ornithine decarboxylase, being active at 5 or 20 mg/kg in Freund's adjuvant-treated rats, and at 20 mg/kg in rats treated with the adjuvant's vehicle. In immunized rats the lower cyclosporine dose was ineffective when injected during the night. Cyclosporine increased lymph node [^{3}H]norepinephrine uptake dose dependently, with significant differences between the 20 mg/kg dose and controls in vehicle-treated rats and between 5 or 20 mg/kg and controls in Freund's adjuvant-treated rats. In immunized rats, 5 mg/kg cyclosporine increased [^{3}H]norepinephrine uptake when injected at 13:00 or 17:00 h. Both doses of cyclosporine augmented lymph node synthesis of [^{3}H]acetylcholine to a similar extent. In immunized and non-immunized rats cyclosporine suppressed the diurnal rhythm of lymph node adrenergic and cholinergic activity found in controls. After unilateral sympathetic denervation (by superior cervical ganglionectomy) and/or unilateral parasympathetic decentralization (by chorda tympani section), cyclosporine (5 mg/kg) decreased Freund's adjuvant-induced activation of lymph node ornithine decarboxylase when injected at 17:00 or 01:00 h. On the sham-operated side, cyclosporine was effective when injected at 17:00 h only. Decentralization, or a combined ganglionectomy plus decentralization, decreased lymph node ornithine decarboxylase activity. The results indicate active regulation of the effects of cyclosporine in submaxillary lymph nodes by local autonomic nerves.

Keywords: Cyclosporine chronopharmacology; Sympathetic nervous system; Parasympathetic nervous system; Submaxillary lymph node; Ornithine decarboxylase; Norepinephrine uptake; Acetylcholine synthesis; Superior cervical ganglion; Chorda tympani

1. Introduction

Cyclosporine is a potent immunosuppressive drug widely used to avert allograft rejection and in the therapy of autoimmune disorders. Both the pharmacology and the toxicology of cyclosporine are dependent on a number of factors, knowledge of which is important for therapeutic optimization. Among these the time of day of administration must be considered as relevant. Indeed, a number of studies have indicated the existence of a circadian modification of cyclosporine pharmacokinetics in experimental animals and in humans (Magnus et al., 1985; Bowers et al., 1986; Canafax et al., 1988; Luke et al., 1988; Sangalli et al., 1988; Ramon et al., 1989; Sabate et al., 1990; Cugini et al., 1991; Levi et al., 1991; Malmary et al., 1991, 1992; Ohlman et al., 1993; Batalla et al., 1994).

Cyclosporine decreases the activity of ornithine decarboxylase in lymphoid and non-lymphoid tissues (Fidelius et al., 1984; Esquifino et al., 1991, 1994, 1995). Ornithine decarboxylase catalyses the initial, rate-limiting, step in polyamine biosynthesis and is often used as an indicator of cell proliferation and growth (Russel, 1985). In lymphoid-competent organs, this enzyme is taken as an indicator for immunomodulatory phenomena (Endo, 1984; Fidelius et al., 1984; Neidhart, 1989).

In previous studies we showed that surgical sympathectomy and/or parasympathectomy of submaxillary lymph nodes in rats modified the suppressive activity of cyclosporine on ornithine decarboxylase activation induced

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by Freund's complete adjuvant (Esquifino et al., 1991, 1994). Conversely, cyclosporine exerted a stimulatory effect on presynaptic adrenergic and cholinergic markers in submaxillary lymph nodes (Esquifino et al., 1991, 1994, 1995; Esquifino and Cardinali, 1994). Since exposure to stressor agents is an effective means to change neural signals in local autonomic nerves (Appenzeller, 1990), we also tested whether the activity of cyclosporine could be modified during stress. The results obtained indicated that the immunosuppressive effects of cyclosporine diminished by stress, in part due to changes in the traffic of neural signals in local sympathetic nerves (Esquifino et al., 1995).

The present study was carried out to search for the existence of neurally dependent dosing time effects of cyclosporine in rat submaxillary lymph nodes. Specifically, we wished to answer the following questions: (a) does the efficacy of cyclosporine to decrease Freund's adjuvant-induced ornithine decarboxylase activation in submaxillary lymph nodes change diurnally?; (b) does cyclosporine exert a time-of-day-dependent effect on presynaptic noradrenergic and cholinergic markers in submaxillary lymph nodes?; (c) does regional sympathectomy and/or parasympathectomy of submaxillary lymph nodes affect time-of-day changes in cyclosporine activity on ornithine decarboxylase activity?

2. Materials and methods

2.1. Chemicals

Methyl [^{3}H]choline chloride (specific activity 85 Ci/mmol), [^{3}H]norepinephrine (norepinephrine, DL, 7-^{3}H, N, specific activity 12.1 Ci/mmol) and L-[1-^{14}C]ornithine hydrochloride (specific activity 58 Ci/mol) were purchased from NEN (Boston, MA, USA). Diphenyloxazole (PPO) and 1,4-bis{2-(5-phenyl)-oxazolyl}benzene (POPOP) were obtained from Serva (Heidelberg, Germany). Freund's complete adjuvant was purchased from Difco (Detroit, MI, USA). All other drugs and reagents used were obtained from Sigma (St. Louis, MO, USA).

2.2. Animals and experimental design

Experiments were carried out in adult male Wistar rats (180–220 g), kept under conditions with lights on between 06:00 and 18:00 h daily. Rats had access to food and water ad libitum.

A first series of experiments was performed to examine the diurnal changes in cyclosporine efficacy to modify ornithine decarboxylase activity, norepinephrine uptake and [^{3}H]choline conversion into [^{3}H]acetylcholine in submaxillary lymph nodes of rats. Cyclosporine was injected s.c. at doses of 5 or 20 mg/kg for 5 days, at one of six time intervals throughout a 24-h cycle, i.e., at 01:00, 05:00, 09:00, 13:00, 17:00 or 21:00 h. Freund's adjuvant or its vehicle (0.5 ml paraffin oil containing 15% mannide monooleate) was injected s.c. on the third day of treatment, at 11:00 h, as described by Neidhart (1989). Animals were killed 2 h after the last injection of cyclosporine, on the fifth day of treatment. Control groups included rats treated in a similar way with vehicle (instead of cyclosporine) and Freund's adjuvant or the adjuvant's vehicle. Groups of 7–8 rats were killed by cervical dislocation and both submaxillary lymph nodes were dissected out. Submaxillary lymph nodes from one side (selected at random) were kept at −20°C until assayed for ornithine decarboxylase (within 2–3 days) while the contralateral lymph nodes were processed immediately for measurement of [^{3}H]norepinephrine uptake or of [^{3}H]choline conversion into [^{3}H]acetylcholine. In a previous study we did not detect time-dependent effects of Freund's adjuvant on day-night differences in ornithine decarboxylase activity of submaxillary lymph nodes, inasmuch as immunization performed during daylight or at night resulted in similar day-night differences in ornithine decarboxylase activity, with higher enzyme values during daylight (Cardinali et al., 1996).

A second series of experiments was performed to examine the effect of local sympathetic denervation (by superior cervical ganglionectomy) and/or a local parasympathetic decentralization (by chorda tympani section) on day-night difference in cyclosporine efficacy to decrease submaxillary lymph node ornithine decarboxylase activity. Surgery was performed unilaterally under light ether anesthesia (Alito et al., 1987). Each rat received a contralateral sham-operation. The criterion for assessing the completeness of the unilateral superior cervical ganglionectomy was the detection of an ipsilateral palpebral ptosis after the operation up to about 10 h after surgery, an ipsilateral palpebral retraction (due to degeneration activity at the level of the periorbital muscles) seen 8–24 h after surgery, and an irreversible ipsilateral palpebral ptosis seen from the 24th h on. Animals that did not exhibit palpebral retraction, and therefore which had received an incomplete ganglionectomy, were discarded. The side of operation was changed at random in each set of experiments.

The surgical procedures, including the assessment of specific markers of noradrenergic or cholinergic activity in submaxillary lymph nodes, were validated previously (Esquifino et al., 1991, 1994, 1995). Unilateral superior cervical ganglionectomy brought about a 93–95% decrease in norepinephrine content of the ipsilateral submaxillary lymph nodes as compared to the contralateral innervated lymph nodes (Esquifino et al., 1991). After chorda tympani section, choline acetyltransferase activity in the ipsilateral submaxillary lymph nodes decreased to 23%, and neuronal [^{3}H]choline uptake to 22%, of contralateral sham-operated controls (Esquifino et al., 1994).

Ten days after surgery groups of 7–8 rats were injected for 5 days with cyclosporine (5 mg/kg) at 17:00 or 01:00 h, and Freund's complete adjuvant as in experiment 1. Animals were killed 2 h after the last injection of cy-

closporine, on the fifth day of treatment. Control groups included rats treated in a similar way with vehicle (instead of cyclosporine) and Freund's adjuvant. Rats were killed by cervical dislocation and both submaxillary lymph nodes were dissected out and kept at −20°C until assayed for ornithine decarboxylase (within 2–3 days).

2.3. Ornithine decarboxylase activity

Submaxillary lymph nodes were homogenized in chilled phosphate buffer (NaH_2PO_4 and KH_2PO_4, 50 mM each (5:1, v/v), pH 7.2, containing 5 mM NaF, 0.1 mM pyridoxal-PO_4, 0.1 mM EDTA-Na and 2 mM dithiothreitol, as described previously (Esquifino et al., 1991, 1994, 1995). The homogenate was centrifuged (2000 × *g* for 10 min at 4°C) and 200-μl supernatant fractions were incubated in glass tubes fitted with rubber stoppers and center wells containing a filter paper disk spotted with 20 μl of 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 0.5 ml citric acid. The $^{14}CO_2$ liberated by the enzymatic reaction was collected on the filter papers, and the radioactivity was counted in 10 ml 30% Triton X-100–toluene phosphor solution. Results were expressed as pmol of $^{14}CO_2$ released/mg supernatant protein/30 min. The reaction was completely inhibited by addition of 0.25 mM α-difluoromethyl ornithine. Blanks including zero-time controls and heated supernatants were used. Enzyme activity was linear with respect to incubation time and enzyme concentration. Protein concentration was measured by the Lowry procedure, using bovine serum albumin as a standard (Lowry et al., 1951).

2.4. Norepinephrine uptake

For [^{3}H]norepinephrine uptake studies, individual submaxillary lymph nodes were incubated for 30 min in 0.5 ml of a solution of 10 mM Hepes (adjusted at pH 7.4 with Tris base) of the following composition (mM): NaCl, 140; KCl, 5; $CaCl_2$, 2.5; $MgCl_2$, 1; ascorbic acid, 0.11; glucose, 1.1. [^{3}H]norepinephrine (1–2 μCi, 0.16–0.32 μM) and 0.1 mM pargyline (to prevent degradation by monoamine oxidase) were also added. The temperature was kept at 37°C and the solution was bubbled with O_2. Ten minutes after the start of the incubations with [^{3}H]norepinephrine, the submaxillary lymph nodes were quickly separated from media and weighed. After 2 successive washes with fresh portions of buffer, the tissue was weighed and digested with hyamine hydroxide and the radioactivity was measured by liquid scintillation spectrometry. Specific uptake, as shown in the results, was defined as that blocked by incubation in the presence of 10 μM de-

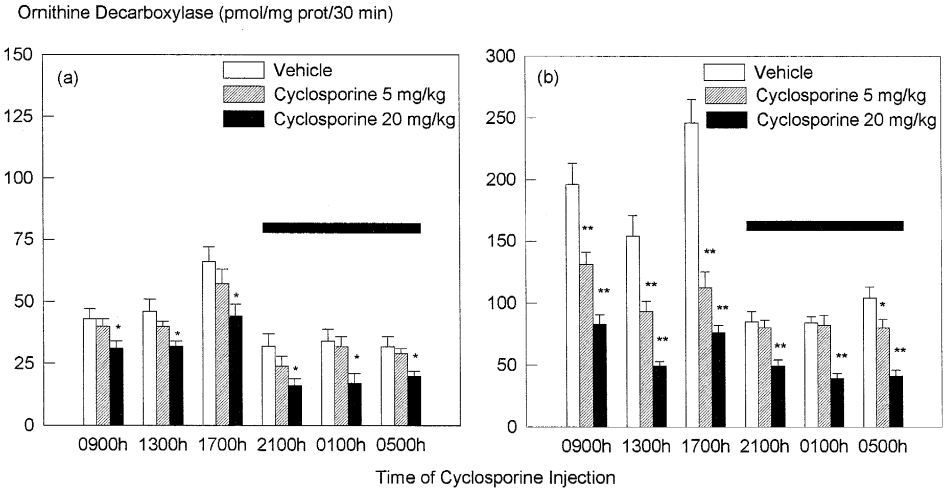

Fig. 1. Effect of cyclosporine (5 or 20 mg/kg), injected for 5 days at one of six different time intervals throughout the 24-h cycle, on ornithine decarboxylase activity in rat submaxillary lymph nodes of rats treated with (a) Freund's adjuvant vehicle or (b) Freund's complete adjuvant. Freund's adjuvant or its vehicle was injected s.c. on the third day of treatment, at 11:00 h. Rats were killed 2 h after the last injection of cyclosporine or cyclosporine vehicle and the submaxillary lymph nodes were dissected out and assayed for ornithine decarboxylase activity as described in Section 2. Shown are the means ± S.E.M. (n = 15–16/group for Freund's adjuvant-treated rats, and n = 7–8/group for vehicle-treated rats). F values in one-way ANOVA at each time interval were: Left panel: 09:00 h: $F(2,19) = 4.91$, $P < 0.02$; 13:00 h: $F(2,19) = 4.32$, $P < 0.03$; 17:00 h: $F(2,19) = 6.24$, $P < 0.009$; 21:00 h: $F(2,19) = 5.87$, $P < 0.02$; 01:00 h: $F(2,19) = 5.24$, $P < 0.02$; 05:00 h: $F(2,19) = 5.24$; $P < 0.02$. Right panel: 09:00 h: $F(2,42) = 22.47$, $P < 0.0001$; 13:00 h: $F(2,40) = 21.41$, $P < 0.0001$; 17:00 h: $F(2,42) = 34.24$, $P < 0.0001$; 21:00 h: $F(2,40) = 9.49$, $P < 0.002$; 01:00 h: $F(2,41) = 19.43$, $P < 0.0001$; 05:00 h: $F(2,43) = 19.15$; $P < 0.0001$. Asterisks designate significant differences in post-hoc Dunnett's t-tests performed at each time interval ($^{*}P < 0.05$; $^{**}P < 0.01$, as compared to the respective vehicle-treated controls). Cyclosporine decreased ornithine decarboxylase activity, as revealed in a factorial ANOVA ($F(2,398) = 133.28$, $P < 0.0001$). A post-hoc Tukey's test indicated significant differences ($P < 0.001$) among the three dose levels. A significant interaction between treatment and time of administration was found in the factorial ANOVA ($F(10,398) = 8.53$, $P < 0.0001$). Ornithine decarboxylase activity was augmented significantly after Freund's adjuvant injection (factorial ANOVA, $F(1,398) = 377.24$, $P = 0.0001$) and regardless of treatment received, it changed diurnally, showing a maximum during the light phase of daily photoperiod ($F(5,398) = 5.08$, $P < 0.0002$).

sipramine (an inhibitor of neuronal norepinephrine uptake). Uptake was linear for up to 20 min. Results were expressed as pmol of [^{3}H]norepinephrine taken up per mg of tissue per 10 min. In the presence of pargyline, more than 92% of medium or tissue radioactivity was identified as authentic norepinephrine after adsorption onto alumina at pH 8.6 and subsequent elution in 0.1 M HCl.

2.5. [^{3}H]Choline conversion to acetylcholine

The conversion of [^{3}H]choline into [^{3}H]acetylcholine was assessed in vitro by a modification of the procedure described by Rand and Johnson (1981), as described elsewhere (Esquifino et al., 1994). After dissection, the tissue was rinsed and homogenized in 100 μl of homogenization buffer (10 mM Tricine, 10 mM sodium thioglycolate, 1 mM EDTA and 1 mM phenanthroline, pH 8.0). Fifty microliters of homogenate (approximately 150 μg of protein) plus 50 μl of incubation buffer (50 mM Tricine, 0.5 mM acetyl coenzyme A, 20 μM eserine and 0.2 μCi of [^{3}H]choline, pH 8.0) were incubated at 37°C for 10 min in 5-ml scintillation vials. One hundred μl of choline kinase solution (100 mM Tris-HCl, 20 mM $MgCl_2$, 10 mM ATP, 0.2 units/ml yeast choline kinase, pH 8.1) was then added and the mixture was incubated for 30 min at 37°C. The reaction was stopped by adding 200 μl of distilled water and 4 ml of toluene-tetraphenylboron scintillation cocktail (0.5% PPO, 0.03% POPOP, 10% isoamylic alcohol, 0.3% sodium tetraphenylboron). With this procedure, unreacted [^{3}H]choline was phosphorylated to phosphoryl-[^{3}H]choline, which remained in the aqueous phase, while newly synthesized [^{3}H]acetylcholine was extracted into the scintillator organic phase. After agitation and a 30-min extraction period, radioactivity was measured in a liquid scintillation spectrometer. Incubations in the absence of tissue or in the presence of boiled submaxillary lymph node homogenates gave identical results and were used as blanks.

2.6. Statistical analysis

Statistical analysis of results was performed by using a factorial analysis of variance (ANOVA) followed by a Tukey's test, by a one-way ANOVA followed by a Dunnett's *t*-test, or by Student's *t*-test, as stated. Each experiment was repeated twice, the combined analysis of results from the 2 independent experiments being shown.

3. Results

Fig. 1 shows the effect of cyclosporine, injected for 5 days at six different time intervals throughout the 24-h cycle, on ornithine decarboxylase activity in rat submaxillary lymph nodes of rats treated with Freund's complete

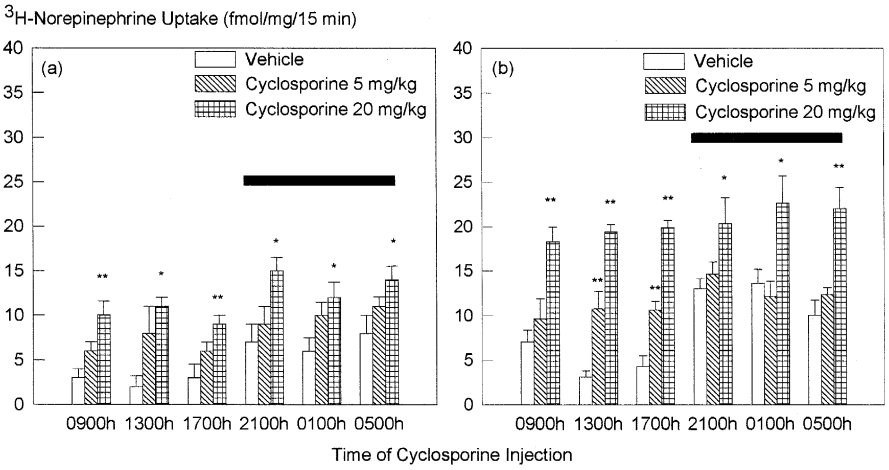

Fig. 2. Effect of cyclosporine (5 or 20 mg/kg), injected for 5 days at one of six different time intervals throughout the 24-h cycle, on [^{3}H]norepinephrine uptake in rat submaxillary lymph nodes of rats treated with (a) Freund's adjuvant vehicle or (b) Freund's complete adjuvant. Animals were treated and killed as described in the legend to Fig. 1. The submaxillary lymph nodes were dissected out and assayed for [^{3}H]norepinephrine uptake as described in Section 2. Shown are the means ± S.E.M. ($n = 7–8$/group). F values in one-way ANOVA at each time interval were: Left panel: 09:00 h: $F(2,20) = 7.70$, $P < 0.003$; 13:00 h: $F(2,20) = 4.99$, $P < 0.02$; 17:00 h: $F(2,20) = 6.52$, $P < 0.007$; 21:00 h: $F(2,20) = 5.13$, $P < 0.02$; 01:00 h: $F(2,20) = 3.60$, $P < 0.04$; 05:00 h: $F(2,20) = 3.76$; $P < 0.04$. Right panel: 09:00 h: $F(2,21) = 10.60$, $P < 0.001$; 13:00 h: $F(2,21) = 39.94$, $P < 0.0001$; 17:00 h: $F(2,20) = 61.88$, $P < 0.0001$; 21:00 h: $F(2,20) = 3.66$, $P < 0.04$; 01:00 h: $F(2,20) = 6.40$, $P < 0.008$; 05:00 h: $F(2,20) = 12.90$, $P < 0.0001$. Asterisks designate significant differences in post-hoc Dunnett's *t*-tests performed at each time interval ($^*P < 0.05$; $^{**}P < 0.01$, as compared to the respective vehicle-treated controls). A factorial ANOVA revealed that cyclosporine augmented uptake in a dose-dependent way ($F(2,261) = 145.97$, $P < 0.0001$), with significant differences among the three dose levels, as shown in a post-hoc Tukey's test ($P < 0.05$). [^{3}H]Norepinephrine uptake was also augmented significantly after Freund's adjuvant injection (factorial ANOVA, $F(1,261) = 121.77$, $P = 0.0001$). Uptake changed diurnally in a significant way, exhibiting a maximum during the dark phase, when analyzed as a global effect in the factorial ANOVA ($F(5,261) = 14.05$, $P < 0.0001$). However, one-way ANOVA comparing time intervals in each experimental group indicated that a diurnal rhythm in lymph node uptake with maxima at night occurred in Freund's adjuvant- ($F(5,39) = 11.93$, $P < 0.0001$) and vehicle-injected control rats ($F(5,39) = 2.54$, $P < 0.05$) only.

adjuvant (right panel) or with its vehicle (left panel). Cyclosporine caused a dose-dependent decrease in enzyme activity, as revealed in a factorial ANOVA performed on the combined results from the 2 experiments carried out ($F(2,398) = 133.28$, $P < 0.0001$). A post-hoc Tukey's test indicated significant differences ($P < 0.001$) among the three dose levels (0, 5 and 20 mg/kg). A significant interaction between treatment and time of administration was found (factorial ANOVA, $F(10,398) = 8.53$, $P < 0.0001$), cyclosporine being less effective when injected during the scotophase. This was further substantiated by a one-way ANOVA performed at each time interval for data from Freund's complete adjuvant-treated rats, which indicated a lack of effect of 5 mg/kg cyclosporine when injected at 21:00 or 01:00 h (Fig. 1, right panel). In the case of vehicle-treated rats (Fig. 1, left panel), only a 20-mg cyclosporine dose was effective at all time intervals to augment ornithine decarboxylase activity (one way ANOVA, Dunnett's *t*-test, $P < 0.05$). Ornithine decarboxylase activity was augmented significantly by Freund's adjuvant injection (factorial ANOVA, $F(1,398) = 377.24$, $P = 0.0001$) and regardless of the treatment received, it changed diurnally, showing a maximum during the light phase of the daily photoperiod ($F(5,398) = 5.08$, $P < 0.0002$).

The time-of-day-dependent effect of cyclosporine on [^{3}H]norepinephrine uptake by rat submaxillary lymph nodes is depicted in Fig. 2. Cyclosporine increased neurotransmitter uptake in a dose-dependent way, as revealed in the factorial ANOVA ($F(2,261) = 145.97$, $P < 0.0001$), with significant differences among the three dose levels, as shown in a post-hoc Tukey's test ($P < 0.05$). In the case of Freund's adjuvant-injected rats (Fig. 2, right panel), a one-way ANOVA performed at each time interval indicated significant effects of 5 mg/kg cyclosporine on [^{3}H]norepinephrine uptake when injected at 13:00 or 17:00 h only ($P < 0.01$, Dunnett's *t*-test). [^{3}H]Norepinephrine uptake was augmented significantly by Freund's adjuvant injection (factorial ANOVA, $F(1,261) = 121.77$, $P = 0.0001$), and when analyzed as a main factor in the factorial ANOVA it was shown to change diurnally, showing a maximum during the dark phase ($F(5,261) = 14.05$, $P < 0.0001$). However, a diurnal rhythm in submaxillary lymph node [^{3}H]norepinephrine uptake with maxima at night was found in Freund's adjuvant- and vehicle-injected control rats (one-way ANOVA, $P < 0.0001$ and $P < 0.05$, respectively) but not in cyclosporine-administered animals (Fig. 2).

Fig. 3 shows the time-of-day-dependent effect of cyclosporine on the conversion of [^{3}H]choline into [^{3}H]acetylcholine by rat submaxillary lymph nodes. Cyclosporine augmented this cholinergic parameter significantly (factorial ANOVA, $F(2,260) = 173.92$, $P < 0.0001$), both doses of cyclosporine being equally effec-

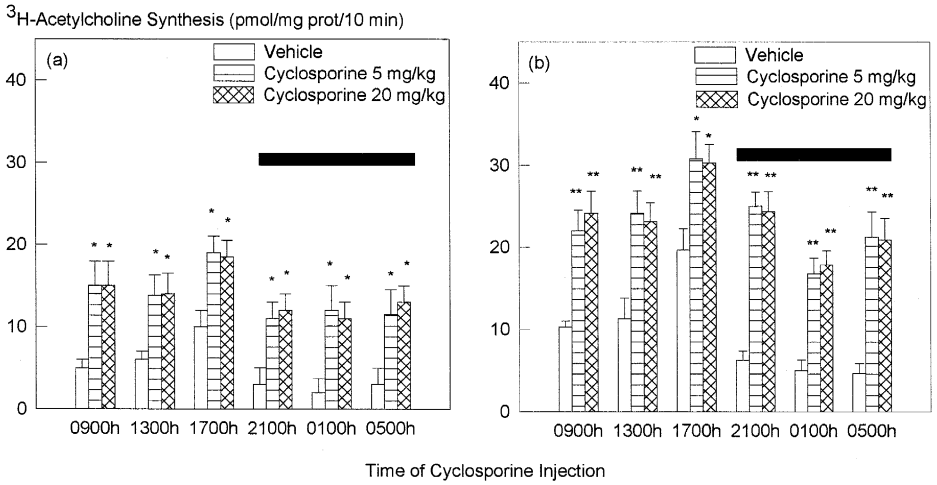

Fig. 3. Effect of cyclosporine (5 or 20 mg/kg), injected for 5 days at one of six different time intervals throughout the 24-h cycle, on [^{3}H]choline conversion into acetylcholine in rat submaxillary lymph nodes of rats treated with (a) Freund's adjuvant vehicle or (b) Freund's complete adjuvant. Animals were treated and killed as described in the legend to Fig. 1. The submaxillary lymph nodes were dissected out and assayed for [^{3}H]acetylcholine synthesis as described in Section 2. Shown are the means ± S.E.M. ($n = 7–8$/group). *F* values in one-way ANOVA at each time interval were: Left panel: 09:00 h: $F(2,19) = 4.91$, $P < 0.02$; 13:00 h: $F(2,19) = 4.32$, $P < 0.03$; 17:00 h: $F(2,19) = 6.24$, $P < 0.008$; 21:00 h: $F(2,19) = 5.87$, $P < 0.01$; 01:00 h: $F(2,19) = 5.24$, $P < 0.02$; 05:00 h: $F(2,19) = 4.66$; $P < 0.02$. Right panel: 09:00 h: $F(2,19) = 8.80$, $P < 0.002$; 13:00 h: $F(2,20) = 7.42$, $P < 0.004$; 17:00 h: $F(2,20) = 4.78$, $P < 0.02$; 21:00 h: $F(2,20) = 29.90$, $P < 0.0001$; 01:00 h: $F(2,20) = 17.05$, $P < 0.0001$; 05:00 h: $F(2,20) = 16.90$, $P < 0.0001$. Asterisks designate significant differences in post-hoc Dunnett's *t*-tests performed at each time interval ($^*P < 0.05$; $^{**}P < 0.01$, as compared to the respective vehicle-treated controls). In the factorial ANOVA cyclosporine augmented significantly acetylcholine synthesis (factorial ANOVA, $F(2,260) = 173.92$, $P < 0.0001$). A post-hoc Tukey's test indicated that both doses of cyclosporine were equally effective to increase this parameter ($P < 0.001$). [^{3}H]Choline conversion into acetylcholine was augmented significantly after Freund's adjuvant injection (factorial ANOVA, $F(1,260) = 205.53$, $P = 0.0001$) and when analyzed as a global effect, it changed diurnally, showing a maximum in the afternoon (i.e., at 17:00 h) ($F(5,260) = 20.58$, $P < 0.0001$). However, one-way ANOVA comparing time intervals in each experimental group indicated that a diurnal rhythm in lymph node uptake with maxima in the afternoon occurred in Freund's adjuvant- ($F(5,39) = 10.86$, $P < 0.0001$) and vehicle-injected control rats ($F(5,38) = 2.91$, $P < 0.03$) only.

tive, as shown by a post-hoc Tukey's test ($P < 0.001$). [^{3}H]Acetylcholine synthesis was augmented significantly by Freund's adjuvant injection (factorial ANOVA, $F(1,260) = 205.53$, $P = 0.0001$), and when analyzed as a main factor it was shown to change diurnally, showing a maximum in the afternoon (i.e., at 17:00 h) ($F(5,260) = 20.58$, $P < 0.0001$). As shown by post-hoc one-way ANOVA, significant 24-h variations in submaxillary lymph node [^{3}H]acetylcholine synthesis, displaying maxima during daylight, were found in Freund's adjuvant- and vehicle-injected control rats ($P < 0.01$ and $P < 0.05$, respectively) but not in animals treated with cyclosporine (Fig. 3).

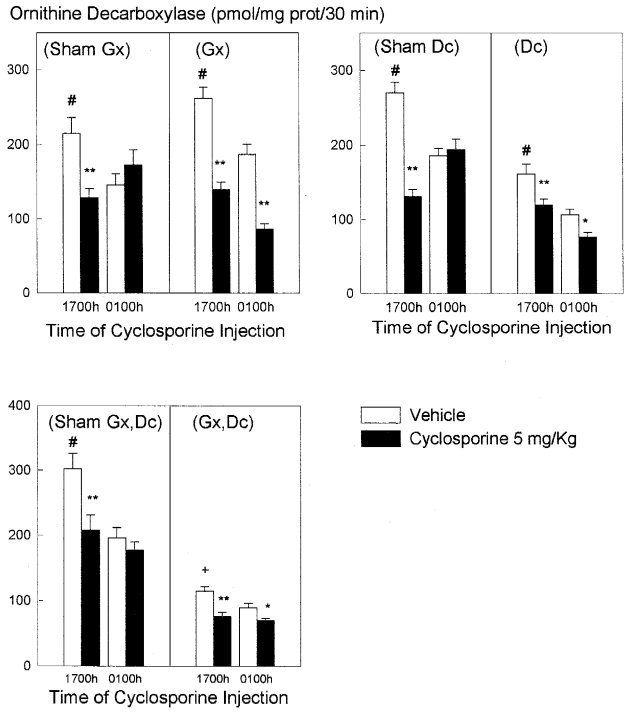

Fig. 4. Effect of unilateral superior cervical ganglionectomy (Gx) and/or unilateral chorda tympani section (parasympathetic decentralization, Dc) on day-night differences in cyclosporine-induced inhibition of Freund's adjuvant-induced submaxillary lymph node ornithine decarboxylase activation. Each rat received a contralateral sham-operation. Ten days after surgery, groups of 15–16 rats were injected for 5 days with cyclosporine (5 mg/kg) at 17:00 or 01:00 h. Freund's adjuvant was injected s.c. on the third day of treatment, at 11:00 h. Animals were killed 2 h after the last injection of cyclosporine. The submaxillary lymph nodes were dissected out and assayed for ornithine decarboxylase activity as described in Section 2. Shown are the means ± S.E.M. Asterisks designate significant differences as compared to vehicle-treated controls, Student's *t*-test ($^*P < 0.05$; $^{**}P < 0.01$). Significant differences in a paired *t*-test analysis between the operated and the sham-operated sides of vehicle-treated controls are also indicated ($^+P < 0.05$; $^\#P < 0.01$, vs. the denervated side). Analyzed as a main factor in a factorial ANOVA, the effect of cyclosporine treatment was significant on the denervated side (Gx: $F(1,56) = 83.05$, $P < 0.0001$; Dc: $F(1,56) = 13.25$, $P < 0.0006$; Gx + Dc: $F(1,56) = 18.44$, $P < 0.001$). Analyzed as a main factor in a factorial ANOVA, the effect of time of day of injection was significant on the denervated side (Gx: $F(1,56) = 27.86$, $P < 0.0001$; Dc: $F(1,56) = 25.94$, $P < 0.0001$; Gx + Dc: $F(1,56) = 5.34$, $P < 0.03$). A factorial ANOVA also indicated significant interactions between cyclosporine treatment and time of day of injection for sham Gx ($F(1,56) = 10.20$, $P < 0.003$), sham Dc ($F(1,56) = 35.05$, $P < 0.0001$) and a marginally significant interaction for the sham Gx-Dc side ($F(1,56) = 3.82$, $P = 0.054$). Dc, or Gx + Dc, decreased significantly submaxillary lymph node ornithine decarboxylase activity, when analyzed as a main global factor (factorial ANOVA: $F(1,113) = 87.56$ and 163.01, $P < 0.0001$, respectively).

Fig. 4 shows the effect of unilateral sympathetic denervation (by superior cervical ganglionectomy) and/or unilateral parasympathetic decentralization (by chorda tympani section) on day-night differences in the efficacy of 5 mg/kg of cyclosporine to decrease submaxillary lymph node ornithine decarboxylase activity in Freund's adjuvant-injected rats. Each animal received a contralateral sham-operation. Five daily cyclosporine injections were performed at 17:00 or 01:00 h. On the sham-operated side cyclosporine decreased significantly ornithine decarboxylase activation when injected at 17:00 h only while on the denervated side, it decreased enzyme activity at both studied times (Fig. 4). Thus, when analyzed as a main factor in a factorial ANOVA, the effect of cyclosporine was significant on the denervated side only (ganglionectomy: $F(1,56) = 83.05$, $P < 0.0001$; decentralization: $F(1,56) = 13.25$, $P < 0.0006$; ganglionectomy + decentralization: $F(1,56) = 18.44$, $P < 0.001$) as was the effect of time of day of injection (ganglionectomy: $F(1,56) = 27.86$, $P < 0.0001$; decentralization: $F(1,56) = 25.94$, $P < 0.0001$; ganglionectomy + decentralization: $F(1,56) = 5.34$, $P < 0.03$).

The time-dependent effect of cyclosporine on Freund's adjuvant-induced ornithine decarboxylase activation was further indicated by the significant interaction in the factorial ANOVA between cyclosporine treatment and time of day of injection for the sham-ganglionectomized and sham-decentralized sides (sham-ganglionectomy: $F(1,56) = 10.20$, $P < 0.003$; sham-decentralization: $F(1,56) = 35.05$, $P < 0.0001$) and a marginally significant interaction for the sham ganglionectomized-decentralized side ($F(1,56) = 3.82$, $P = 0.054$). Chorda tympani section, or combined superior cervical ganglionectomy plus chorda tympani section, decreased significantly submaxillary lymph node ornithine decarboxylase activity, when analyzed as main global factor in the factorial ANOVA ($F(1,113) = 87.56$ and 163.01, $P < 0.0001$, respectively) (Fig. 4).

4. Discussion

These experiments were carried out to examine the existence of dosing time-dependent effects of cyclosporine on a number of rat submaxillary lymph node parameters, including ornithine decarboxylase activity and labeled norepinephrine uptake and acetylcholine synthesis. Cyclosporine caused a dose-dependent decrease in lymph node ornithine decarboxylase activity, the lower dose (5

mg/kg) being ineffective when injected during most of the scotophase in Freund's adjuvant-treated rats. A dose-dependent effect of cyclosporine on lymph node [^{3}H]norepinephrine uptake was found in immunized and non-immunized animals. Only in immunized rats did a 5 mg/kg dose of cyclosporine increase [^{3}H]norepinephrine uptake when injected at 13:00 or 17:00 h. A stimulatory effect of cyclosporine on [^{3}H]choline conversion into [^{3}H]acetylcholine by submaxillary lymph nodes was found at both doses examined and regardless of time of administration.

Cyclosporine chronopharmacology has been examined in animals (Magnus et al., 1985; Bowers et al., 1986; Luke et al., 1988; Sangalli et al., 1988; Levi et al., 1991; Malmary et al., 1991, 1992; Batalla et al., 1994).as well as in patients (Canafax et al., 1988; Ramon et al., 1989; Sabate et al., 1990; Cugini et al., 1991; Ohlman et al., 1993). For example, studies on intraindividual variability and circadian variation in oral cyclosporine pharmacokinetics in renal transplant recipients indicated a significantly longer half-time during the night than during the day (Ohlman et al., 1993). Repeated cyclosporine blood concentration measurements at steady state showed lower concentrations during the day than the night, suggesting higher cyclosporine clearance during daytime (Cipolle et al., 1988; Ramon et al., 1989).

The documented time-of-day variation in immunosuppressive action of cyclosporine is presumably related to the efficacy of the drug to induce effects as the plasma concentrations of the drug showed no periodic variations over the 24-h span (Cugini et al., 1991). Indeed, the in vitro response of murine splenocytes to cyclosporine strongly depended upon circadian time of exposure (Levi et al., 1991).

With regard to cyclosporine toxicity, studies in rats indicated that the safest time for drug administration is about 10 h after lights are switched on, a time when rats usually begin their diurnal rest and/or sleep span (Magnus et al., 1985). For example, the nephrotoxic effect of cyclosporine in rats is higher during the night (Malmary et al., 1991, 1992; Batalla et al., 1994). Indeed, cyclosporine is less well tolerated when given during the active period (daytime in humans and nighttime in rodents). As shown in genetically hyperlipidemic rats, the increased non-fasting serum triglyceride levels following the active period most likely reduce cyclosporine distribution into sensitive tissues like the kidney (Luke et al., 1988).

Several interpretations can be entertained to explain the diurnal variation in cyclosporine effects on submaxillary lymph nodes reported in the present study (Reinberg, 1992). Besides possible diurnal changes in submaxillary lymph node sensitivity to cyclosporine, the changes could be due to chronodependent variations in the rate of absorption of cyclosporine after i.p. administration, in the interaction of cyclosporine with other molecules (e.g., binding), in the level of cyclosporine metabolism and in the eventual activity of cyclosporine metabolites. Further studies in vitro are needed to document the existence of time-of-day changes in lymph node cell sensitivity to cyclosporine.

One of the possible effects of cyclosporine on circadian rhythmicity could be to modify the periodicity of the circadian oscillator. In this sense, the diurnal rhythm in [^{3}H]norepinephrine uptake and [^{3}H]choline conversion into [^{3}H]acetylcholine documented herein in submaxillary lymph nodes of immunized and non-immunized rats disappeared after cyclosporine treatment. Several other 24-h rhythms have been shown to change following cyclosporine administration. For example, the 24-h mean levels of atrial natriuretic peptide, plasma renin activity and plasma aldosterone were significantly increased, while the concentrations of plasma cortisol were reduced, together with an abolished circadian pattern of plasma concentrations of vasoactive intestinal peptide, in patients with heart transplants treated with cyclosporine (Cugini et al., 1991, 1993a,b). The 24-h blood pressure profile was altered in liver transplanted or heart transplanted patients under cyclosporine treatment (Dart et al., 1992; Van de Borne et al., 1993). As shown by Sehested et al. (1992), repeated determination of circulating catecholamines, neuropeptide Y, pancreatic polypeptide, calcitonin gene-related peptide, plasma renin activity, aldosterone, atrial natriuretic factor and cortisol in patients treated with cyclosporine showed abnormalities in levels or circadian rhythmicity, or both. It must be noted, however, that in the present study the 24-h rhythm in submaxillary lymph node ornithine decarboxylase activity still persisted in cyclosporine-injected rats, although with a considerably lower amplitude.

The stimulatory effect of cyclosporine on norepinephrine uptake by rat submaxillary lymph nodes suggests that after drug administration an increase in sympathetic activity can be expected. It is known that cyclosporine induces hypertension after transplantation (Lipkin et al., 1993; Van de Borne et al., 1993; Textor et al., 1994). Soon after immunosuppression with cyclosporine and corticosteroids, hypertension develops in most patients who undergo transplantation. The blood pressure increases, which are usually moderate, occur universally because of increased peripheral vascular resistance. However, the changes in blood pressure sometimes are severe and associated with rapidly developing target injury, including intracranial hemorrhage, left ventricular hypertrophy, and microangiopathic hemolysis. The complex mechanisms that underlie this disorder include alterations in vascular reactivity which cause widespread vasoconstriction (Lanese et al., 1994) as well in postsynaptic adrenergic receptors (Brodde et al., 1995). Within this context, the stimulation of local noradrenergic activity by cyclosporine treatment reported herein deserves to be considered. In addition, data from the literature support the existence of changes in norepinephrine dynamics in lymphoid tissue in the course of the immune reaction or as a consequence of cytokine adminis-

tration (Ader et al., 1990; Besedovsky and Del Rey, 1992; Rothwell and Hopkins, 1995).

Because of its very short half-life and the speed with which it responds to regulatory stimuli, ornithine decarboxylase has often been used as an indicator for immunomodulatory phenomena in lymphoid-competent organs (Endo, 1984; Fidelius et al., 1984; Russel, 1985; Neidhart, 1989). For example, Neidhart (1989) reported a 75–190% increase in ornithine decarboxylase activity in bone marrow, thymus, spleen and lymph nodes of rats injected with Freund's adjuvant and the existence of a prominent peak in thymic enzyme activity at night that could be attributed to high circulating levels of prolactin. As reported herein, significant diurnal variations in ornithine decarboxylase activity of submaxillary lymph nodes were found in vehicle-treated rats, with a maximal activity in the middle or late portion of the photophase. These results confirm results reported in a previous study (Cardinali et al., 1996). Indeed, there is evidence that a number of immunological indicators exhibit diurnal rhythmicity and that the overall immunological effectiveness changes diurnally in both experimental animals and humans (Carandente et al., 1988; Ader et al., 1990; Angeli et al., 1994).

Submaxillary lymph nodes receive sympathetic innervation from neurons located in the superior cervical ganglia (Felten et al., 1992), whose activity shows a strong circadian functional organization (Barontini et al., 1988; Gonzalez Burgos et al., 1994). Taking this experimental evidence into account, we wished to test the hypothesis that the diurnal changes in the submaxillary lymph node ornithine decarboxylase response to cyclosporine found in Freund's complete adjuvant-treated rats could be related to a circadian signal conveyed via the sympathetic neurons arriving at the lymph nodes. To do this, we unilaterally removed one superior cervical ganglion, leaving the contralateral one as a control to correct for the possible contribution of humoral signals to the changes observed.

We also tested the role of the parasympathetic innervation of the submaxillary lymph nodes, which is conveyed via the facial nerve and the lingual nerve-chorda tympani trunk (Appenzeller, 1990). As in many other organs, this parasympathetic pathway is preganglionic, innervating local ganglia that play the role of intermediate relay stations in parasympathetic control (Appenzeller, 1990; Felten et al., 1992).

The effect of unilateral sympathetic denervation and/or unilateral parasympathetic decentralization on day-night differences in the efficacy of 5 mg/kg cyclosporine to decrease submaxillary lymph node ornithine decarboxylase activation by Freund's complete adjuvant was tested. Each surgical procedure augmented the effect of cyclosporine in that the lack of effect on enzyme activity at night found in sham-operated rats changed into a significant decreasing effect when assessed in denervated rats. Whether or not these observations have clinical implications deserves to be tested. For example, it could be possible to amplify cyclosporine's immunosuppressive activity by concomitant adrenergic and/or cholinergic pharmacological blockade.

In summary, the questions posed in the Introduction may now be answered. Dosing time-dependent effects of cyclosporine on ornithine decarboxylase in rat submaxillary lymph nodes were demonstrated, with a maximal effect during the second half of the daily photoperiod in Freund's adjuvant-treated rats. Time-of-day-dependent effects of cyclosporine on [^{3}H]norepinephrine uptake in submaxillary lymph nodes of immunized animals were also found, cyclosporine being ineffective at low doses when injected at night, but augmenting [^{3}H]choline conversion into [^{3}H]acetylcholine by submaxillary lymph nodes regardless of time of administration. Regional sympathectomy and/or parasympathectomy of submaxillary lymph nodes affected partially, but did not abolish, time-of-day changes in the effect of cyclosporine on ornithine decarboxylase activation, supporting the view that regulation of dosing time-dependent effects of cyclosporine in submaxillary lymph nodes may derive from the autonomic innervation to the immunocompetent tissue. Collectively, the results suggest that cyclosporine treatment, e.g., antirejection therapy of transplanted subjects, can be adjusted so that cyclosporine can be given at a time that would promote immunosuppression without altering rhythmic performance (Liu et al., 1986).

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