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Effect of stress and cyclosporine on ornithine decarboxylase activity in rat submaxillary lymph nodes

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Abstract

This study was performed: (1) to assess whether the stress produced in rats by daily turpentine oil injections for 5 days, or by restraining the animals for 30 min during 5 days, affected basal and Freund's adjuvant-stimulated submaxillary lymph node ornithine decarboxylase activity, an indicator of cell proliferation; (2) to analyze whether the activity of the immunosuppressive drug cyclosporine on submaxillary lymph node ornithine decarboxylase activation after Freund's adjuvant injection was modified in stressed rats; (3) to examine the mediation of stress effects on submaxillary lymph node ornithine decarboxylase activation by regional sympathetic or parasympathetic nerves. Animals subjected to a unilateral superior cervical ganglionectomy, or to a unilateral chorda tympani section, together with a contralateral sham-operation were employed. After turpentine oil or restraint stress, a significant decrease in submaxillary lymph node ornithine decarboxylase was found. A unilateral sympathetic denervation of submaxillary lymph nodes counteracted in part the inhibitory effect of stress on ornithine decarboxylase activation, as well as augmented the enzyme response in innervated submaxillary lymph nodes. Ornithine decarboxylase activation attained similar values in parasympathetic decentralized or intact submaxillary lymph nodes and the unilateral parasympathetic decentralization did not interfere with the inhibition of enzyme activity found in turpentine oil-stressed rats. Cyclosporine administration (5 or 20 mg/kg) significantly decreased Freund's adjuvant-induced ornithine decarboxylase activity in the submaxillary lymph nodes of control rats, but failed to modify it in turpentine oil-stressed animals. In this latter group, a higher (40 mg/kg) dose of cyclosporine decreased ornithine decarboxylase activity on the innervated side only. A diminished inhibitory response to cyclosporine was found in the parasympathetic decentralized submaxillary lymph nodes of unstressed rats. The results support the view that the immunosuppressive effects of cyclosporine may diminish during stress, in part due to changes in the traffic of neural signals in local sympathetic nerves.

Keywords: Neuroimmunomodulation; Cyclosporine; Stress; Sympathetic nervous system; Parasympathetic nervous system; Submaxillary lymph node; Superior cervical ganglion; Chorda tympani

1. Introduction

The immunosuppressive drug cyclosporine is widely employed as a therapeutic agent in organ transplantation (Borel, 1983). Cyclosporine decreased the activity of ornithine decarboxylase in lymphoid and non-lymphoid tissues (Fidelius et al., 1984; Esquifino et al., 1991, 1994b). Ornithine decarboxylase catalyses the initial, rate-limiting, step in polyamine biosynthesis and is oftenly 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 by Freund's complete adjuvant (Esquifino et al., 1991, 1994b). Since exposure to stress agents is an effective means to change traffic neural signals in local autonomic nerves (Appenzeller, 1990), we considered it worthwhile to assess whether the activity of cyclosporine could be modified in stressed rats. Indeed, a

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variety of stressors have been found to alter immune responsiveness and stress is considered a potent immunosuppressor (Shavit et al., 1985; Dorian and Garfinkel, 1987; Dunn, 1989; Irwin et al., 1990; Besedovsky and Del Rey, 1992).

The present study was undertaken to answer the following questions: (1) does turpentine oil or restraint stress affect the activity of cyclosporine on Freund's adjuvant-induced ornithine decarboxylase activation in submaxillary lymph nodes?; (2) does regional sympathectomy or parasympathectomy of submaxillary lymph nodes affect stress-induced changes of cyclosporine activity?

2. Materials and methods

2.1. Chemicals

L-[^{14}C]-Ornithine hydrochloride (specific activity 58 Ci/mol) was purchased from NEN Research Products, Boston, MA, USA. Diphenyloxazole (PPO) and 1,4-bis-{2-(5-phenyl)-oxazolyl}benzene (POPOP) were obtained from Serva Feinbiochemica, Heidelberg, Germany. Cyclosporine was obtained from Sandoz, Basel, Switzerland. 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.

2.2. Animals and experimental design

Adult male Wistar rats (180–220 g) were kept under light from 06:00 to 20:00 h daily, and were given access to food and water ad libitum. A superior cervical ganglionectomy or a parasympathetic decentralization of submaxillary lymph nodes (obtained by severing the chorda tympani), was performed unilaterally under light ether anesthesia as described previously (Alito et al., 1987). Each rat received also a contralateral sham-operation. Validation of the surgical procedures, including the assessment of specific markers of noradrenergic or cholinergic activity in submaxillary lymph nodes, was published before (Esquifino et al., 1991,1994b). The side of operation was changed at random for each set of experiments. The studies were conducted in accord with the principles and procedures outlined in the NIH guide for the Care and Use of the Laboratory Animals.

A first series of experiments was performed to assess the effect of stress on basal or Freund's adjuvant-induced ornithine decarboxylase activity in submaxillary lymph nodes. Stress was induced either by giving five daily s.c. injections of 5 μl turpentine oil/g body weight (Stern et al., 1993) or by restraining the rats for 30 min in a small flexible wire mesh container where the animals had no room to move, once daily during 5 days. Exposure to stress was performed at 09:00 h each day.

Freund's complete adjuvant was injected s.c. (0.5 mg heat-killed Mycobacterium butyricum/rat) 1 h before the third exposure to stress. The dose of Freund's adjuvant was that described by Neidhart (1989). Rats were killed 2 h after the last exposure to stress, on the fifth day of treatment, at 11:00 h. The time of killig was selected on the basis of a preliminary experiment indicating that a maximal inhibition of submaxillary lymph node ornithine decarboxylase activity occurred at this time (data not shown). Control groups included undisturbed rats as well as rats treated with 5 μl of saline (instead of turpentine oil) and rats injected with the Freund's adjuvant vehicle (0.5 ml paraffin oil containing 15% mannide monooleate).

A second series of experiments was performed to examine the effect of turpentine oil stress in rats subjected to a unilateral superior cervical ganglionectomy or to a unilateral section of the chorda tympani 10 days earlier. Animals were treated with turpentine oil or saline, and with Freund's complete adjuvant, being killed 2 h after the last turpentine oil or saline injection on the fifth day, as in experiment 1.

A third series of experiments was performed to examine the capacity of cyclosporine to modify submaxillary lymph node ornithine decarboxylase activation in turpentine oil-stressed rats. Cyclosporine was injected s.c. 5 min after turpentine oil or saline administration as in experiment 1. Cyclosporine was given at doses of 5 or 20 mg/kg for 5 days. Freund's adjuvant was injected s.c. 1 h before the third injection of cyclosporine. Animals were killed 2 h after the last injection of cyclosporine, on the fifth day of treatment, at 11:00 h. Control groups included rats treated in a similar way with vehicle (instead of cyclosporine) and Freund's adjuvant.

A fourth series of experiments was performed to examine the capacity of cyclosporine to modify submaxillary lymph node ornithine decarboxylase activation in stressed rats subjected to unilateral superior cervical ganglionectomy. Ten days after surgery, cyclosporine was injected s.c. 5 min after turpentine oil or saline administration as in experiment 1. Cyclosporine was given at doses of 5 or 20 mg/kg for 5 days. Freund's adjuvant was injected s.c. 1 h before the third injection of cyclosporine. Animals were killed 2 h after the last injection of cyclosporine, on the fifth day of treatment, at 11:00 h. Control groups included rats treated in a similar way with vehicle (instead of cyclosporine) and Freund's adjuvant. In a fifth series of experiments the effect of higher doses of cyclosporine (20 and 40 mg/kg) on submaxillary lymph node ornithine decarboxylase activation in turpentine oil-stressed, unilaterally superior cervical ganglionectomized rats was examined as in experiment 4.

A sixth series of experiments was performed to examine the capacity of cyclosporine to modify submaxillary lymph node ornithine decarboxylase activation in turpentine oil-stressed rats subjected to unilateral chorda tympani section. Ten days after surgery, cyclosporine was s.c. injected 5 min after turpentine oil or saline administration as in experiment 1. Cyclosporine was given at doses of 5 or 20 mg/kg for 5 days. Freund's adjuvant was injected s.c. 1 h before the third injection of cyclosporine. Animals were killed 2 h after the last injection of cyclosporine, on the fifth day of treatment, at 11:00 h. Control groups included rats treated in a similar way with vehicle (instead of cyclosporine) and Freund's adjuvant.

2.3. Ornithine decarboxylase activity

Submaxillary lymph nodes were removed after death, and were kept at −20°C until assayed (within 2–3 days). The tissues 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 phosphate, 0.1 mM EDTA-Na and 2 mM dithiothreitol, as described previously (Esquifino et al., 1991). The homogenate was centrifuged (at 2000 × *g* for 10 min at 4°C) and 200-ml supernatant fractions were incubated in glass tubes fitted with rubber stoppers and center wells containing a filter paper disk spotted with 20 ml of hyamine hydroxide. L-[1-^{14}C]-Ornithine hydrochloride (1 mCi/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 from the enzymatic reaction was collected on the filter papers, and the radioactivity was counted in 10 ml 30% Triton X 100-toluene phosphor solution. The results were expressed as pmol of $^{14}CO_2$ released/mg supernatant protein/30 min. The reaction was completely inhibited by the addition of 0.25 mM α-difluoromethyl ornithine. Blanks including zero-time controls and heated supernatants were also run. Enzyme activity was linear with respect to incubation time and enzyme concentration. Protein concentration was measured by the Lowry's procedure using bovine serum albumin as a standard (Lowry et al., 1951).

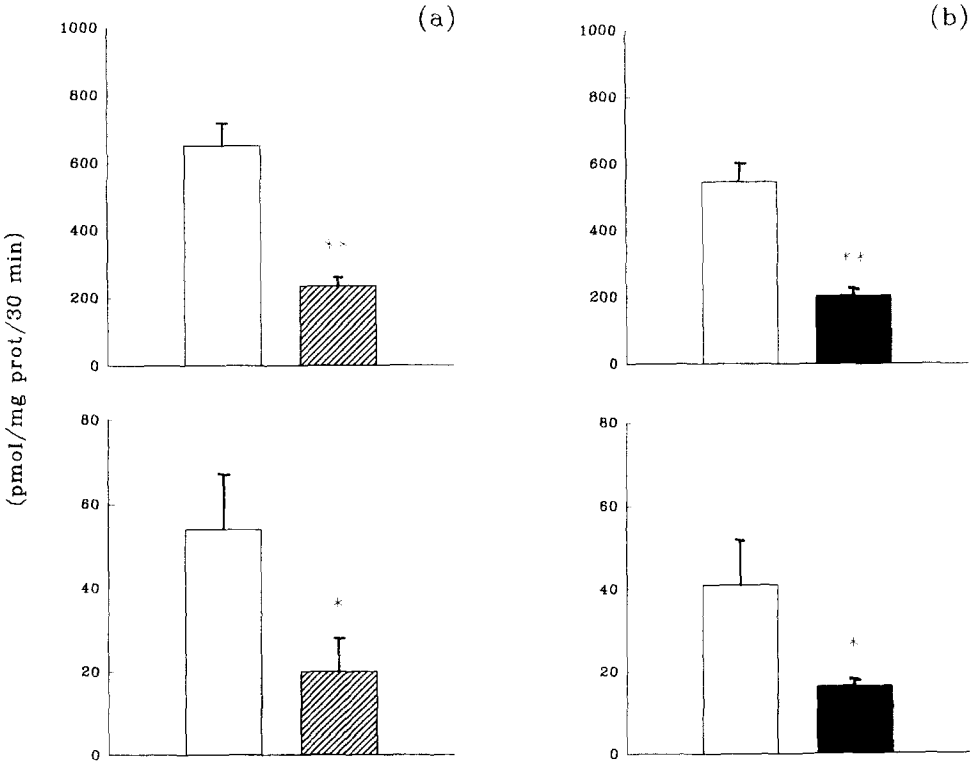

Fig. 1. Ornithine decarboxylase activity in submaxillary lymph nodes of turpentine oil-stressed (a) or restraint rats (b). Groups of seven rats received five daily s.c. injections of 5 μl turpentine oil/g body weight or 5 μl/g of saline (control) (turpentine oil experiment), or were subjected to immobilization for 30 min in a small flexible wire mesh container (or left unrestrained) once daily during 5 days. Freund's complete adjuvant (0.5 mg heat-killed Mycobacterium butyricum/rat) (upper panel) or adjuvant's vehicle (0.5 ml paraffin oil containing 15% mannide monooleate) (lower panel) was injected 1 h before the third exposure to stress. Rats were killed 2 h after the last exposure to stress, on the fifth day of treatment, at 11:00 h. Ornithine decarboxylase activity in submaxillary lymph nodes was measured as described in Methods. Shown are the means ± S.E of values in control (open bars), turpentine oil-injected rats (hatched bars) or restrained rats (solid bars). Significance values correspond to a Student's *t*-test, * $P < 0.05$, ** $P < 0.01$.

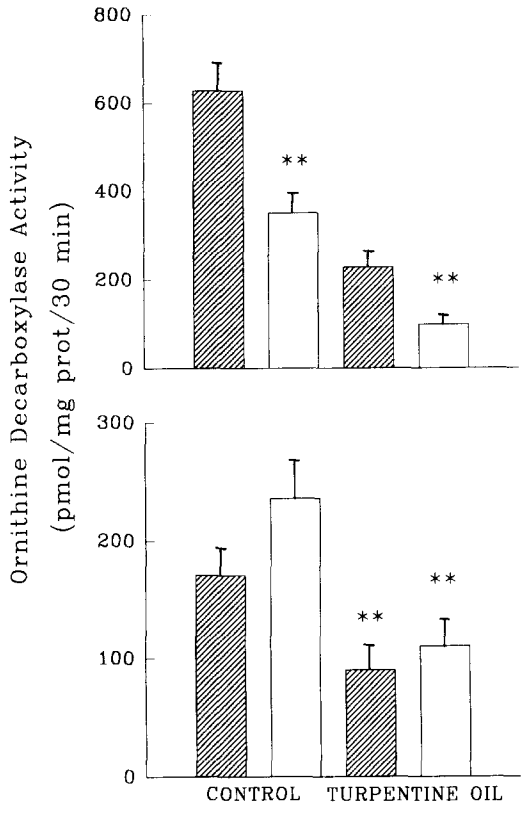

Fig. 2. Effect of sympathetic denervation by superior cervical ganglionectomy (upper panel), or of parasympathetic decentralization by chorda tympani section (lower panel), on Freund's adjuvant-induced ornithine decarboxylase activity in submaxillary lymph nodes of turpentine oil-stressed rats. Groups of seven to eight rats, subjected to a unilateral superior cervical ganglionectomy or chorda tympani section, and to a contralateral sham-operation 10 days earlier, received turpentine oil, saline and Freund's complete adjuvant as described in the legend to Fig. 1. Ornithine decarboxylase activity in submaxillary lymph nodes was measured as described in Methods. Shown are the means ± S.E of values in sympathetically denervated or parasympathetically decentralized submaxillary lymph nodes (hatched bars), and innervated (open bars) submaxillary lymph nodes. Significant values in the upper panel correspond to a paired Student's t-test (** $P < 0.01$, as compared to the contralateral sympathetically denervated lymph nodes). Significant values in the lower panel correspond to an analysis of variance followed by a Student-Newman-Keuls multiple comparisons test, ** $P < 0.01$, as compared to enzyme activation at the decentralized lymph nodes of saline-treated rats.

2.4. Statistical analysis

Statistical analysis of results was performed by employing a one way analysis of variance (ANOVA) followed by a Student-Newman-Keuls multiple comparisons test, or by a factorial ANOVA. Each series of experiments was repeated twice with essentially similar results. The results of only one of the two replicate experiments are shown ($n = 7$–8 animals/group), except for experiments 4 and 5, which are depicted as the combined data from all the experiments done ($n = 15$–32 animals/group).

3. Results

Rat submaxillary lymph node ornithine decarboxylase activity increased by about 10 times after the s.c. injection of Freund's complete adjuvant (Fig. 1). Treatment of rats with turpentine oil caused a significant decrease in submaxillary lymph node ornithine decarboxylase, both in rats injected with Freund's complete adjuvant or with its vehicle (Fig. 1a). When immobilization for 30 min was employed as a stressor maneuver, similar results as those following turpentine oil injection were obtained (Fig. 1b).

Fig. 2 depicts the effect of turpentine oil stress on Freund's adjuvant-induced activation of submaxillary lymph node ornithine decarboxylase in rats previously subjected to a unilateral superior cervical ganglionectomy or to a unilateral section of the chorda tympani section. Sympathetic denervation augmented ornithine decarboxylase response to Freund's adjuvant injection (Fig. 2, upper panel). After turpentine oil injection, ornithine decarboxylase activation decreased at both the sympathetically innervated and denervated side. The difference between enzyme activation at the innervated lymph nodes of saline-treated rats and that at the denervated lymph nodes of turpentine oil-stressed rats were not significant (Fig. 2, upper panel). As shown in

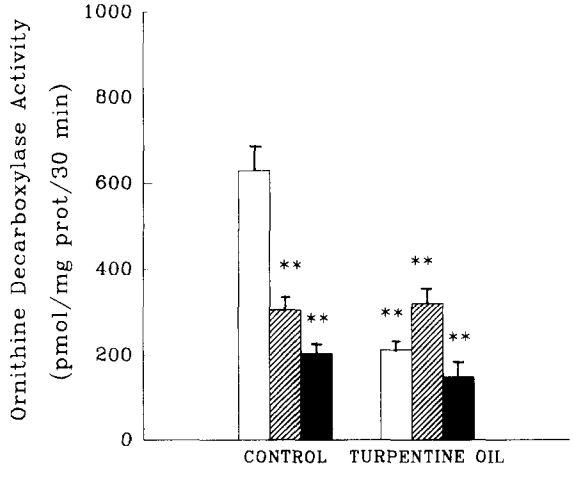

Fig. 3. Effect of cyclosporine (5 mg/kg: hatched bars; 20 mg/kg: solid bars) or vehicle (open bars) on Freund's adjuvant-induced ornithine decarboxylase activity in submaxillary lymph nodes of turpentine oil-stressed rats. Groups of seven to eight rats received 5 daily s.c. injections of turpentine oil or saline as described in the legend to Fig. 1. Cyclosporine or its vehicle was s.c. injected 5 min after turpentine oil or saline administration. Freund's complete adjuvant was injected s.c. 1 h before the 3rd injection of cyclosporine or vehicle. Animals were killed 2 h after the last injection of cyclosporine or vehicle, on the 5th day of treatment. Ornithine decarboxylase activity was measured in submaxillary lymph nodes as described in Methods. Shown are the means ± S.E. Significant values correspond to an analysis of variance followed by a Student-Newman-Keuls multiple comparisons test, ** $P < 0.01$, as compared to enzyme activation in vehicle-injected control rats.

the lower panel of Fig. 2, a unilateral parasympathetic decentralization did not interfere with ornithine decarboxylase responses to Freund's adjuvant, nor with turpentine oil effect on it.

Fig. 3 shows the effect of cyclosporine (5 or 20 mg/kg) on submaxillary lymph node ornithine decarboxylase activity during the immune reaction in rats treated or not with turpentine oil. Cyclosporine decreased effectively Freund's adjuvant-induced ornithine decarboxylase activation in control rats. However, it failed to modify enzyme activity, even at a 20 mg/kg dose, in turpentine oil-stressed animals.

The effect of cyclosporine (5, 20 or 40 mg/kg) on submaxillary lymph node ornithine decarboxylase activity in stressed rats subjected to a unilateral superior cervical ganglionectomy is depicted in Fig. 4. Cyclosporine decreased effectively Freund's adjuvant-induced ornithine decarboxylase activation in control rats, while it failed to modify enzyme activity at a 20 mg/kg dose, in turpentine oil-stressed animals, either at the innervated or denervated side. A higher dose of cyclosporine (40 mg/kg), injected to unilaterally ganglionectomized, stressed rats, decreased ornithine decarboxylase activation at the innervated side only. A significant interaction between sympathetic denervation and the higher dose of cyclosporine was detected in a factorial ANOVA (F ratio for the interaction = 4.134, $P = 0.045$).

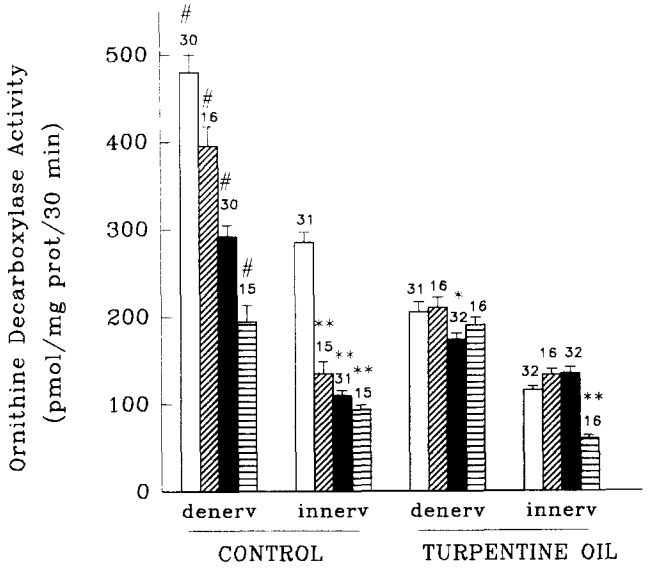

Fig. 4. Effect of cyclosporine (5 mg/kg: diagonally hatched bars; 20 mg/kg: solid bars; 40 mg/kg: horizontally hatched bars) or vehicle (open bars) on Freund's adjuvant-induced ornithine decarboxylase activity in submaxillary lymph nodes of unilaterally superior cervical ganglionectomized, stressed rats. Animals were subjected to a unilateral superior cervical ganglionectomy and a contralateral sham-operation 10 days earlier. Cyclosporine, saline and Freund's complete adjuvant were injected, and the animals further treated, as described in the legend to Fig. 3. Shown are the means ± S.E. of values of combined experiments (number of animals is quoted). Ornithine decarboxylase data in ipsilateral denervated (denerv) and contralateral innervated (innerv) submaxillary lymph nodes are shown. Significance values correspond to an analysis of variance followed by a Student-Newman-Keuls multiple comparisons test, # $P < 0.001$ vs. the remaining groups in denervated side; ** $P < 0.01$, * $P < 0.05$, as compared to the respective vehicle-injected group. A factorial ANOVA indicated a significant interaction between denervation and the 40 mg/kg dose of cyclosporine ($F = 4.134$, $P = 0.045$).

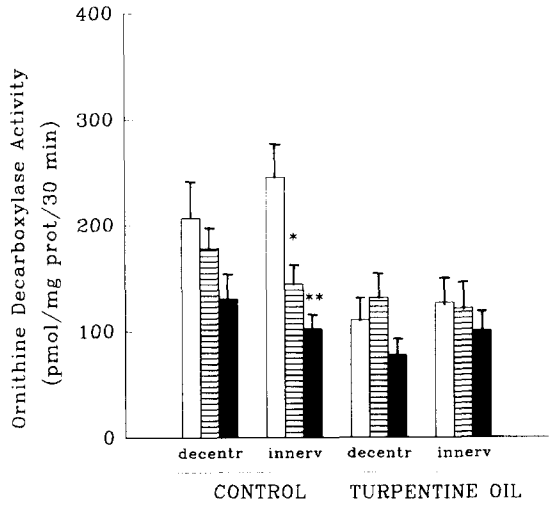

Fig. 5. Effect of cyclosporine (5 mg/kg: horizontally hatched bars; 20 mg/kg: solid bars) or vehicle (open bars) on Freund's adjuvant-induced ornithine decarboxylase in parasympathetically decentralized submaxillary lymph nodes of turpentine oil-stressed rats. Groups of seven to eight rats were subjected to a unilateral chorda tympani section and a contralateral sham-operation 10 days earlier. Cyclosporine, saline and Freund's complete adjuvant were injected, and the animals further treated, as described in the legend to Fig. 3. Shown are the means ± S.E. Ornithine decarboxylase data in ipsilateral parasympathetically decentralized (decentr) and contralateral innervated (innerv) submaxillary lymph nodes are shown. Significance values correspond to an analysis of variance followed by a Student-Newman-Keuls multiple comparisons test, * $P < 0.05$, ** $P < 0.01$ as compared to the respective vehicle-injected group.

Fig. 5 shows the effects of 5 or 20 mg/kg cyclosporine on submaxillary lymph node ornithine decarboxylase activity in stressed rats subjected to a unilateral parasympathetic decentralization. In control rats, cyclosporine decreased enzyme activity at the innervated side only. In stressed rats, cyclosporine failed to affect ornithine decarboxylase activation at both the innervated or decentralized submaxillary lymph nodes (Fig. 5).

4. Discussion

The immunosuppressive effects of cyclosporine are amenable to modulation by a number of endocrine and neural factors. For example, the activity of cyclosporine is influenced by changes in the secretion rate of several hormones, some of them caused by the drug itself (Sikka et al., 1988; Villanúa et al., 1992; Esquifino et

al., 1994a). Another modulating factor for cyclosporine immunosuppression is the autonomic innervation of the immunocompetent organs, currently considered as a channel for the neural regulation of immunity (Perez-Polo et al., 1988; Ader et al., 1990; Esquifino and Cardinali, 1994). In the absence of an intact sympathetic innervation, the effect of cyclosporine on rat submaxillary lymph node ornithine decarboxylase activation by Freund's adjuvant became attenuated (Esquifino et al., 1991). A similar impairment of cyclosporine activity on submaxillary lymph node ornithine decarboxylase activation (an indicator of cell proliferation and growth, Russel, 1985) was found in rats subjected to a regional unilateral parasympathetic decentralization of submaxillary lymph nodes obtained by chorda tympani section (Esquifino et al., 1994b).

A major objective of the present study was to examine whether the effect of cyclosporine on ornithine decarboxylase could be affected by autonomic nervous system activation during stress. Rat submaxillary lymph node ornithine decarboxylase activity increased after the s.c. administration of Freund's complete adjuvant and this increase was impaired by turpentine oil or restraint stress, in vein with the widely held view that stress is a potent immunosuppressor (Shavit et al., 1985; Dorian and Garfinkel, 1987; Dunn, 1989; Irwin et al., 1990; Besedovsky and Del Rey, 1992). A comparable degree of inhibition of ornithine decarboxylase activity was found in stressed rats regardless of the type of stress employed, suggesting that under this condition an on-off response in enzyme response may be generated.

Turpentine oil stress reduced ornithine decarboxylase activation in submaxillary lymph nodes, either in the presence or in the absence of intact sympathetic nerves. However, turpentine oil stress-induced inhibition of enzyme activity found at the sympathetically denervated submaxillary lymph nodes was less than that observed at the innervated contralateral side. Indeed, enzyme activation in sympathetically denervated lymph nodes of stressed rats attained values that did not differ statistically from those found in innervated lymph nodes of unstressed rats. The results are compatible with the view that the turpentine oil stress-induced decrease of immune response is partly dependent on the integrity of the sympathetic innervation to the lymph nodes. Assuming that ornithine decarboxylase activation is an indicator of immunocompetent cell proliferation in lymphoid organs (Endo, 1984; Fidelius et al., 1984; Neidhart, 1989), it can be concluded that activation of the sympathetic nervous system during stress may produce immunosuppression both by direct local effects at lymphoid tissue level and by indirect effects through changes in hormone secretion from the hypothalamic-pituitary axis (Murakami et al., 1989; Irwin et al., 1990).

A unilateral parasympathetic decentralization of submaxillary lymph nodes did not interfere with ipsilateral ornithine decarboxylase responses to Freund's adjuvant, nor with turpentine oil stress effect on it. Hence, the results tend to rule out a significant participation of the local parasympathetic nerves in immune response changes that follow stress.

It should be stressed that the activity of ornithine decarboxylase in the submaxillary lymph nodes of intact rats was about twice that in sham-operated submaxillary lymph nodes of unilaterally sympathectomized or parasympathetically decentralized rats. We do not have any satisfactory explanation for this difference. One possibility is that some fibers from the superior cervical ganglion or, less feasibly, from the facial nerve, could have a bilateral distribution. The influence of surgical stress could presumably be ruled out by the time elapsed after the operation (i.e., 10 days). The operations did not interfere with normal feeding as far as animals' weight did not differ from intact controls.

Cyclosporine administration, at a dose of 5, 20 or 40 mg/kg, decreased ornithine decarboxylase activation in innervated and sympathetically denervated submaxillary lymph nodes of unstressed rats (Esquifino et al., 1991, 1994b). On the contrary, the inhibitory effect of 5 or 20 mg/kg of cyclosporine on ornithine decarboxylase in stressed rats became blunted regardless of the integrity of sympathetic innervation. When a higher cyclosporine dose (i.e., 40 mg/kg) was administered to turpentine oil-stressed rats, a decrease in ornithine decarboxylase activation was found at the sympathetically innervated side only. The results suggest that the increase in traffic signal in sympathetic nerves during stress modulates in part the effect of cyclosporine on lymphoid tissue. In this regard, we recently reported that cyclosporine treatment modified catecholamine metabolism at the hypothalamic level (Esquifino et al., 1994a), suggesting that the effect of the drug can be partly mediated by changes in adrenergic transmitter release.

As shown before (Esquifino et al., 1994b) cyclosporine exhibited an impaired effect on ornithine decarboxylase activity in parasympathetically decentralized submaxillary lymph nodes. Hence, although of little importance in mediating stress-induced changes, the data suggest that cell proliferation in lymphoid tissue can be positively influenced by the local parasympathetic innervation.

Summarizing, the questions posed in the Introduction can now be answered. Turpentine oil or restraint stress does impair the activity of cyclosporine on Freund's adjuvant-induced ornithine decarboxylase in lymphoid tissue and the effect seems to be mediated in part via changing traffic signals in local sympathetic nerves. This is a hitherto unknown aspect of cy-

closporine activity whose therapeutical relevance deserves to be further explored. It might be envisioned that the therapeutical immunosuppressive effect of the drug can be substantially changed in situations that modify significantly activity in the sympathetic nerves, like psychological or surgical stress. The clinical importance of such investigations seems to be warranted.

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