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Interferon-Gamma Release in Sympathetically Denervated Rat Submaxillary Lymph Nodes

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Key Words

Interferon-γ release · Mitogenic responses · Submaxillary lymph nodes · Superior cervical ganglion · Sympathetic nerves · Norepinephrine

Abstract

Objective: To examine the regulation of interferon (IFN)-γ release by cells derived from submaxillary lymph nodes of rats subjected to an acute or chronic superior cervical ganglionectomy (SCGx). ***Methods:*** A unilateral SCGx and a contralateral sham operation were performed. Twenty hours or 7 days later cells from submaxillary lymph nodes were incubated for 24 h without any additional treatment (experiment 1), after adding lipopolysaccharide or concanavalin A (experiment 2) or after adding norepinephrine (NE, 10^{-8} *M*; experiment 3). IFN-γ concentration in the culture media was measured by ELISA. ***Results:*** Compared to controls, cells obtained from lymph nodes at a time of degeneration of sympathetic nerve terminals released more IFN-γ, whereas those derived from chronically SCGx lymph nodes released less IFN-γ. Stimulation of IFN-γ release by mitogens was detectable in the innervated or acutely denervated lymph nodes, but not in chronically denervated lymph nodes. When the effect of 10^{-8} *M* NE on IFN-γ release was tested, the neurotransmitter augmented cytokine release in cells prepared from chronically denervated lymph nodes only. ***Conclusion:*** The microenvironment provided by local sympathetic nerves is essential to enable an appropriate IFN-γ release by submaxillary lymph node cells to occur.

Introduction

There have been major efforts to understand the influence of the nervous system on the immune and inflammatory responses, with a number of critical phenomena emerging, including innervation of the immune system, molecular communication pathways between the brain and immune organs, and complex events like conditioning of immune mechanisms of host defenses [1–4]. Within this network, the sympathetic innervation of lymphoid organs plays a major role. For example, genetically manipulated mice that lacked dopamine β-hydroxylase, and therefore norepinephrine (NE) synthesis in sympathetic nerve terminals were more susceptible to infection, and exhibited an extreme thymic involution and impaired T cell function, including Th1 cytokine production after immune challenge [5].

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Among cytokines, interferon (IFN)-γ had antitumor and antiviral activity and is involved in the regulation of cytotoxicity, as well as in the expression of surface antigens, cellular differentiation, antibody production and macrophage activation [6–8]. During the early phase of host defense, production of IFN-γ by natural killer cells plays an important role in mediating acute inflammation. In a subsequent phase of the immune response, IFN-γ acts as a regulator of antigen presentation and of proliferation and differentiation of lymphocyte populations [6–8].

In a study designed to assess the participation of local sympathetic nerves in the regulation of immune responses in rat submaxillary lymph nodes we examined the promotive effect of IFN-γ on mitogenic responses to lipopolysaccharide (LPS) and concanavalin A (Con A) in unilaterally denervated lymph nodes. For mitogenic responses to LPS and Con A, the promotive effect of IFN-γ was found in the innervated side only, indicating that it needed absolutely the integrity of local sympathetic nerves [9].

As a continuation to those studies we considered it worthwhile to analyze to what extent the in vitro IFN-γ release by submaxillary lymph node cells is modified by severing the local sympathetic nerves. Rats were subjected to a unilateral superior cervical ganglionectomy (SCGx) and a contralateral sham operation 20 h or 7 days earlier. These times were selected to include both the chronic deprivation sequels of SCGx and the transient postsynaptic activation that occurs during the early phase of anterograde (wallerian) degeneration of nerve endings after superior cervical ganglion (SCG) ablation. Such a complementary approach has enabled us to analyze the neuroendocrine consequences of an increased or decreased postsynaptic exposure to an adrenergic transmitter in neuroendocrine territories of the SCG, like the thyroid and parathyroid glands, the median eminence and the posterior pituitary [10, 11].

Materials and Methods

Chemicals

All drugs and reagents employed were obtained from Sigma, St. Louis, Mo., USA.

Animals and Experimental Protocol

Wistar rats bred in our animal facilities were used. Animals were kept under standard conditions of light from 08:00 to 20:00 h daily and temperature (22 ± 2 °C) and with access to food and water ad libitum. Surgery was performed under tribromoethanol anesthesia (250 mg/kg, i.p.). 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.

Rats were subjected to a unilateral SCGx and to a contralateral sham operation, as described in detail elsewhere [12]. The left or right side of the operation was changed at random. Twenty hours or 7 days later, the rats (8 animals/group) were killed and the submaxillary lymph nodes were removed aseptically from both sides.

Evaluation of IFN-γ Release by Submaxillary Lymph Nodes

The submaxillary lymph nodes were placed in Petri dishes containing balanced salt solution and the cells were 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 m*M* *L*-glutamine, 0.02 m*M* 2-mercaptoethanol and gentamicin (50 mg/ml). Cells (10^5/100 µl) were incubated for 24 h without any additional treatment (experiment 1), after adding LPS or Con A (1 µg/50 µl; experiment 2) or after adding NE (10^{-8} *M*; experiment 3). The time of incubation was found as optimal in preliminary studies; neither treatment affected viability of the cells.

IFN-γ concentration in culture media was measured after centrifugation to remove adherent cells. An ELISA commercial kit from Endogen (Woburn, Mass., USA), which has been previously validated in our laboratory, was employed. The assay was as follows: 100 µl of standards or unknown samples were added to each antibody-coated well, and the plates were incubated overnight at room temperature. The reaction was stopped by washing thrice with wash buffer (2% Tween 20 in 50 m*M* Tris, pH 3.6). The wells were incubated with 100 µl of biotinylated detecting antibody at the titer previously tested. After 1 h at room temperature the reaction was stopped by washing thrice with wash buffer. One hundred microliters of streptavidin-HRP solution (in Dulbecco's phosphate-buffered saline, pH 7.4) was then added and the samples were incubated for 30 min. The reaction was stopped by adding 100 µl of 0.18 *M* sulfuric acid. The plates were read within 30 min in an ELISA reader set at 450 and 550 nm. Values were obtained by subtracting the reading at 550 nm from the reading at 450 nm, to correct for any optical defect of microtiter plate. IFN-γ release was expressed as picograms per milliliter per 24 h of incubation. The sensitivity of the assay was 100 pg/ml.

Statistical Analysis

Statistical analysis of the results was performed by Student's t test, a one-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls test or Dunnett's t test. Mean values were considered significantly different if $p < 0.05$.

Results

Figure 1 shows the release of IFN-γ after a 24-hour incubation of cells derived from submaxillary lymph nodes of rats subjected to a unilateral SCGx and to a contralateral sham operation 20 h or 7 days earlier. Lymph node cells obtained at the time of degeneration of sympathetic nerve terminals exhibited a significantly higher IFN-γ release than those derived from the innervated lymph nodes. In contrast, cells obtained from chronically sympathectomized lymph nodes released significantly

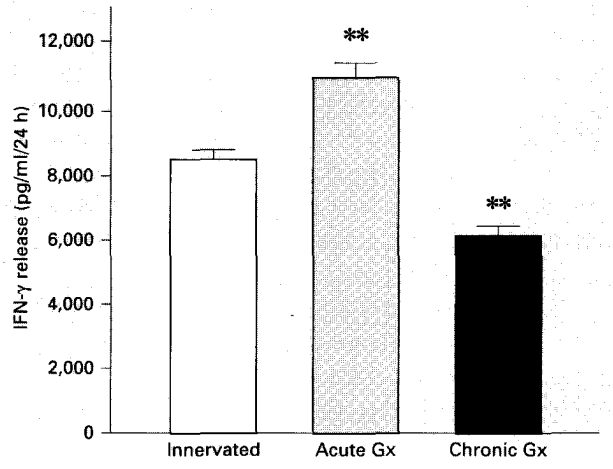

Fig. 1. Release of IFN-γ by cells from submaxillary lymph nodes of rats subjected to a unilateral SCGx and a contralateral sham operation 20 h or 7 days earlier. Means ± SEM of 8 animals, except for the control group (n = 16) for which sham-operated values of both time intervals were pooled after verifying that they did not differ significantly. ** $p < 0.01$ as compared to controls, one-way ANOVA followed by a Dunnett's t test.

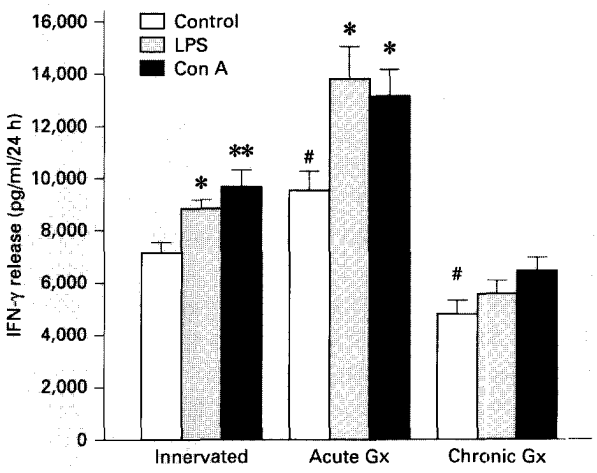

Fig. 2. Effect of mitogenic stimulation (LPS, Con A) on IFN-γ release by cells from submaxillary lymph nodes of rats subjected to a unilateral SCGx and a contralateral sham operation 20 h or 7 days earlier. Means ± SEM of 8 animals, except for the control group (n = 16) for which control sham-operated values of both time intervals were pooled after verifying that they did not differ significantly. * $p < 0.05$, ** $p < 0.01$ as compared to controls, one-way ANOVA followed by a Dunnett's t test in each experimental group; # $p < 0.05$ as compared to the innervated control group, ANOVA, Dunnett's t test.

Table 1. Effect of NE on IFN-γ release by cells from submaxillary lymph nodes of rats subjected to a unilateral SCGx and a contralateral sham operation 20 h or 7 days earlier

	IFN-γ-release, pg/ml/24 h	
	vehicle	10^{-8} *M* NE
Sham operation	6,556 ± 502	6,377 ± 805
Acute SCGx	9,012 ± 698[c]	7,561 ± 876
Chronic SCGx	4,298 ± 396[b]	17,626 ± 1,864[a]

Means ± SEM of 8 animals, except for the control group (n = 16) for which control values of both time intervals were pooled after verifying that they did not differ significantly.

[a] $p < 0.001$ as compared to vehicle, Student's t test.

[b] $p < 0.05$ compared to the innervated control group, ANOVA, Dunnett's t test.

[c] $p < 0.01$ as compared to the innervated control group, ANOVA, Dunnett's t test.

less IFN-γ ($p < 0.01$; fig. 1). There were no significant differences in the weight of denervated lymph nodes as compared to the controls (results not shown).

The effect of LPS or Con A stimulation on IFN-γ release by cells obtained from submaxillary lymph nodes of rats subjected to acute or chronic SCGx is depicted in figure 2. Stimulation by mitogens was only apparent in cells prepared from innervated or acutely denervated lymph nodes, with significant effects for both LPS and Con A. As in figure 1, higher levels of IFN-γ release after 24 h were found in cells from acutely sympathectomized lymph nodes, and lower levels in those from chronically denervated lymph nodes, when data in unstimulated lymph nodes were analyzed by a one-way ANOVA (fig. 2).

Table 1 summarizes the effect of 10^{-8} *M* NE on IFN-γ release by cells obtained from innervated or denervated lymph nodes. In the chronically denervated lymph nodes, exposure to NE augmented significantly IFN-γ release, whereas in the innervated or acutely denervated groups the addition of NE did not modify IFN-γ release.

Discussion

The foregoing results further contribute to the concept that the sympathetic nervous system is an active efferent pathway through which the CNS regulates immune responses. Cells obtained from submaxillary lymph nodes at the time of degeneration of local sympathetic nerve terminals (20 h after SCGx) released significantly more IFN-γ than controls after a 24-hour incubation, whereas those prepared from chronically sympathectomized lymph nodes (7 days after SCGx) released significantly less IFN-γ. At the two time intervals studied, essentially different phenomena occurred in the vicinity of sympathetic varicosities, namely a transient hyperactivity of the denervated organ as a consequence of the supraliminal transmitter release from degenerating nerve terminals 20 h after ganglionectomy and an irreversible postsynaptic paralysis 7 days after operation [13]. Stimulation of IFN-γ release by LPS or Con A was apparent in cells derived from innervated or acutely denervated lymph nodes, while it was obliterated in cells prepared from chronically denervated lymph nodes. A stimulatory effect of a physiological concentration of NE on IFN-γ release was found in chronically denervated lymph nodes only.

The existence of a link between IFN-γ release and the sympathetic nervous system has been suggested by several studies. NE added in vitro at physiological concentrations to infected human lymphocytes accelerated HIV-1 replication by decreasing production of several cytokines, including IFN-γ [14]. In the present study we could not detect any effect of NE on IFN-γ release by cells derived from innervated rat submaxillary lymph nodes. Whether species differences exist regarding NE-induced IFN-γ release awaits further investigation.

One obvious question about the present results concerns as to whether the changes found after denervation are due to changes in the cellular composition of the nodes in response to denervation. We can partly answer this question. In a recent study, the submaxillary lymph nodes of rats that received a SCGx or sham operation and that were injected 10 days later with Freund's complete adjuvant or its vehicle were examined [15]. In rats injected with adjuvant vehicle, mean values for 24-hour rhythms of relative size of B, T, B-T, CD4+, CD8+ and CD4+CD8+ cell lymphocyte subsets in lymph nodes were not affected by chronic SCGx. Further studies are needed to ascertain whether this is also true for acute SCGx lymph nodes.

Data on the effect of sympathetic denervation on IFN-γ synthesis and release are conflictive. For example, several days after the surgical denervation of the rat spleen, a suppression of the effect of Con A on splenic IFN-γ gene expression was observed [16]. This indicates, in agreement with the present study, the existence of a long-term depressive effect of sympathetic denervation on IFN-γ release. However, an increase in Con-A-induced IFN-γ production by lymph node cells, and a decrease in Con-A-induced IFN-γ production by spleen cells, occurred after chemical sympathectomy of adult mice injected with the adrenergic neurotoxin 6-hydroxydopamine [17]. It must be noted that a number of systemic reflexes arise after systemic 6-hydroxydopamine injection, e.g. induction of Fos protein in several brain nuclei or activation of corticotrophin releasing factor-containing neurons in the hypothalamic paraventricular nucleus [18]. This may mask, through a number of endocrine side effects, the local influence of suppressing sympathetic nerve activity.

Further studies are needed to explore the effect of a local (surgical) sympathetic denervation on IFN-γ release in different lymphoid organs before a definitive picture of the phenomenon is obtained. In addition, a more extensive time course than the 24-hour and 7-day time points reported herein would be useful, although at least for tissue NE levels, a steady decrease to 5–10% of initial values is observed within 24–48 h of SCGx and remained unmodified thereafter [10–13]. It is interesting to note that LPS, a T-cell-independent mitogen, caused a significant release of IFN-γ in cells derived from submaxillary lymph nodes. Indeed, IFN-γ is produced by cells other than T cells, e.g. natural killer cells [19] which could be present in the submaxillary lymph node cultures. Moreover, after LPS exposure, a number of Th2 cytokines are produced that can stimulate T cells to produce IFN-γ [20–22]. The origin of the IFN-γ produced in cell cultures by mitogen exposure deserves to be further explored.

The present study indicated that while NE was ineffective to modify IFN-γ release in innervated or in acutely denervated lymph nodes, it augmented IFN-γ release in chronically denervated lymph nodes. Therefore, changes in adrenoceptor density at different time intervals after sympathetic denervation of the submaxillary lymph nodes could be responsible for the differential response to NE observed.

In a previous study we examined the acute (after 18 h) and the chronic effects of SCGx (after 12 days) on the in vitro mitogenic responses of lymphocytes from submaxillary lymph nodes [23]. In acutely denervated rats, LPS or Con A produced an increased mitotic response as compared to the innervated lymph nodes, whereas in chronic

SCGx rats, the mitogen effect remained unchanged. Our present results on IFN-γ release can partly explain those observations since an augmentation of IFN-γ release by cells derived from acutely denervated lymph nodes was found. However, the inhibition of IFN-γ release detected in cells derived from chronically sympathectomized lymph nodes did not correlate with the unhampered effect of Con A or LPS reported earlier [23]. Apparently, the reduced amounts of IFN-γ found in lymph nodes from chronic SCGx rats are enough to elicit a normal response to mitogens. In view of the significant changes in immune parameters that occur as a function of the time of day the tissue is obtained [11], additional studies are needed, including cells collected throughout the 24-hour cycle, before a definitive picture of the incumbent responses is obtained.

A last aspect of the present study merits comment. Studies employing NE indicated that the neurotransmitter was ineffective to modify IFN-γ release in innervated or in acutely denervated lymph nodes while it augmented IFN-γ release in chronically denervated lymph nodes. The extent of changes of adrenoceptor populations at different time intervals after sympathetic denervation of the submaxillary lymph nodes deserves to be further explored, both by examining the concentration-dependent effects of NE in the presence of antagonists as well by binding characterization of the adrenoceptors involved. In a preliminary study the effect of NE on IFN-γ release in chronically denervated lymph nodes was blocked by adding the α-blocker prazosin or the β-blocker propranolol, alone or in combination [Castrillón et al., unpubl. results].

In conclusion, the present and foregoing discussed studies, together with reports dealing with alterations in antibody responses of submaxillary lymph nodes following local sympathectomy [12, 24], with the evidence that the diurnal changes in submaxillary lymph node cell proliferation is related to a circadian signal conveyed by the local autonomic neurons [11] and with indication that immunosuppression diminished in lymph nodes during acute stress in rats partly due to changes in signal trafficking in SCG nerves [25] support the concept that the sympathetic innervation of submaxillary lymph nodes is an important channel for neural regulation of immune responses in this tissue. To what extent the release of other cytokines besides IFN-γ (i.e. tumor necrosis factor or interleukins) is affected by sympathetic denervation of lymph nodes awaits further investigation.

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