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Effect of Interleukin 1β on rat thymus microenvironment

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SUMMARY

The effect of interleukin 1β on the thymus of control and chemically sympathectomized adult and aged rats was studied with the aim of assessing the importance of adrenergic nerve fibres (ANF) in the regulation of some immunological functions.The whole thymus was removed from normal, sympathectomized (with the neurotoxin 6-OH-dopamine) and treated (interleukin 1β) rats. Thymic slices were stained with eosin orange (for the recognition of microanatomical details of the thymic microenvironment) and with Bodian's method for staining of nerve fibres. Histofluorescence microscopy was employed for staining ANF and immunofluorescence was used for detecting NPY-like immunoreactivity. All images were submitted to quantitative morphometrical analysis and statistical analysis of data. Moreover, the amount of proteins and noradrenaline was measured on thymic homogenates. The results indicate that in normal conditions the formation of the thymic nerve plexi in the rat is complex: the majority of ANF are destroyed after chemical sympathectomy with 6-OH-dopamine and do not change after treatment with interleukin 1β; on the contrary, treatment with interleukin 1β induces substantial changes in the fresh weight of the thymus, the thymic microenvironment, thymic nerve fibers, ANF, NPY-like positive nerve fibres, and on the total amount of proteins and noradrenaline in rat thymic tissue homogenates.Immunostimulation with interleukin 1β induces substantial changes in the whole thymus, in its microenvironment and in ANF and NPY-like nerve fibres. After chemical sympathectomy, no significant immune response was evoked by interleukin 1β, since the majority of ANF was destroyed by chemical sympathectomy.

INTRODUCTION

As early as 1976, Singh and Owen (1976), studying the maturation of thymus stem cells, demonstrated that catecholamines exert a role on the expression of T alloantigens. Sympathetic innervation of the thymus is inhibitory to the immunoreactivity of T lymphocytes and to the proliferative response of the mouse thymus, as confirmed in earlier reports (Besedowsky *et al.*, 1979; Singh, 1979; Singh *et al.*, 1979). Moreover, the sympathetic nervous system modulates the antibody response to thymus-independent anti-

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gens (Miles *et al.*, 1981). This effect demonstrates the existence of a functional link between the nervous and immune systems. The sympathetic nervous system exerts a selective modulation of antibody response. Sympathetic nerve endings can be destroyed with 6-OH-dopamine (6-OH-DA). The sympathectomy induces a different response to thymus-dependent and -independent antigens (Williams *et al.*, 1981). In fact, sympathectomy induces modifications in the maturation of thymocytes (Singh, 1985a; Singh, 1985b).

In antigenically-stimulated rats, the mitotic cells present in the thymic cortex are reduced.

A statistically significant increase in the density of innervation was found for the vessels of the capsula and septa, but could not be found in either the cortex or medulla of the thymic parenchyma (Novotny and Hsu, 1993).

Interleukin 1ß was first employed by Niijima *et al.* (1991) to study its effects on autonomic nerves. Interleukin 1ß was able to enhance sympathetic nerve activity (Ichijo *et al.*, 1994) and induce noradrenaline release (Shimizu *et al.*, 1994) in rats.

Moreover, interleukin 1ß was also able to enhance the efferent activity of the vagus nerve to the thymus. In fact, an iv. injection of 10 ng of interleukin 1ß in rats induced activation of efferent electrical activities of the vagal branches to the thymus. These branches are involved in the neural modulation of thymic functions (Niijima *et al.*, 1995).

We studied the effects of immunostimulation with inteleukin 1ß on GABA-transaminase (Cavallotti *et al.*, 2000c) and on acetylcholinesterase activities in rat thymus (Cavallotti *et al.*, 2000d).

We have studied the occurrence of adrenergic nerve fibres (ANF) in the human thymus in normal conditions (Cavallotti *et al.*, 2000a) and after immune response induced by treatment with interferon β (Cavallotti *et al.*, 2000b). Moreover, we studied the occurrence of GABA-transaminase (Cavallotti *et al.*, 1999a) and ANF (Cavallotti *et al.*, 1999b) in young and old rats. These data served as a basis for the present paper, where we present the results obtained on (ANF), neuropeptide Y (NPY)-like immunoreactivity and noradrenaline (NA) content in rat thymus during immune response. Treatment with 6-OH-DA completely destroyed adrenergic innervation and in this experimental condition rats did not respond to treatment with interleukin 1β.

MATERIALS AND METHODS

Male Wistar rats, weighing 180-360 gr and aged 12-24 months were used. The animals were caged in groups of three and housed in controlled lighting (lights on from 08:00 to 20:00). Food and water were provided ad libitum. The animals were treated in conformity with the Helsinki accord on the use of animals in research approved by all Institutional Review Boards.

The procedures performed in this study included: 1) experimental groups of animals; 2) treatment with interleukin 1β; 3) treatment with 6-OH-DA; 4) removal of the thymus and sections of the samples; 5) staining of the thymus; 6) staining of nerve fibres; 7) histofluorescence microscopy; 8) immunostaining of NPY reactivity; 9) estimation of protein content; 10) dosage of NA content; 11) quantitative analysis of images; 12) statistical analysis of the data.

1) *Experimental groups.* Group A, rats aged 12 months (n=18) from which A1 rats without treatment served as normal controls (n=6); A2 rats, treated with interleukin 1β (n=6) and A3 rats, treated for one week before sacrifice with 6-OH-DA (n=6, 6 survived out of 10 treated). Also, rats of group B (24 months old, n=18) were divided into three subgroups B1,B2,B3 and treated in the same manner as those of group A.

2) *Treatment with interleukin 1β.* The animals of groups A2 and B2 were treated with interleukin 1β, a promoter of the immune response (Niijima *et al.*, 1995). The preparations used were recombinant human interleukin 1ß (RH-IL-1, 15.7 Kda Genzyme) and human fraction interleukin 1ß (HFr-IL-1ß,1005 Kda Peninsula). Both preparations were dissolved in physiological saline just before use and injected i.v. in a volume of 0.1 ml at a rate of 0.1ml/30 sec. at a dose of 10 ng. Treatment was administered daily for six weeks to 6 rats for each age group. Both fractions of interleukin 1ß were used in the same sample at the same concentration.

3) *Treatment with 6-OH-DA.* The animals of groups A3 and B3 were subjected to chemical sympathectomy. Destruction of thymic sympathetic nerve fibres by 6-OH-DA was obtained following and partially modifying the guidelines proposed by Johnson (1980) and Angeletti and Levi-Montalcini (1970). Each rat received 6-OH-DA i.p. in a solution of 0.5% ascorbic acid, at doses of 100

mg/kg/day for 3 days. After 3-5 days, this treatment leads to the destruction of almost all ANF.

4*) Drawing of thymus and sections of the samples.* One week after the end of the treatment by immunostimulating drugs or by 6-OH-DA all the animals were killed by a sharp below on the head and decapitated. The thymus glands were then carefully and rapidly removed. The dissected organs were weighed and measured using a gauge. The thymuses were rapidly transported to our laboratories under dry ice for the experimental procedures.

Serial sections (10 μm) thick were cut through the whole thymus on a criostat (–20°C). Each section was mounted on a pre-weighed slide. The slide was post-weighed for determining the weight of each section.

Ten consecutive thymic sections were mounted on ten numbered slides (from 1 to 10).

The first was treated with Eosin to provide histological orientation, to identify the microanatomical details and to define the thymic compartments.

The second one was treated with Bodian's method to identify the nerve fibres.

Another three sections (from 3 to 5) were treated for histofluorescence microscopy (blank without glyoxylic acid, blank with denatured section and whole reaction).

The last five sections (from 6 to 10) were used for immunostaining of NPY (four blank plus one slide for whole reaction).

Once the sections had been cut, a small slice (200 μm thick) was cut, weighed and used for biochemical assay. The same procedure (cutting in serial sections) was used for the whole length of the thymus.

5) *Staining of thymus.* The microanatomical details of thymic tissues were detected with Eosin-orange. After fixation, sections were treated with a working solution of Eosin-orange (Eosin gamma water soluble 10 gr dissolved in 1000 ml of distilled water plus 2 ml of glacial acetic acid) which represents the stock solution. The working solution consists of 25 ml of stock solution + 75 ml of H2O + 0.5 ml of glacial acetic acid (Townsend, 1960).

6) *Staining of nerve fibres.* Nervous structures were coloured using the method of Bodian (1936). This method can be used to verify that a stained structure is nervous in nature: in fact, it stains nerve fibres and neurofibrils. After fixation in Bouin's fluid sections were treated with: 1) 1% protargol solution (colloidal silver), 2) reducing solution (hydroquinone+ sodium sulphite), 3) 1% gold chloride solution, 4) 2% oxalic acid solution and counterstained with 0.03 % aniline blue. The nerve fibres and neurofibrils were coloured in black. Further details regarding this staining are reported in the cited work of Bodian (Bodian, 1936).

7) *Histofluorescence microscopy.* For the staining of adrenergic nerve fibres, a glyoxylic acid-induced fluorescence technique was used as described by Qayyum and Fatani in 1985 (Qayyum and Fatani, 1985). Briefly, the staining solution was prepared immediately before the use by adding to a solution of 0.236 M potassium phosphate monobasic (pH 7,4) 0.2 M sucrose and 1% glyoxylic acid; this staining solution is named sucrose, phosphate, glyoxylic acid (SPG). The slides with thymic samples were immediately dipped in this solution for 5 minutes. To assure a comparable fluorescence, it is important to standardize times and temperatures without intervals. After staining, the sections must be drained, covered with non-autofluorescent immersion oil, heated at 95°C for 5 min, and coverlipped. The sections must then be immediately observed, analyzed and photographed to prevent the diffusion and the photodecomposition of the fluorescence.

The sections were examined and photographed under a Zeiss photomicroscope equipped with exciter and barrier filters and with a mercury lamp for observation of the fluorescence.

8) *Immunostaining of NPY reactivity.* The immunohistochemical method used for the detection of the NPY-positive nerve fibres was proposed by Uddman *et al.* (Uddman *et al.*, 1985). Owing to the thickness of the sections (10 μm), the samples were incubated for a long time (18-24 hours) at room temperature, so that the antibodies completely penetrated the sections, with the rabbit anti-NPY serum (Cambridge RB-CRB-U.K.) diluted 1: 600 in PBS.

Five slides (each containing one slice of sample) were used for each immunostaining procedure.

The first four slides contained: 1) the first blank: primary or secondary antiserum omitted or denatured or previously absorbed with an excess of corresponding peptide; 2) the second blank: primary or secondary antiserum replaced by a non-immune serum; 3) the third blank: sample previously fixed by immersion in a 4% solution of formaldehyde in PBS that does not preserve the immunoreactive

sites; 4) the fourth blank: sample denatured with formaldehyde before or after treatment with primary antiserum or before treatment with secondary antiserum. All these procedures showed the absence of any immunoreaction. Positive immunostaining was only observed in the last (fifth) slide that contained the normally treated sample.

After treatment with the specific antibodies (rabbit anti-NPY) the samples were washed in PBS and incubated with fluorescein isothyocyanate-conjugated antiserum (goat anti-rabbit IgG-Nordic Immunological Reagents: NIR, The Netherlands) diluted 1:100 in PBS for 18-24 hours at room temperature to allow complete penetration of the fluorescent IgG into the thick sections (10 μm). The samples were washed in PBS and observed using a Zeiss III photomicroscope equipped with epi-illumination and Neofluar objectives. Once the samples had been stained with NPY, it was always possible to identify the total fluorescent area of the nerve fibres stained by this neurotransmitter under light microscopy. Morphometrical quantification of the density of nerve fibres was performed using a Quantimet Leica® 500 image analyzer.

The identification of NPY in peripheral tissues such as the thymus requires considerable care.

Immunohistochemical techniques are able to show the "in situ" localization of numerous neuropeptides including NPY. Immunohistochemistry uses fluorescent antibodies to stain neuropeptides in specific structures on tissue sections. Fluorescein isothiocyanate is a common fluorescent marker used to visualize the immunohistochemical reaction. However, other visualization techniques also are suitable. Owing to the potential for antibody cross-reactivity to chemically related antigens, together with other non-specific antigens, an immunohistochemical staining is never unequivocal and absolute. The specific staining of a neuropeptide requires numerous controls (Coons *et al.*, 1955).

Even after all these controls (such as those we employed) have been performed, absolute identification of a specific neuropeptide still requires a biochemical analysis. For these reasons, in all immunohistochemical results, the descriptive suffix "NPY-like immunoreactivity" should be used.

9) *Estimation of protein content.* In all experiments samples of thymus coming from the autopsies were weighed and placed on dry ice, (specimens for histochemical staining) or into an ice-cold homogenization buffer (samples for estimating the protein content and the biochemical activity). Tissue protein concentration was determined by the method described by Lowry *et al.* using bovine serum albumin (BSA) as standard and Folin phenol as reagent (Lowry *et al.*, 1951).

10) *Determination of noradrenaline content.* The NA content of the thymic tissue was determined by high pressure liquid chromatography (HPLC) as reported by Keller *et al.* (1976).

Briefly, the tissue samples (with previously determined weight and protein content) were homogenized in a 1:10 solution of perchloric acid (0,1 ml/L) with sodium metabisulphite (0.5 ml/L) used to prevent the oxidation of NA. The homogenate was centrifuged at 3000 r.p.m. for 20 min. The supernatant was injected into the chromatographic system in aliquots of 10, 20 and 30 μl.

Electrochemical detection was performed by means of a glassy carbon electrode versus Ag/AgCl reference electrode at 0,75 V. The mobile phase was formed by sodium phosphate (50mmol/L) citric acid (25mmol/L, pH 3,6), EDTA (0.25 mmol/L), octane sulphonic acid (sodium salt 0,75 mmol/L) and 3% acetonitrile. The results are expressed as ng/mg protein ± SD.

11) *Quantitative analysis of images (QAI).* In order to evaluate the amount of staining, a quantitative analysis of the intensity of the histochemical staining was performed on photographs (to avoid the photodecomposition of the fluorescence) by means of a Quantimet Analyzer Leica®. The values of control photographs (from samples incubated without glyoxylic acid or incubated without anti-NPY) were considered as "zero". Each photograph was examined separately, evaluating the standard error of the mean (S.E.M.).

QAI may provide incorrect results. In fact, the main choices (i.e. the instructions for software) are ordered by each research-worker, according to personal preferences. For these reasons the data tend to be partial rather than impartial and it is necessary to follow very careful rules. The counts must be repeated at least three times using the technique of the double masked. All the counts should be performed by different research-workers, on different analysers, and with samples identified only by a number or by a letter. Final results must be obtained by another research-worker, who examines experimental protocols to identify each sample and attribute specific values. Final values

must be submitted to the statistical analysis of data. The values reported in this paper represent the intensity of staining for each sample and are expressed in conventional units (C.U.) ±SEM.

Further details on QAI are reported in the Book of methods of Quantimet Leica 500 image analyzer (Manual of Methods for Quantimet 500, 1997).

12) *Statistical analysis of data.* The preliminary studies of each value were performed with the aid of basic sample statistics. Mean values, maximum and minimum limits, variations, standard deviation (S.D.), standard error of the mean (S.E.M.) and correlation coefficients were performed according to Serio (1986).

The relationship between each pair of variables was studied using the respective correlation coefficients grouped in a correlation matrix, thus enabling us to study the existence of a linear (values next +1 or -1) or non-linear (values next +0) dependency. Finally, a correlative analysis of the morphological and biochemical data was performed by comparing the significant differences for each group with the corresponding values of the other homogeneous groups.

Correlation coefficients denote a significant level less than 0.001 ($P<0.001$), while the correlation coefficient is not significant when $P>0.05$ (n.s.). This correlation coefficient was calculated according to Castino and Roletto (1992).

RESULTS

QAI, performed after staining with eosin orange for the detection of the microanatomical details in thymic microenvironment, shows that both age and treatment with interleukin 1ß induces substantial opposite changes in the thymic microenvironment. In fact, in aged rats the thymus shows the signs of an involution (decrease of thymocytes, increase of Hassall's corpuscles, increase of connective tissue and of fatty cells). After treatment with interleukin 1ß we can observe an upward trend in the whole thymic microenvironment. Moreover, the total nerve fibres stained by Bodian's method appear unchanged in their morphology and in their number both in aged and interleukin 1ß treated rats. After chemical sympathectomy with neurotoxin 6-OH-dopamine the majority of thymic ANF are destroyed and consequently the thymic tissue is not responsive to treatment with interleukin 1ß (Table 1).

In adult and old rats, treatment with interleukin 1ß induces an increase in all values tested in our experiments (in comparison to untreated rats).

The fresh weight of lobes (in mg) was 108±0.16 in six untreated rats of 12 months of age rising to 138±0.21 in a group of six rats of the same age after immune-response (treated with interleukin β). Comparing the significant differences between treated versus untreated rats we can observe $P<0.001$.

Table I
Experimental values found in rat thymus after immune response

Thymus	Untreated (18 months, n=6)	Treated (18 months, n=6)	Untreated (24 months, n=6)	Treated (24 months, n=6)
Weight of lobes mg±SD	68±0.15	98±0.22*	149±0.14	61±0.27*
Protein content mg/g fresh tissue weight±SD	9.9±0.16	12.3±0.12(ns)	9.6±0.13	11.7±0.14*
Biochemical dosage of NA ng/mg protein±SD	447.5±18.9	491.4±16.3*	576.8±21.3	684.3±32.1
Total nerve fibres^ C.U.±SEM	15.6±1.3	15.2±1.4	15.9±1.6	15.8±1.7

Each value is the mean of independent determinations from six animals (standard deviation -SD- for biochemical values and standard error of the mean -SEM- for morphological values). P was calculated by comparing the significant differences in treated versus untreated rats. Thymic microenvironment: the signs of a thymic involution are: decrease of number of thymocytes, increase of number of fatty cells, increase of number of Hassall's corpuscles. Treatment with 6-OH-dopamine versus respective untreated group induces a total disorder of ANF and the thymic values cannot be detected; (ns)= not significant *$P<0.001$ n=number of animals for each experimental group. ^Stained with Bodian's method and counted by QAI. C.U.= conventional units.

In untreated rats of 24 months of age the fresh weight of the whole thymus was 49 mg ±0.14. It rose to 61 mg ±0.27 in treated rats of 24 months after the administrtion of interleukin 1ß. In this case, P of treated versus untreated rats is <0.001.

The protein content of the thymus expressed as mg/gr. tissue fresh weight was 11.2±0.14 in untreated rats of 12 months of age rising to 14.3 ±0.11 in treated rats of the same age.

In untreated rats (24 months of age) this value was 9.6±0.13 mg/gr tissue and rose to 11.7±0.14 mg/gr tissue in treated rats of the same age after administration of interleukin 1β.

In both age-groups (12 and 24 months) the significant differences calculated by comparing the values obtained in treated versus untreated rats are highly positive (P<0.001). Moreover, the protein content of the thymus decreased with age.

The NA amount, expressed as ng/mg protein and measured in the supernatant of the whole thymus homogenate, is 224.0±15.9 ng/mg protein in untreated rats (n=6 12 months old). It increases to 311.4±18.3 ng/mg protein in rats of the same age previously treated with interleukin 1ß (n=6, 12 months old). The coefficient of significance between these two groups is highly positive with P<0,001.

In untreated elderly rats (n=6, 24 months old) the amount of NA increases to 576.8±21.3 ng/mg protein; in rats of the same age previously treated with interleukin 1ß (n=6, 24 months old) the amount of NA arises to 684.3±32.1 ng/mg protein. In this case the coefficient of significance between treated versus untreated is also highly positive with P<0,001.

The results of NPY-like immunoreactivity and those of ANF fluorescent staining are discussed in relation to Table 2 and 3.

In fact, in Table 2 we can observe the experimental values of QAI performed after the immunofluorescent staining for NPY-like activity.

The values changes after interleukin 1ß treatment expecially in the thymic microenvironment where we can observe a staining increase from 10.8±2.2 CU in untreated rats to 31.8±1.2 in treated rats (n=6 12 months old). In structures resembling nerve fibres, the differences between untreated and treated rats (n=6, 12 months old) are not significant (n.s.) passing from 41.9±3.4 to 43.3±3.1 C.U.

Neither were the differences significant in the thymic arteries and in thymic veins in untreated and treated rats with the following values: 18.4±1.1 in veins of untreated rats (n=6, 12 months old) and 20.4±1.1 in treated rats. In the thymic arteries the values for untreated rats are 24.1±1.2 while in treated rats the values are 25.5±2.1 (n=6, 12 months old): in this case too the differences are not significant (n.s.). In the whole thymus the experimental values are 18.4±1.6 C.U. in normal untreated rats, while they go up to 26.7±3.4 C.U. in treated rats. Considering the same values in untreated and treated elderly rats (n=6, 24 months old) we can observe the following quantitative results: thymic microenvironment from 6.6±1.6 C.U. to 17.3±1.6 C.U. In nerve

Table II

Experimental values of the quantitative analysis of images after immunofluorescent staining for NPY and after immune response

Thymus	Untreated (18 months, n=6)	Treated (18 months, n=6)	Untreated (24 months, n=6)	Treated (24 months, n=6)
NPY C.U. ± S.E.M.	11.4±1.3	18.7±3.1	10.5±1.4	16.4±1.3
Vessels	13.3±1.5	22.41±1.1(ns)	16.3±1.3	19.6±1.7(ns)
Nerve fibres	47.4±2.8	49.8±3.1(ns)	22.4±1.9	23.1±1.8
Microenvironment	9.82±2.2	33.4±1.2*	6.6±1.6	17.3±1.6*

The values represent the intensity of the staining for NPY and are expressed in Conventional Units (C. U.) as described in the methods ± standard error of the mean (S.E.M.). The analyzer was calibrated considering as "zero" the values of control sections incubated without antigen or in the absence of the specific antiserum. Each value represents the mean of many determinations carried out in double masked as reported in methods. P was calculated by comparing the significant differences in treated versus untreated rats; (ns)= not significant. *P<0.001 Treatment with 6-OH dopamine causes a strong decrease of NPY-positive structures. The thymic values of these structures cannot be detected and therefore the related values are not reported in this table.

Table III
Experimental values of the QAI following histofluorescent staining for ANF after immune response

Thymus	Untreated (18 months, n=6)	Treated (18 months, n=6)	Untreated (24 months, n=6)	Treated (24 months, n=6)
ANF C.U. ± S.E.M.	40.3±1.6	43.2±1.8	38.6±1.1	41.2±1.5*
Vessels	27.1±1.5	29.7±(ns)	16.4±1.2	26.8±1.6(ns)
Nerve fibres	48.1±3.1	53.1±3.9*	40.3±3.8	46.1±3.1*
Microenvironment	18.8±1.6	29.4±2.1*	11.4±0.9	20.6±1.1*

The values represent the intensity of the staining for ANF and are expressed in Conventional Units (C. U.) as described in the methods ± standard error of the mean (S.E.M.). The analyser was calibrated considering as "zero" the values of the control sections incubated without glyoxylic acid. Each value represents the mean of many determinations carried out in double masked. P was calculated by comparing the significant differences in treated versus untreated rats; (ns)= not significant; *P<0.001; w.t.°= whole thymus. Treatment with 6-OH Dopamine causes a strong decrease of NPY-positive structures. The thymic values of these structures cannot be detected and therefore the related values are not reported in this table.

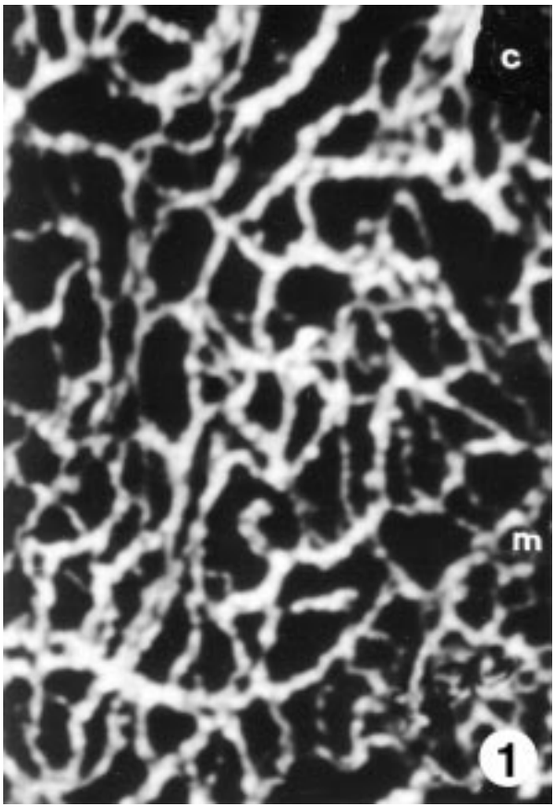

Fig. 1 - Thymus NPY-like positive nerve fibres in an untreated young rat. Magnification 100X. In normal conditions we can observe many fluorescent subcapsular nerve fibres that penetrate the corticomedullary boundaries. These nerve fibres show an irregular course with varicosities, swellings and crossings.

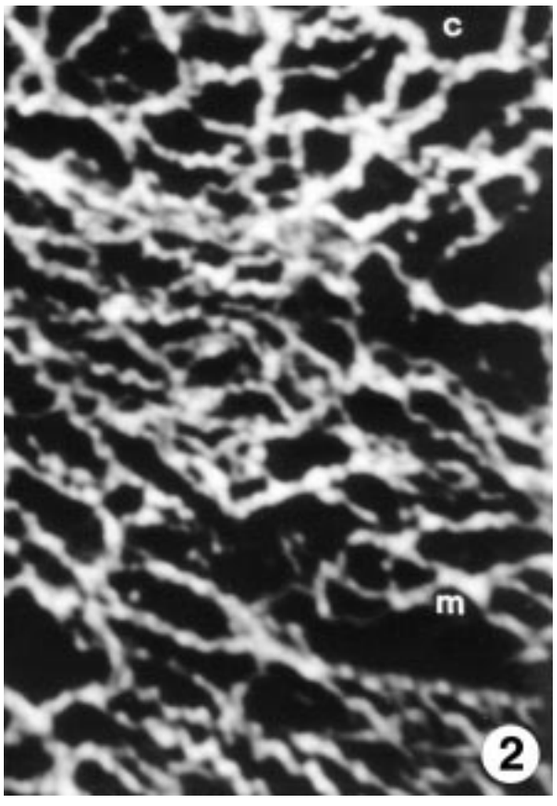

Fig. 2 - Thymus NPY-like positive nerve fibres in a young rat previously treated with interleukin 1β. Magnification 100X.After treatment with interleukin 1β the fluorescent nerve fibres are increased in number and in thickness.

fibres the values are 22.4±1.9 in untreated and 23.1±1.8 in treated rats.The coefficient significance is not positive (n.s.). Thymic veins and arteries gave these results: 16.3±1.3 in six untreated rats of 24 months of age and 19.6±1.7 in six rats of the same age treated with interleukin 1ß. In arteries the following results were observed: 21.6±0.9 in untreated and 22.5±2.3 in treated rats. Finally, whole thymus values were 10.5±1.4 C.U. in untreated rats, increasing to 16.4±1.3 in treated rats of 24 months of age.

Examining Table 3 we can observe the following results of ANF in treated and untreated rats aged both 12 months and 24 months. ANF in the whole thymus from 42.3±1.6 C.U. (n=6 age 12 months untreated) to 58.2±3.1 C.Us (n=6 age 12 months treated) from 38.6±1.1 C.U.(n=6 age 24 months untreated) to 41.2±1.5 C.U. (n=6 age 24 months treated).

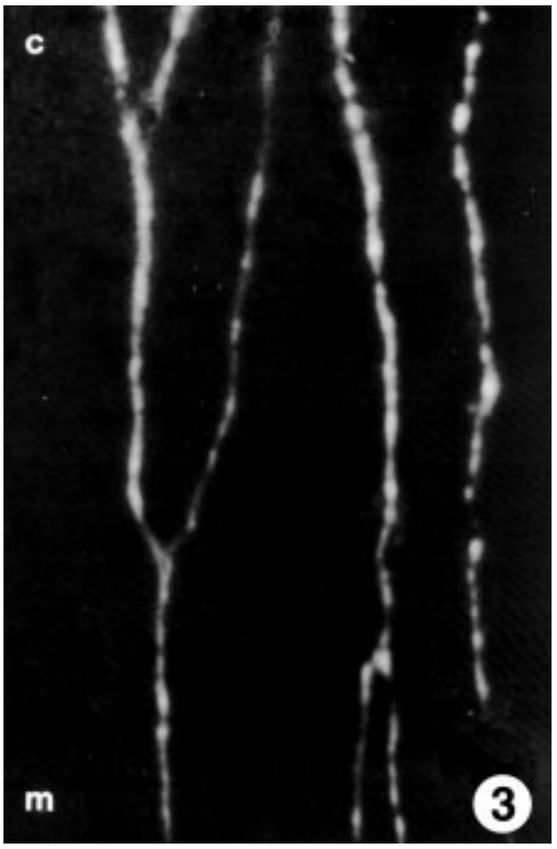

Fig. 3 - Thymus NPY-like positive nerve fibres in an untreated old rat. Magnification 100X. In old age the fluorescent NPY-like positive nerve fibres are decreased in number and thickness. Moreover, the amount of fluorescence is also decreased.

Thymic arteries, veins and structures resembling nerve fibres do not present substantial variations while in the thymic microenvironment there are marked differences between untreated and treated rats, from 31.8±2.7 C.U. to 59.8±3.9 (in 12 months old rats) and from 11.4±0.9 C.U. to 20.6±1.1 (in 24 months old rats).

Careful examination of figures 1-4 shows that: in young, untreated rats many fluorescent subcapsular nerve fibres run in the cortico-medullary zones. The course of these fibres is irregular and presents varicosities and crossings (Fig. 1). After treatment with interleukin 1β these fluorescent nerve fibres appear to be increased both in number and in thickness (Fig. 2). In an untreated old rat only a few nerve fibres containing a small amount of NPY-like immunoreactivity can be observed (Fig. 3). After treatment with interleukin 1ß, the fluorescent nerve fibres are increased in number and thickness (Fig. 4). Initially, their course is irregular with numerous swellings and varicosities. The amount of fluorescence is also increased.

DISCUSSION

The interleukines 1ß are natural proteins related to the class of cytokines (such as interferons, lymphokines, thymomodulines, thymostimulines, thymopentines and others). All these substances exert an immunostimulating and/or an immunomodulating action (Gurney *et al.*, 1986).

Interleukines 1ß exert their effects on adrenal, splenic and renal tissues through the sympathetic nerves (Niijima *et al.*, 1991). Interleukines 1ß also induce NA release in the spleen (Shimizu *et al.*, 1994).

Sympathetic nerves are able to suppress the cytotoxicity of natural killer cells (Katafuchi *et al.*, 1993a; Katafuchi *et al.*, 1993b) and interleukin 1ß enhances splenic sympathetic nerve activity in rats (Ichijo *et al.*, 1994). Through the action of sympathetic nerve fibres interleukines are capable of suppressing the cytotoxicity of natural killer cells (Katafuchi *et al.*, 1993a; Katafuchi *et al.*, 1993b).

In the rat thymus treatment with interleukin 1ß induces an increase of protein content, biochemically tested level of NA, histochemically stained amounts of ANF, and NPY-like immunoreactivity. Staining of different structures of the thymus in

treated and untreated rats shows that the greatest modifications concern the parenchyma, the structures resembling nerve fibres and the whole thymus.

The nerve fibres that supply the thymus are numerous and contain many neurotransmitters.

Thymic innervation arises from three sources: 1) parasympathetic or cholinergic source (Xth cranial nerve) 2) sympathetic or adrenergic source (plexi surrounding the origin of the main vessels emerging from the heart) 3) somatic source (by means of phrenic nerve). At their origin, all these nerve fibres run parallel to the vessels: subsequently, they form periadventitial plexi and penetrate into the vascular wall, entering the capsule of the thymus and spreading to the connective tissue of the thymus. Finally, they pass between the lobules so that small, thin nerve fibres penetrate the medulla where they form beads, varicosities, swellings and spreadings of neurotransmitters. Only a few nerve fibres coming from the medulla penetrate the cortex where they form a relationship with thymic cells and thymic microenvironment. From careful observation of our results, the statistical analysis of data shows that only the values related to the whole thymus, nerve fibres and parenchyma are significant with $P<0.001$, while the values found in thymic arteries and in veins are not significant. Concerning the vascular component of the thymus we can affirm that the changes found with age are probably related to the global involution of the thymus and the parenchyma/vessels ratio may be modified owing to the real loss of parenchyma (remaining substantially unchanged the vascular component). Moreover, immunostimulated rats show a really unchanged vascular component versus the untreated ones.

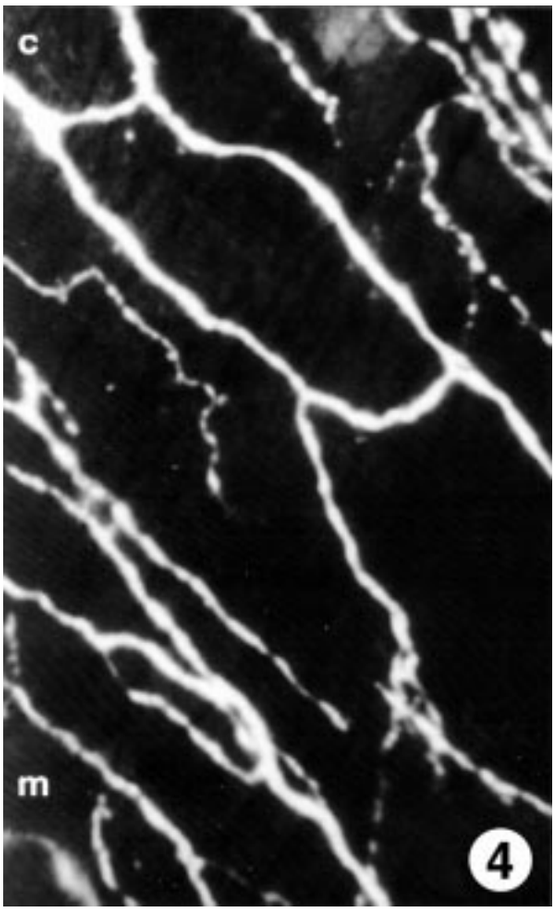

Fig. 4 - Thymus NPY-like positive nerve fibres in an old rat previously treated with interleukin 1β. Magnification 100X. After treatment with interleukin 1β the fluorescent nerve fibres are increased in number, in thickness and in the amount of the fluorescence.

Nevertheless, all values in the rat thymus increase after treatment with interleukin 1β. In all our experimental procedures, control sections incubated without the specific antibodies and/or in presence of competitive inhibitors, gave negative results. All our results confirm that in old rats all values are modified in comparison to adult rats. In rats treatment with interleukin 1β induces a strong increase of all tested values if compared to untreated rats of the same age. On the strenght of these results, we can hypothesize that interleukin 1β is a promoter of the thymic immunomodulation and is also able to induce biochemical and morphological changes in the thymic microenvironment.

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