

# Hormonal function and proliferative activity of thymic cells in humans: Immunocytochemical correlations

Igor M. Kvetnoy,<sup>1</sup> Victoria O. Polyakova,<sup>2</sup> Alexander V. Trofimov,<sup>2</sup> Vadim V. Yuzhakov,<sup>3</sup> Alexander A. Yarilin,<sup>4</sup> Emma S. Kurilets,<sup>3</sup> Ludmila N. Mikhina,<sup>3</sup> Nina I. Sharova<sup>4</sup> & Maria F. Nikonova<sup>4</sup>

1. Ott Institute of Obstetrics and Gynecology, St. Petersburg, Russia
2. St. Petersburg Institute of Bioregulation and Gerontology, St. Petersburg, Russia
3. Medical Radiological Research Center, Obninsk, Russia
4. Institute of Immunology, Moscow, Russia

*Correspondence to:* Prof. Igor M. Kvetnoy, M.D., Ph.D.  
Ott Institute of Obstetrics and Gynecology  
3 Mendeleevskaya linia, St. Petersburg, 199034, RUSSIA  
TEL: +7 812 328 98 30 FAX: +7 812 230 00 49  
EMAIL: kvetnoy@gerontology.ru

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## Abstract

**OBJECTIVES:** The immunocytochemical study of the localization of hormones in thymic cells has been performed to clarify possible correlations of their expression with proliferative activity of thymocytes.

**METHODS:** We used commercial antibodies to serotonin, melatonin, somatostatin, glucagon, gastrin, beta-endorphin and histamine, and ABP or BSP kits for visualization of reaction. Computer image analysis was used to find correlations between hormone production and proliferative activity of thymocytes.

**RESULTS:** Different subpopulations of thymocytes are able to produce hormones: precursors of T-lymphocytes (CD4<sup>-</sup>CD8<sup>-</sup>) contain serotonin and melatonin, immature cortical cells (CD4<sup>+</sup>CD8<sup>+</sup>) produce only serotonin, mature medullar cells (CD4<sup>+</sup>CD8<sup>-</sup>) show immunoreactivity to serotonin, melatonin, beta-endorphin and histamine. The expression of serotonin, somatostatin and gastrin is localized in thymic epithelial cells, formatting Gassal's bodies. Proliferative activity of thymocytes depends from the expression of serotonin and somatostatin in thymic cells.

**CONCLUSION:** The data received testify the expression of different hormones in human thymic cells and showing by this fact high endocrine activity of thymus. The presence of correlations between hormonal expression and cell proliferative activity could be considered as the bright illustration of important role of neuroimmunoendocrine mechanisms in the regulation of local thymic homeostasis.

## Introduction

It is well known, that thymus is the central organ of immune system crucial for T-lymphocytes development. Thymus is lympho-epithelial gland, which is specialized exclusively for the development of T-lymphocytes and myeloid elements of its own microenvironment. Epithelial thymic cells are the stable component of local origin, but lymphoid cells are transitory elements, their precursors migrate in thymus from the bone marrow and the major part of mature T-lymphocytes then migrates to the peripheric compartment of immune system. Cellular and humoral factors of the microenvironment, formatted by stromal elements and thymic epithelium effect on T-lymphocytes precursors and control T-cell maturing, their differentiation in subpopulation and clonal selection [1, 2]. There are some data, testifying that additionally to the key role of thymus in the regulation of immune function, this gland also effects non-immunological organs including neuroendocrine system [7, 9, 12].

It is well known that immune system together with the nervous and endocrine systems plays very important role in homeostasis regulation and adaptation of organism to the changes of external environment. Currently, it is well established that immune system cells may express cytokines, neuropeptides, tissue growth factors, and also receptors to many signal molecules [2,3]. The presence of receptors to hormones, neuro-mediators and neuropeptides in different target cells supports close interactions of nervous, endocrine, and immune systems in homeostasis regulation, what allows to introduce the term “*neuroimmunoendocrinology*” into the scientific lexicon [1].

Many data about localization of peptide hormones like, ACTH, endorphins, TTG and also growth factors in immune system cells have been received earlier [5,6]. In 1994 it was shown that not only mast cells produce histamine – Zwadlo-Klarwasser *et al.* [14] using radioimmunological analysis obtained macrophages, monocytes and lymphocytes as alternative sources of histamine in human organism. Later Kubo and Nakana [13] have determined the possibility of histamine synthesis in subpopulations of T-lymphocytes – CD4<sup>+</sup> and CD8<sup>+</sup> populations in mice. It was established that mice T-lymphocytes produce histamine *de novo* in response to mitogenic effect. In this situation granulocyte/macrophage colony-stimulating factor and interleukin-3 essentially enhance concavalin A induced histamine production. Data about neuroendocrine differentiation of thymic cells were confirmed at the study of the expression and ectopic secretion of synaptophysin, chorionic gonadotropin, ACTH, calcitonin, calcitonin-gene-related peptide, and serotonin in thymic carcinomas and thymomas (11).

This paper is devoted to immunocytochemical analysis of the localization of biogenic amines and regulatory peptides in thymic cells to clarify possible correlations of their expression with the proliferative activity of thymocytes.

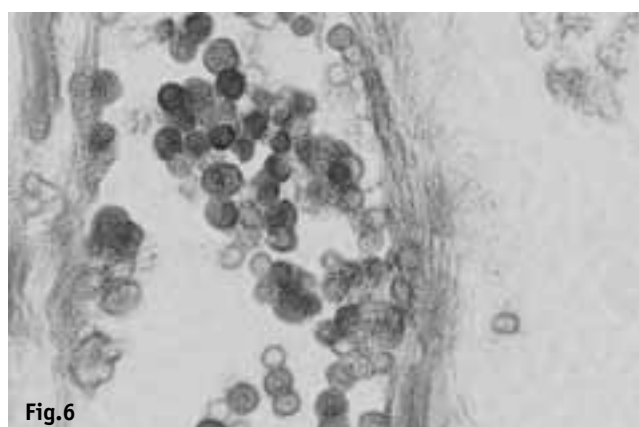
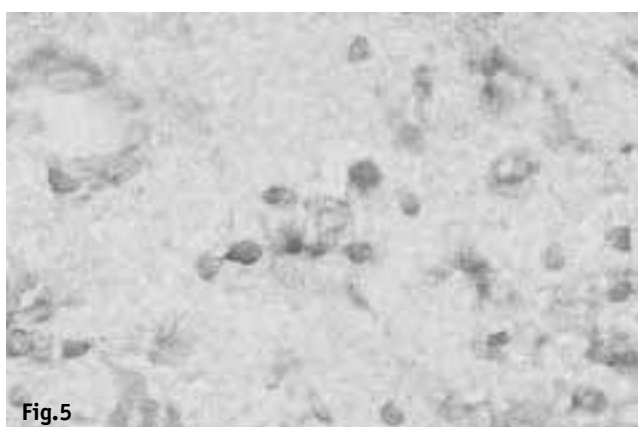
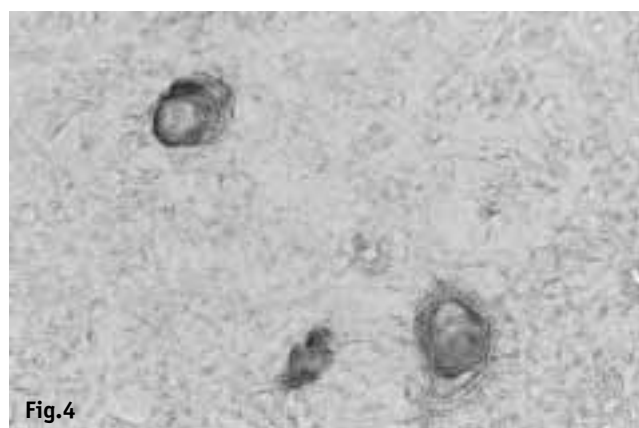
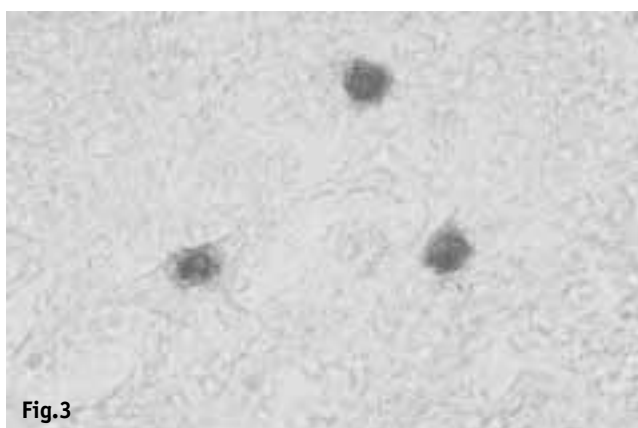
## Materials and Methods

The morpho-fuctional study has been performed in thymus of children at the ages of 6 months, and 1–4 years old. Material was taken through surgery of congenital heart’s defects. Also thymocytes and epithelial thymic cells cultures have been investigated. Thymocytes were excreted from the thymic fragments of 20-week’s human embryos which were received from abortion material. Thymocytes were divided on subpopulations using kits for positive CD4 and CD8 secretion. In this case magnetic microglobules (Dyna’s) carrying antibodies to CD4 or CD8 markers were added into cellular suspension (concentration 10<sup>7</sup>/ml) cooled till 4°C, in ratio 4–10 cells for 1 carrier and were incubated for 20 minutes into refrigerator, where they were constantly agitated. All stacked cells were cleaned with magnet use. The cells were removed from the globules by specific enzyme’s solution (Denach&Bead). The CD4<sup>+</sup> or CD8<sup>+</sup> fractions correspondingly remained in the solution after all flushes. The expression of membrane molecules for identification of thymocyte’s subpopulations was determined by flow cytofluorometry with using kits of monoclonal antibodies to surface antigens (Catlag). Flow cytofluorometry was performed on flow cytometer FACSCalibur (Becton Dickinson).

For immunocytochemical study the cellular suspension was preliminarily fixed in mixture of 4% formaldehyde and 0.1% glutaraldehyde on the phosphate-saline buffer, pH 7.4 (PSB) for 3–5 min and then, by sequent centrifugation—resuspension was washed off by PSB. After elimination fixative admixture 0.03% triton X-100

**Table 1.** Immunocytochemical study of hormone’ expression in human thymus

Antibodies	Immunostaining kits*
Rabbit Anti Histamine ,1:50 ICN	Anti Rabbit, BSP ICN
Rabbit Anti Serotonin, 1:50 BioGenex	Anti Rabbit, BSP BioGenex
Mouse Anti Serotonin, 1:15 DAKO	Anti Mouse, BSP ICN
Rabbit Anti Melatonin, 1:10 SID Tech Res	Anti Rabbit, BSP BioGenex
Rabbit Anti β-Endorphin, 1:100 Amersham	Anti Rabbit, BSP BioGenex
Rabbit Anti Somatostatin, 1:40 DAKO	Anti Rabbit, ABP Sigma
Rabbit Anti Somatostatin, 1:100 Novocastra	Anti Rabbit, BSP ICN
Rabbit Anti Gastrin, 1:40 DAKO	Anti Rabbit, ABP Sigma
Rabbit Anti Gastrin, 1:100 Novocastra	Anti Rabbit, BSP ICN
Rabbit Anti Glucagon, 1:40 DAKO	Anti Rabbit, ABP Sigma
Rabbit Anti Glucagon, 1:25 Novocastra	Anti Rabbit, BSP ICN
Mouse Anti Insulin, 1:100 Novocastra	Anti Mouse, BSP ICN
Rabbit Anti Calcitonin, 1:40 DAKO	Anti Rabbit, ABP Sigma



or 0.025% saponine were added at the suspension for 20 min for cellular membrane permeabilization. Cellular suspension were incubated in refrigerator for 4 °C and periodically resuspended. After triton/saponine elimination the cells washed twice by PSB for 3 min and prepared on glasses.

As thymic epithelial cells (TEC) we used the cells of suspensioal line VTEC2.H/S, which were received earlier by transformation of TEC of embryonal human thymus using the material containing SV40 virus and by sequent transformed cell cloning. More than 90% of VTEC2.H/S cells contain cytokeratins 8/18, which confirms their epithelial nature. Immunocytochemical study has been performed with using antibodies to different hormones (*Table 1*). Antibodies to PCNA

and to cyclin A (Novocastra) were used as markers of proliferative cell activity.

Computer analysis of microscopic images was performed by using of Morphostar-2 system (Imstar S.A.). Indexes of proliferation ( $I_{PCNA}$  and  $I_{cyclin A}$ ) were calculated by the following ratio:

$$I_{marker} (\%) = \frac{N \text{ nuclei, staining by antibody to marker}}{N \text{ nuclei, staining by hematoxylin-eosin}} \times 100$$

The program ANOVA (Kruskal-Willis) was used for correlation analysis, and the program Statistica (Statsoft) was used for statistical analysis.

**Table 2.** Computer analysis of proliferative activity in human thymus

Number of case	Age	CORTEX		MEDULLA	
		I <sub>PCNA</sub> <sup>*</sup>	I <sub>cyclin A</sub> <sup>*</sup>	I <sub>PCNA</sub> <sup>*</sup>	I <sub>cyclin A</sub> <sup>*</sup>
1	6 months	23.21	13.11	8.76	1.63
2		21.07	12.19	7.28	1.97
3		20.12	14.18	8.11	1.81
4		19.10	11.59	8.20	1.94
M ± m for 6 months (n=4)		<b>20.8 ± 0.9</b>	<b>12.7 ± 0.6</b>	<b>8.1 ± 0.3</b>	<b>1.8 ± 0.1</b>
5	1–2 years	29.82	17.37	5.64	1.12
6		28.57	16.21	6.45	0.99
7		32.91	15.27	3.84	1.18
M ± m for 1–2 years (n=3)		<b>30.3 ± 1.3</b>	<b>16.3 ± 0.6</b>	<b>5.3 ± 0.8</b>	<b>1.1 ± 0.06</b>
8	2–4 years	40.59	15.70	3.04	0.93
9		40.12	17.93	4.77	0.83
10		36.72	16.87	5.10	0.85
M ± m for 2–4 years (n=3)		<b>39.1 ± 1.2</b>	<b>16.8 ± 0.6</b>	<b>4.3 ± 0.6</b>	<b>0.87 ± 0.03</b>

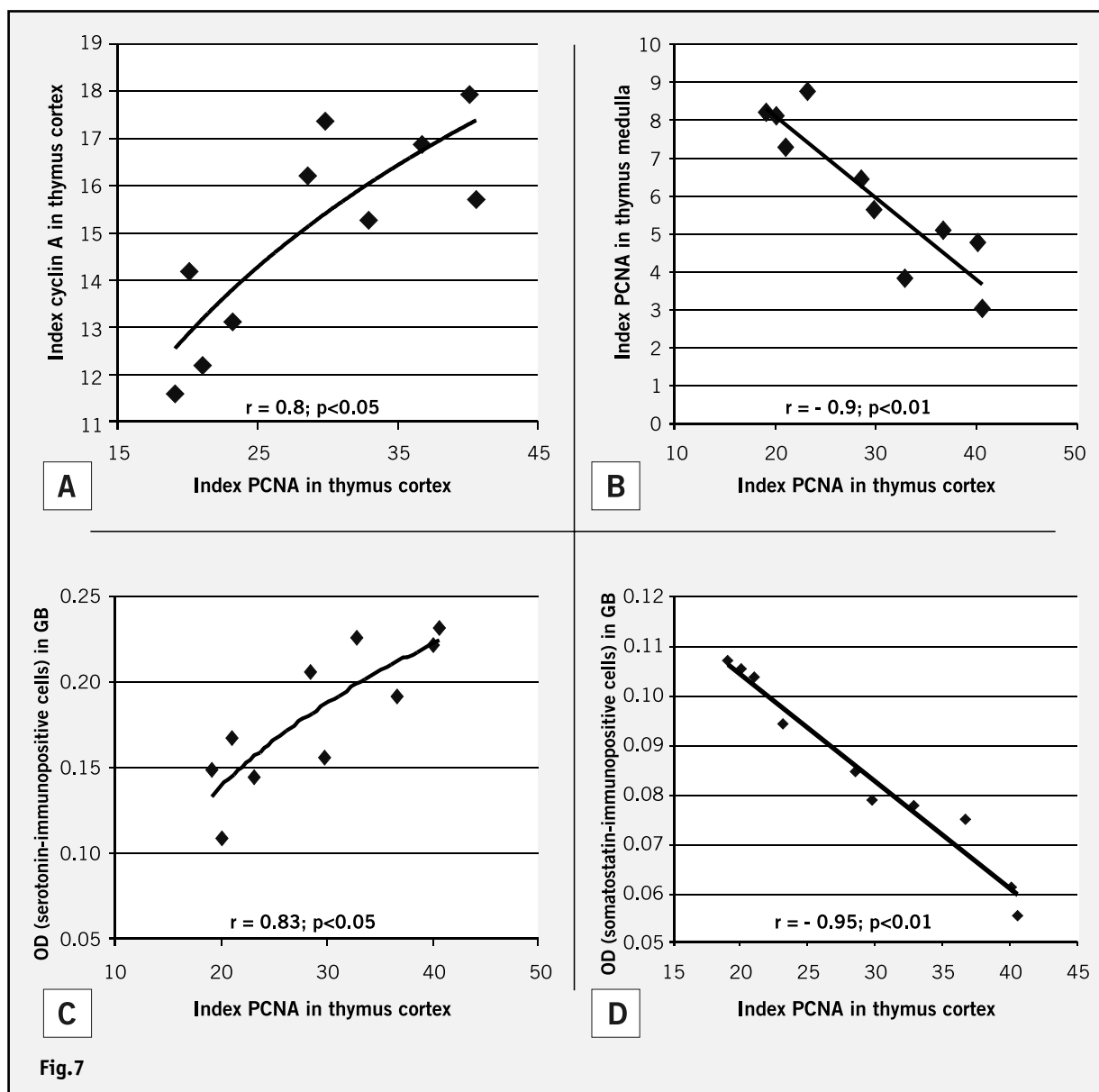
**Table 3.** Computer analysis of serotonin and somatostatin expression in epithelial cells of Gassal's bodies in human thymus (calculation has been performed on Gassal's bodies' square)

Number of case	Age	SEROTONIN		SOMATOSTATIN	
		Volume density	Optical density	Volume density	Optical density
1	6 months	0.182	0.144	0.062	0.095
2		0.194	0.168	0.095	0.105
3		0.190	0.108	0.185	0.106
4		0.251	0.149	0.081	0.108
M ± m for 6 months (n=4)		<b>0.204 ± 0.015</b>	<b>0.142 ± 0.012</b>	<b>0.106 ± 0.027</b>	<b>0.104 ± 0.003</b>
5	1–2 years	0.171	0.156	0.025	0.079
6		0.251	0.206	0.110	0.085
7		0.341	0.226	0.105	0.078
M ± m for 1–2 years (n=3)		<b>0.254 ± 0.049</b>	<b>0.196 ± 0.021</b>	<b>0.080 ± 0.028</b>	<b>0.081 ± 0.002</b>
8	2–4 years	0.307	0.231	0.048	0.055
9		0.256	0.221	0.052	0.061
10		0.301	0.191	0.138	0.075
M ± m for 2–4 years (n=3)		<b>0.288 ± 0.016</b>	<b>0.214 ± 0.012</b>	<b>0.079 ± 0.029</b>	<b>0.064 ± 0.006</b>

**Table 4.** Expression of biogenic amines and peptide hormones in subpopulations of thymocytes.

Antibodies	Immunostaining kits <sup>*</sup>	Immunopositive reaction		
		Pre-T CD4 <sup>-</sup> CD8 <sup>-</sup>	Im-T CD4 <sup>+</sup> CD8 <sup>+</sup>	M-T CD4 <sup>+</sup> CD8 <sup>-</sup>
Rabbit Anti Histamine 1:50 ICN	Anti Rabbit BSP ICN	–	–	+
Rabbit Anti Serotonin 1:50 BioGenex	Anti Rabbit BSP BioGenex	+	+	+
Mouse Anti Serotonin 1:15 DAKO	Anti Mouse BSP ICN	+	+	+
Rabbit Anti Melatonin 1:10 SID Tech Res	Anti Rabbit BSP BioGenex	+	–	+
Rabbit Anti β-Endorphin 1:100 Amersham	Anti Rabbit BSP BioGenex	–	–	+

**Pre-T** – Thymocytes precursors; **Im-T** – Immature thymocytes; **M-T** – Mature thymocytes.



**Fig. 7.** Correlations between proliferative activity of thymocytes and the expression of serotonin and somatostatin in epithelial cells of Gassal's bodies. **OD** - optical density ; **GB** - Gassal's bodies

## Results and Discussion

Human thymus has the lobular structure on histologic sections stained by hematoxylin and eosin. Bond-tissue septums pass from the thin capsula into tissue, which divides it to lobes. The darker internally-located cortical substance and the lighter centrally-located medulla substance are legibly shown in the lobes. Dark color of cortex caused by high density of thymocytes filling of epithelial space. Few layers of subsheath lymphoblasts are located in surface part of cortex, which belongs to sheath and septums. Small thymic lymphocytes located between epithelial cells in the area of deep cortex and cortex-medullar zone. Cortical epithelial cells are not numerous, they have extended triangle or star-shaped shape because of long and thin cytoplasmic branches creating the "network" in which cortical thymocytes are located. The nuclear saturation of thymic cortex on slides stained by hematoxylin is  $41014 \pm 462$

cells in  $1 \text{ mm}^2$  of section's square in 6 months children;  $41948 \pm 1147$  – for 1–2 years old age group, and  $43137 \pm 762$  – for 2–4 years old age group. The main population of proliferative cells in children thymus are located in cortex and has tendency to concentrate in peripheral lobular area.

In medulla the quantity of lymphocytes is significantly lower than in cortex. It is explained by the fact that mature thymocytes stay in medulla and don't leave thymus. Epithelial cells have a big light nucleus and in some cases formatted well shown Gassal's bodies. The cellular density is high in medulla:  $32262 \pm 1423$  in 6 months old children;  $34835 \pm 911$  – for 1–2 years old age group, and  $33667 \pm 490$  – for 2–4 years old age group, but only single nuclei give a positive reaction with antibodies to PCNA and cyclin A. The indexes of proliferative activity parameters in thymus of different aged children is represented in *Table 2*.

Thin layers of connective tissue locate between thymic lobes, they contain small blood vessels and mast cells. Mast cells in children thymus compound small cellular clusters (10–25 at 1 mm<sup>2</sup>) and are concentrated like chains. According to immunocytochemical study serotonin and somatostatin expression is brightly verified in epithelial thymic cells, forming Gassal's bodies (Table 3, Fig. 1, 2). Immunostaining of single reticuloepithelial cells and Gassal's bodies with antibodies to gastrin was shown also in some cases (Fig. 3, 4). Immunopositive reaction with antibodies to glucagon was shown in reticuloepithelial cells also (Fig. 5).

Computer analysis of microscopic images shows the presence of positive correlation ( $r = +0.8$ ;  $p < 0.05$ ) between PCNA and cyclin A expression in nuclei of thymocytes in cortex (Fig. 6, 7A) and negative correlation ( $r = -0.9$ ;  $p < 0.01$ ) between indexes of proliferation in cortex and medulla of thymus gland (Fig. 7B). Moreover, there are correlations between serotonin and somatostatin expression in epithelial cells of Gassal's bodies and proliferative activity of human thymocytes (Fig. 7B, G). Probably, these correlations show the process of morpho-functional maturation of thymus. Also clear tendency to age-related increase of serotonin expression in epithelial cells, proliferative thymocyte activity in cortex, reduction of proliferation indexes in brain substance has been shown. At the same time the reduction of somatostatin production by epithelial cells occurs in the process of postnatal ontogenesis. The data received support the view about mitogenic effect of endogenous serotonin on lymphoid cell proliferation and about inhibiting somatostatin effect on this process.

The immunocytochemical study allows to find, in different subpopulations of human thymocytes on different stages of its differentiation, following hormones: T-lymphocytes precursors (CD4<sup>-</sup>CD8<sup>-</sup>) contain serotonin and melatonin; immature cortical cells (CD4<sup>+</sup>CD8<sup>+</sup>) contain only serotonin, mature medullar cells contain serotonin, melatonin, beta-endorphin, and histamine (Table 4).

The data received testify the expression of different hormones in human thymic cells, and by this fact, showing high endocrine activity of this gland. The presence of correlations between the hormonal expression and cell proliferative activity could be considered as the bright illustration of important role of neuroimmunoendocrine paracrine mechanisms in the regulation of local thymic homeostasis.

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