

# Modulating Effects of Epithalamin and Epithalon on the Functional Morphology of the Spleen in Old Pinealectomized Rats

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 132, No. 11, pp. 586-591, November, 2001  
Original article submitted October 9, 2001

Immunohistochemical and morphometric analysis showed that epithalamin and epithalon produced similar effects on the functional morphology of the spleen in pinealectomized rats. Both peptides prevented hyperplasia of lymphoid cells in follicular germinative centers induced by pinealectomy and potentiated the decrease in extramedullary hemopoiesis. These findings confirm the data on functional relationships between the pineal gland and immune system. The effects of epithalamin and epithalon on cell and tissue homeostasis in the spleen of old pinealectomized rats can be regarded as a manifestation of the general regulatory effect of these peptides.

**Key Words:** *peptide bioregulators; pinealectomy; spleen; PCNA; immunoglobulins*

The stability of media in the organism is maintained by a complex of intricate reactions requiring close relationships between the nervous, endocrine, and immune regulatory mechanisms. The role of the pineal gland (PG) in hormonal and immune regulation attracts special attention due to a wide spectrum of biological effects of its main hormone melatonin and some low-molecular-weight polypeptides [2,4,8,9,11].

It was established that cytomedins, peptide bioregulators mediating information exchange between various cell groups and modulating their functional activity, are present in PG [3]. Polypeptide preparation of PG epithalamin demonstrated high biological activity towards the reproductive, neuroendocrine, and immune systems [1]. Epithalon, a tetrapeptide (Ala-Glu-Asp-Gly) synthesized on the basis of epithalamin amino acid composition, exhibits higher biological activity [5]. Previous studies showed that epithalon

inhibits metabolic processes in the duodenal mucosa and hemo- and lymphopoiesis in the spleen [7]. It also regulates the function of gastric endocrine cells in pinealectomized rats [6].

We investigated the effects of epithalamin and epithalon on cell and tissue homeostasis in the spleen, a central organ of the immune system, under conditions of separated effects of pinealectomy (PE) and these drugs.

## MATERIALS AND METHODS

The study was carried out on 35 male Wistar rats (7 groups, 5 animals each) aged 18 months, kept under standard vivarium conditions with natural day/night cycle and balanced rations in November-December. Control group consisted of intact animals. In groups 1-6 PE was carried out under ether narcosis as described previously [6]. Starting from day 21 postoperation, the animals of groups 1 and 2 received daily (for 10 days) subcutaneous injections of isotonic NaCl (0.5 ml). Groups 3 and 4 animals were injected with epithalamin (0.5 mg) in an equivalent volume according

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to the same protocol, and groups 5 and 6 received epithalon (0.5 µg/rat). Controls were sacrificed simultaneously with groups 1, 3, and 5 animals. Functional morphology of the spleen in groups 1, 3, and 5 was examined on day 33 after PE (day 3 after the end of drug treatment) and in groups 2, 4, and 6 on day 42 after PE (day 12 after drug treatment). The material (spleen) was collected in the morning (10.00-12.00) at natural light under Nembutal narcosis (50 mg/kg), after which the animals were decapitated. Spleen fragments were fixed for 24 h in acid Bouin fluid. Paraffin sections (7 µ) were mounted onto slides covered with poly-L-lysine film (Sigma).

Spleen histology was studied on sections stained with hematoxylin and eosin. Murine monoclonal antibodies to proliferating cell nuclear antigen (PCNA) diluted 1:50 (clone PC10, Calbiochem) and biotin-streptavidin-peroxidase kit for detection of mouse immunoglobulins (ICN) were used for immunostaining of proliferating cells. Ig-containing cells were identified with a kit for detection of rat immunoglobulins (BioGenex).

Morphometric studies were carried out using Imstar S.A. system for computer analysis of microscopic images with applied Morphostar-2 and Colquant-2 software (Imstar S.A.) in accordance with the basic stereology principles in morphometry. The following stereological parameters were used: test area of 1 field (mm<sup>2</sup>); total test area (test area of 1 field multiplied by the number of fields); total field of structure sections (mm<sup>2</sup>); volumic density of structures (integral indicator of the content of structures in a volume of tissue, %), determined as the ratio of total area of structure section to total tested area); mean area of the structure section (µ<sup>2</sup>); total number of structure sections on a section area; numerical density (number of structure sections per unit of section area). The structures were counted in each animal in at least 60 visual test fields for 3 sections of each tested organ.

The mean area of lymphatic follicles, reactive centers, and width of the marginal zone were evaluated at the level of the maximum follicular diameters determined by serial sections of the spleen. A test area for study of the numerical density of PCNA-positive nuclei, mitoses, and apoptosis of lymphocytes in centers of follicle multiplication included at least 2000 cell nuclei stained with hematoxylin. The ratio of mitotic activity and apoptotic death of lymphocytes was evaluated using  $I_{M/A}$  index (ratio of dividing cells to cells dying by apoptosis). Lymphocytes dying by apoptosis in follicular germinative centers were evaluated by morphological criteria [14]. Test area for evaluating numerical density of PCNA-positive nuclei in zones of extramedullary hemopoiesis was at least

1 mm<sup>2</sup> and for volumic density of Ig-positive cells at least 2.5 mm<sup>2</sup>.

The results were statistically processed using non-parametrical Mann-Whitney's *U* test.

## RESULTS

Functional morphology of the spleen in control animals corresponded to normal (Fig. 1, Table 1). The white pulp was presented by lymphoid periarteriolar lymphatic sheath and round lymphatic follicles (Fig. 1, *a*). PCNA-positive nuclei were weakly immunostained (Fig. 1, *b*). The marginal zones was presented by perifollicular rings (70-90 µ wide) densely packed with reticular cells, large lymphocytes and macrophages. Small foci of myeloid hemopoiesis were situated in the subcapsular zone of the red pulp and along the trabecules (Fig. 1, *c*) with high intensity of immunostaining of PCNA-positive nuclei (Table 1). Ig-containing cells were detected virtually in the entire parenchyma with a trend to concentrate along the periphery of marginal zones and in chains along the trabecules (Fig. 1, *d*).

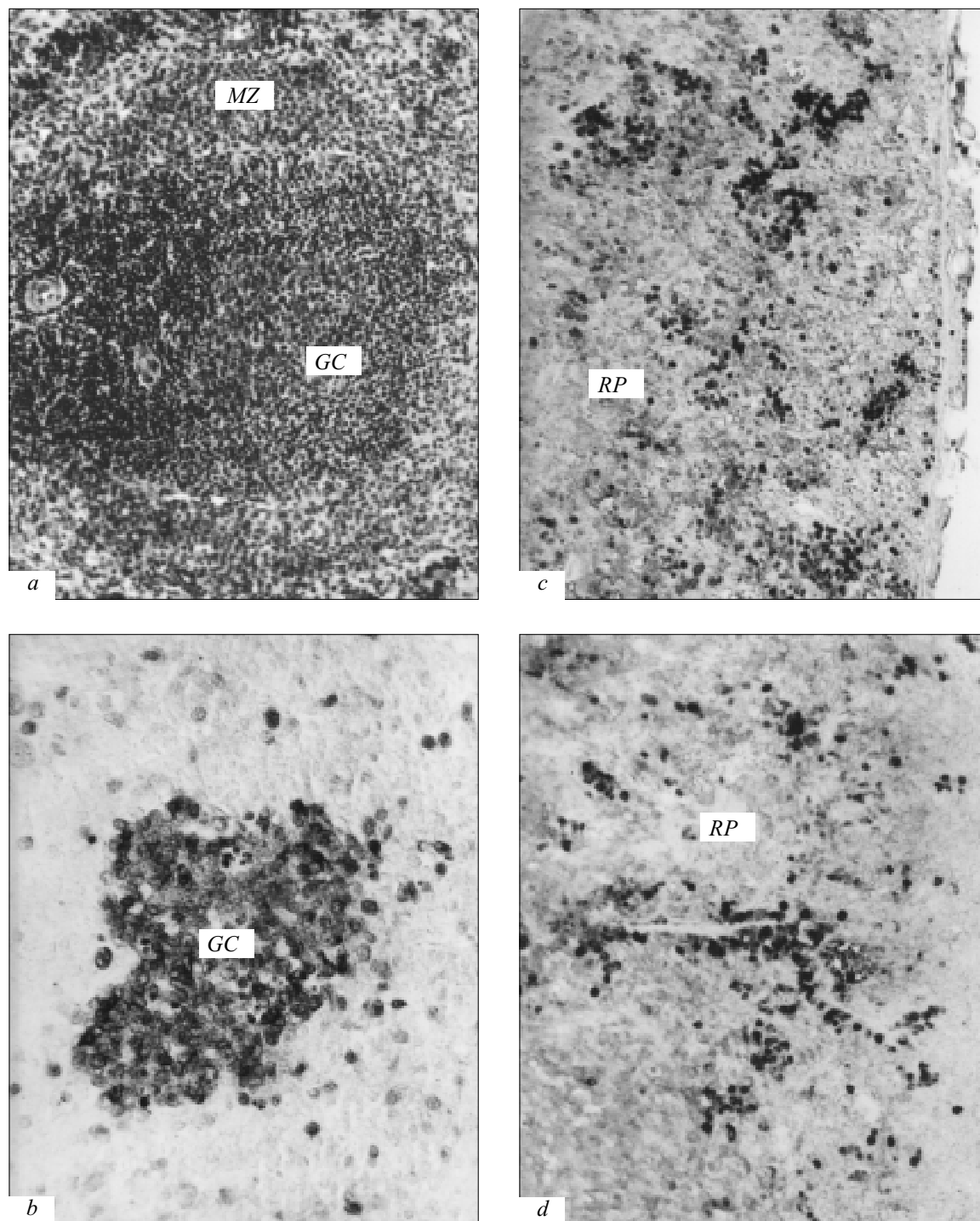
After removal of PG the most significant histological changes were observed in the white pulp. One month after PE the follicles and their germinative zones increased in size, and by day 42 periarterial connections and individual follicles fused and formed ramified cords. By this time the follicles and their multiplication centers enlarged almost 2-fold in comparison with the control. Numerical density of dividing lymphocytes and the content of apoptotic cells essentially increased in the follicular germinative centers.  $I_{M/A}$  increased by day 42 (Table 1), indicating a significant shift of the dynamic equilibrium towards intensification of lymphoid cell multiplication. Other picture of myeloid hemopoiesis and spontaneous antibody-producing cells was observed in the spleen of pinealectomized animals. According to computer analysis, 1.5 months after PE the quantitative density of PCNA-positive cells in zones of extramedullary hemopoiesis decreased by 32% and of Ig-containing cells in the red pulp by 28%.

In groups 3 and 5 the structure of the spleen tended to normal on day 3 after termination of epithalamin and epithalon treatment. Lymphatic follicles became round and smaller than in group 1. In animals receiving epithalamin numerical density of dividing lymphoblasts during this period decreased by 37% compared the corresponding parameter in group 1. In animals receiving epithalon  $I_{M/A}$  returned to normal (Table 1). On the other hand, according to the results of quantitative analysis both drugs seem to potentiate a decrease in the cellular proliferative activity in zones of extramedullary hemopoiesis (Table 1).

**TABLE 1.** Quantitative Characteristics of the Studied Parameters in the Rat Spleen ( $M \pm m$ )

Parameter	Control	PE					
		no correction		+epithalamin		+epithalon	
		day 33	day 42	day 33	day 42	day 33	day 42
Area of lymphoid follicle section, mm <sup>2</sup>	0.058±0.005	0.096±0.005*	0.108±0.006*	0.070±0.005 <sup>+</sup>	0.082±0.005* <sup>o</sup>	0.076±0.002 <sup>+</sup>	0.092±0.003*
Area of follicular GC, mm <sup>2</sup>	0.013±0.002	0.020±0.001*	0.025±0.002*	0.016±0.003	0.023±0.001*	0.022±0.002*	0.029±0.002*
Number of PCNA-positive cells in GC per mm <sup>2</sup>	7680±315	7873±238	8150±420	7400±570	8490±560	6870±460	10,300±690* <sup>o</sup>
Number of mitoses in GC per mm <sup>2</sup>	61±12	154±14*	197±17*	97±31	151±16*	120±6*	164±25*
Number of apoptotic cells in GC per mm <sup>2</sup>	105±4	162±11*	150±14*	103±10	217±31*	199±59*	201±41*
I <sub>M/A</sub>	0.56±0.09	0.97±0.05*	1.32±0.07*	0.92±0.10*	0.71±0.03 <sup>o</sup>	0.77±0.07	0.85±0.06* <sup>o</sup>
Width of marginal zone, μ	81±3	70±4	75±2	78±5	67±3*	85±4	51±2*
Number of PCNA-positive cells in zones of extramedullary hemopoiesis per mm <sup>2</sup>	1390±119	1270±124	950±121*	1150±134	843±163*	1020±70	860±63*
Volumic density of Ig-positive cells, %	4.71±0.28	3.83±0.23	3.40±0.35*	3.12±0.40*	3.03±0.23*	3.01±0.31*	2.82±0.20*

**Note.**  $p < 0.05$ : \*compared to the control, <sup>+</sup>compared to day 33 after pinealectomy without correction, <sup>o</sup>compared to day 42 after pinealectomy without correction.



**Fig. 1.** Functional morphology of the spleen in intact rat. Staining with hematoxylin and eosin (a) and biotin-streptavidin-peroxidase complex and poststaining with diaminobenzidine (b-d);  $\times 175$  (a, c, d),  $\times 350$  (b). a) histological structure of lymphatic follicle; b, c) immunohistochemical reaction of cells with anti-PCNA antibodies in follicular germinal center (b) and in zones of myeloid hemopoiesis (c); d) immunoglobulin-containing cells. GC: germinal center; MZ: marginal zone; RP: red pulp.

In group 4 (PE+epithalamin) histological structure of the spleen and a number of quantitative parameters on day 42 corresponded to the variants observed in group 3. There was just a trend to an increase in the lymphocyte proliferative activity in follicular germi-

nate centers, which was seen by the results of staining for PCNA and numerical density of mitoses. However an increase in the count of apoptotic cells was paralleled by a decrease in  $I_{M/A}$  in these animals (Table 1). The intensity of myeloid hemopoiesis and

count of Ig-positive cells decreased. Similar morpho-functional changes in the spleen was observed in group 6 animals treated with epithalon. On the other hand, on day 12 after the end of epithalon treatment, degenerative changes were detected in the marginal zones. Perifollicular rings were narrowed (Table 1), often looked blurred, somewhere with only reticular skeleton. Decreased intensity of myeloid hemopoiesis in the red pulp of these animals was paralleled by a reciprocal increase in the numerical density of PCNA-positive cells and a corresponding direction of  $I_{MA}$  in follicular germinative centers (Table 1).

Hence, hyperplasia of lymphoid cells, inhibition of myeloid hemopoiesis, and decreased count of Ig-containing cells are objectively recorded effects of PE in the spleen. Complex analysis showed that the effects of epithalamin and epithalon on the functional morphology of pinealectomized animals were similarly directed, and according to some quantitative parameters identical. Both peptide regulators inhibited lymphoid cell hyperplasia and possibly potentiated a decrease in extramedullary hemopoiesis. Comparative analysis of these drugs efficiency suggests that epithalamin is longer acting. It is probable that the increase in the lymphocyte proliferative activity and degenerative changes in the marginal zones 12 days after the end of epithalon treatment are caused by decompensatory reaction of lymphopoiesis on the inhibitory effect of this drug in pinealectomized animals.

These results confirm the findings of other authors on the functional relationships between PG and immune system [10,12,13]. It is now well known that immunocompetent cells can express cytokines, neuro-

peptides, tissue growth factors, and receptors to many signal molecules. This confirms the concept that the immune system is an important and indispensable component in the maintenance of homeostasis. We consider that effects of epithalamin and epithalon on the morphology and function of the spleen in pinealectomized old animals can be regarded as a manifestation of the general regulatory effect of these peptides.

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