MORPHOLOGY AND PATHOMORPHOLOGY

Immunohistochemical and Morphometric Analysis of Effects of Vilon and Epithalon on Functional Morphology of Radiosensitive Organs

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny,* Vol. 131, No. 3, pp. 338-346, March, 2001
Original article submitted October 4, 2000

Studies of the effects of vilon and epithalon on functional morphology of the thymus, spleen, and duodenum in intact rats and rats exposed to single whole-body γ-irradiation in a dose of 6 Gy showed that vilon stimulated proliferative activity of thymocytes and enhanced proliferative potential of stem cells in the intestine, thus stimulating the postradiation recovery of critical organs. Epithalon decelerated metabolic processes in the duodenal mucosa and suppressed hemopoiesis and lymphopoiesis in the spleen.

Key Words: *peptide bioregulators; ionizing radiation; proliferating cell nuclear antigen; serotonin; mast cells*

Lympho- and hemopoietic organs and intestinal epithelium are the most radiosensitive systems of the organism. Radiobiological and pathomorphological studies showed that the main events determining the pathogenesis of radiation damage develop early after irradiation. Recovery of radiosensitive organs depends on the number of survived stem cells and the rate of their proliferation. Apart from decreased proliferative potential of stem cells, damage to vascular and stromal elements and impairment of neurohumoral regulation, hemopoiesis, and immunity are also involved in the mechanism of radiation reactions. Vascular reaction in the irradiated organs is a typical symptom of radiation disease. Recent studies showed that ionizing radiation induces the release of biogenic amines from cells, which, in turn, trigger early morphofunctional vascular reactions [5]. Being a component of systemic response to ionizing radiation mast cell are among the most radiosensitive cells [11].

Complex therapy is used for the treatment of radiation-induced diseases [3]. Therapy with interleukins and growth factors stimulating proliferation of stem cells is a promising approach to the treatment of these conditions [6,10,13]. Another perspective approach is immunotherapy [1]. Experimental and clinical studies demonstrated high efficiency of peptide drugs isolated from immunocompetent organs of experimental animals in preventing and therapy of radiation-induced immunodeficiency [2].

The purpose of this study was to evaluated the effects of two peptide bioregulators, vilon and epithalon, on cell reparation in the thymus, spleen, and intestinal mucosa after radiation exposure. The agents were synthesized at St. Petersburg Institute of Bioregulation and Gerontology.

Vilon (Lys-Glu) was synthesized on the basis of amino acid sequencing of thymaline, a polypeptide preparation from the thymus, amino acid sequences in

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0007-4888/03/1313-0285$25.00

other thymic peptides and cytokines [9], and epithalon (Ala-Glu-Asp-Gly) was synthesized on the basis of amino acid analysis of epithalamine, a polypeptide preparation from the pineal gland.

MATERIALS AND METHODS

Experiments were carried out on male Wistar rats weighing 90-100 g. Some animals (n=30) were exposed to single whole-body γ-irradiation in a dose of 6 Gy on a cobalt GUB 20,000 device (200 rad/min power). After irradiation, 12 rats were not treated with regulatory peptides, and 12 and 6 animals were injected with vilon and epithalon (5 μg/kg, intraperitoneally) every other day for 5 days starting from day 2 postirradiation. Control groups consisted of 6 intact rats and 6 nonirradiated rats injected with vilon or epithalon (3 rats per group) according to the same protocol as irradiated animals.

The effects of vilon, epithalon, and their combination with γ-irradiation were studied on preparations of the thymus, spleen, and proximal portion of the duodenum. The organs were isolated under Nembutal narcosis 3 days after drug therapy and on day 8 after γ-irradiation. Tissue fragments were fixed for 24 h in Bouin fluid, dehydrated, and embedded in paraplast. Microtome sections (7 μ) were put onto slides coated with poly-L-lysine films (Sigma).

The histological studies were carried out on hematoxylin and eosin-stained sections. Murine monoclonal antibodies to proliferating cell nuclear antigen (PCNA) diluted 1:50 (clone PC10, Calbiochem) and avidin-biotin-peroxidase kit for detecting murine immunoglobulin (Vectastain) were used for visualization of proliferating cells. Serotonin-positive cells were detected using rabbit polyclonal antibodies to serotonin and biotin-streptavidin-peroxidase immunostaining system (BioGenex). Ig-containing cells were identified with a kit for detection of rat immunoglobulins (BioGenex). Mast cells were selectively stained with 1% toluidine blue (Fluka) in 0.5 M HCl (pH 0.5).

Morphometric studies were carried out using IMSTAR image analysis system and Morphostar-2 and Colquant-2 software in accordance with basic principles of stereology and morphometry. The following stereological parameters were used: A_T, total tested area; total number of sections of structures on A_T; numerical density (number of sections of structures per unit of section area); summary area of structure sections on A_T; volume density of structures (integral index of content of structures in the bulk of tissue); optical density of immunohistochemically stained structures on section.

For evaluation of proliferative activity of cells, the mitotic index (MI) was estimated and proliferative index by PCNA (I_{PCNA}) was calculated as the ratio of numerical density of PCNA-positive nuclei to numerical density in nuclei of cells stained with hematoxylin. For each animal, the structures were counted in 10-15 test fields in three sections of an organ. MI and I_{PCNA} in the duodenum were evaluated in 10-15 cryptal sections with the total count of at least 1000 enterocyte nuclei. A_T for all other parameters was at least 3 mm^2. The data were processed statistically using nonparametrical Mann—Whitney U test.

RESULTS

Histological pattern of organs in control animals corresponded to normal. The thymus had a lobular structure, the lobules consisted of the cortex and medulla. On hematoxylin-stained preparations, the cortical zone of the thymus contained more than 29,000 thymocytes per mm^2 section area (nuclei) (Table 1). The majority PCNA-positive nuclei occurred in the cortical layer (Fig. 1, *a*), where I_{PCNA} was 26%. In the medulla only solitary PCNA-positive nuclei were seen. Toluidine blue staining visualized few mast cells (MC) in the interlobular connective tissue (Fig. 1, *e*).

The white pulp of the spleen in control animals presented as periarteriolar lymphoid sheaths and round follicles. In follicular germinative centers virtually all nuclei were PCNA-positive, though with different intensity of immunostaining (Fig. 2, *a*). Their numerical density was more than 8000/mm^2. The red pulp consisted of cords and venous sinuses. Small foci of myeloid hemopoiesis with high intensity of PCNA-specific immunostaining were seen in the subcapsular zone and along trabecules (Fig. 2, *a*). Ig-positive cells were detected virtually in the entire splenic parenchyma and were concentrated along the periphery of marginal zones and along trabecules (Fig. 2, *e*). Numerical density of Ig-producing cells was more than 700/mm^2 splenic section area.

The intestinal epithelium is a constantly renewing cell population due to proliferation and differentiation of stem cells in the generative zone (Fig. 3, *a*). High I_{PCNA} and MI reflected high level of physiological regeneration of duodenal epithelium in control animals (Table 2). Immunohistochemical studies showed a positive reaction of enterochromaffine cells (EC) in the epithelium of intestinal crypts and villi and some MC in the lamina propria (Fig. 3, *e*). About 80% EC were found in crypts, and the majority of serotonin-positive MC in the lamina propria of intestinal villi. Differential analysis showed that the summary volume density of serotonin-positive cells in crypts is 0.64% at EC numerical density 162/mm^2 of mucose area. Duodenal MC are topographically situated subepithelially and concentrated near blood and lymph vessels.

In intact animals the most pronounced changes in proliferative activity after vilon injection were seen in the thymus and duodenum. Thymic lobules were enlarged mainly due to widening of the cortical layer. In the peripheral zones the intensity of PCNA-specific immunostaining of thymocyte nuclei increased, characteristic microfollicular structures appeared (Fig. 1, *b*). I_{PCNA} increased to 37% against the background of decreased summary cell density in the cortical matter (due to accumulation of large lymphoblasts and reduced content of mature thymocytes). These findings probably reflect the effect of vilon on T lymphocyte differentiation and the rate of their migration from the cortical layer to the medulla. In the duodenum a 3.4% increase of I_{PCNA} in the crypt generation zone was paralleled by a 1.5-fold increase in the number of cells entering mitosis. All other parameters in the thymus, spleen, and duodenum were virtually the same as in the control.

Epithalon produced no appreciable changes in the histoarchitectonics of organs on hematoxylin-eosin-stained sections. However staining for PCNA showed low intensity of immune reaction of proliferating cells in the spleen (Fig. 2, *b*) and duodenum, (Fig. 3, *b*). Computer analysis showed that numerical density of proliferating cells in the spleen decreased virtually 2-fold both in lymphoid follicles and in zones of myeloid hemopoiesis (Table 1). It is noteworthy that despite the significant decrease of I_{PCNA} in the duodenal crypts, MI little differed from normal (Table 2). Moreover, the integral content of serotonin-positive cells in the intestine of rats injected with epithalon tended to decrease against the background intensive immunostaining of EC. The intensity of MC immunostaining to serotonin increased in some sections of the mucosa (Fig. 3, *f*). No significant changes in the studied parameters were detected in the thymus of rats treated with epithalon.

Three rats survived by day 8 after γ-irradiation without treatment with regulatory peptides. Six rats died on days 3-5 corresponding to the enteric phase of the radiation sickness. Morphofunctional parameters in organs of rats survived after irradiation were as follows. Pronounced atrophic changes were seen in the thymus, thymic lobules were decreased with blurred boundary between the cortical and medullary layers. Numerical density of cells in the cortical zone decreased almost 2-fold, and PCNA-positive nuclei were concentrated mainly at the periphery of reduced lobules (Fig. 1, *c*). High I_{PCNA} (33%) and increased count of MC indicated the beginning of postradiation recovery of the thymus. In the spleen proliferative activity of cells was very low both in the extramedullary zones and in reduced follicles (Fig. 2, *c*). Numerical density of Ig-positive cells decreased more than 2-fold in comparison with the control (Fig. 2, *f*). I_{PCNA} in intestinal crypts increased to 46.5% and MI to 4.2%. These data indicate that epithelium of the intestinal mucosa in survivors rapidly recovered, and the pool of stem cells of intestinal epithelium underwent hyperregeneration

TABLE 1. Quantitative Characteristics of Rat Thymus and Spleen (*M*±*m*)

Parameter	Control (*n*=6)	Vilon (*n*=3)	Epithalon (*n*=3)	γ-Irradiation		
				without correction (*n*=3/12*)	+vilon (*n*=5/12*)	+epithalon (*n*=1/6*)
Thymus						
Number of hematoxylin-stained nuclei per mm²	29,227±857	26,800±173	28,450±967	16,982±1079^{+}	12,846±635^{+o}	16,400
Number of PCNA-positive cells per mm²	7518±314	9933±1197^{+}	7173±535	5420±845^{+}	7135±577	6110
I_{PCNA}, %	26±1	37±4	26±3	33±7	55±3^{+o}	37
Number of MC per mm²	18±2	22±7	24±5	44±11^{+}	71±18^{+}	29
Spleen						
Number of PCNA-positive cells per mm²						
follicular germinative centers	8082±79	7880±253	3230±853^{+}	3630±276^{+}	5908±1011^{+}	3400
zones of extramedullary hemopoiesis	2217±159	2387±711	1168±259^{+}	482±30^{+}	1962±691^{o}	513
Ig-positive cells per mm²	728±44	715±78	567±54	328±34^{+}	558±38^{+o}	412

Note. Here and in Table 2 $p<0.05$: $^{+}$compared to the control, ^{o}compared to γ-irradiation without correction. *Number of survivors (on day 8) among irradiated animals.

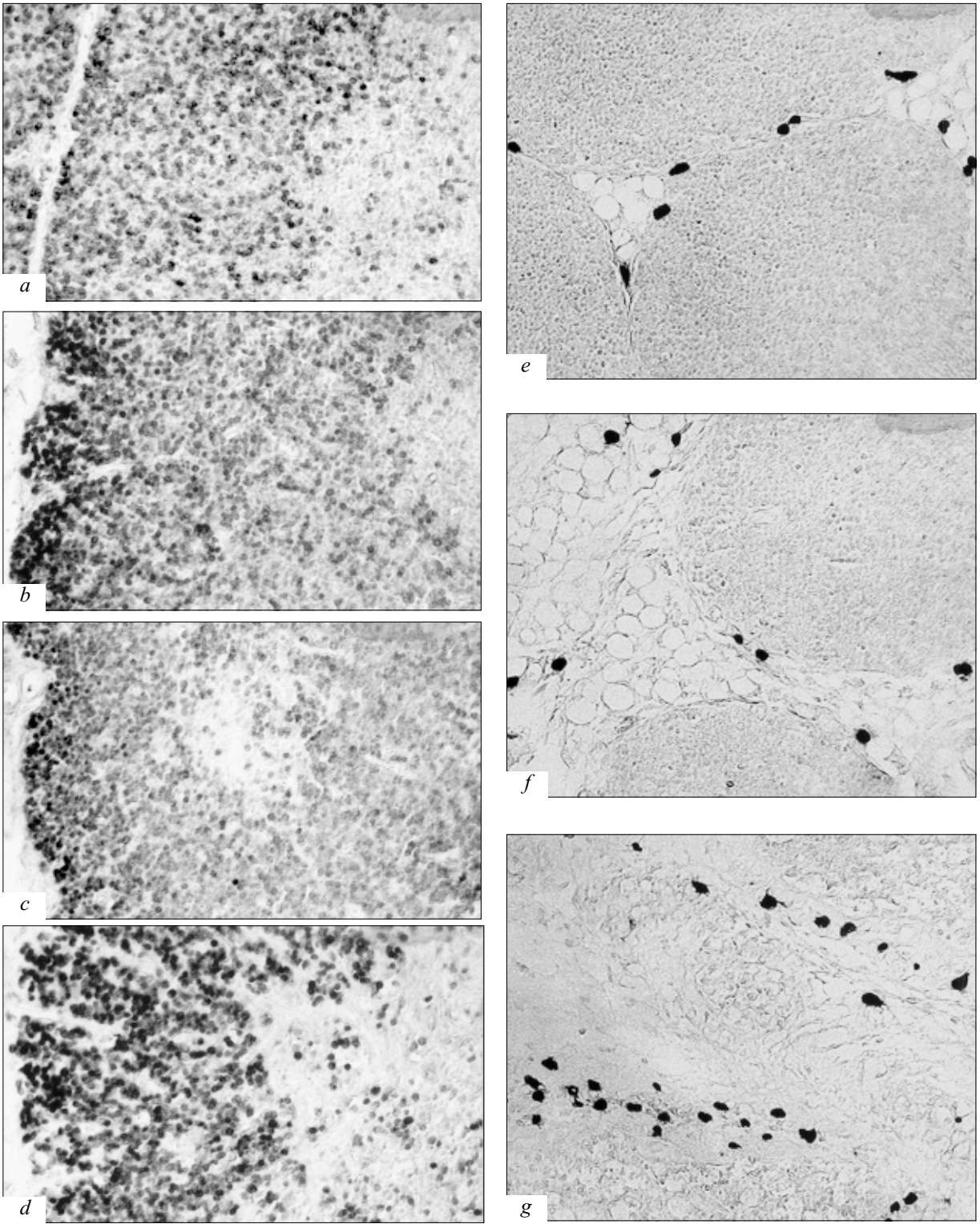

Fig. 1. Proliferative activity and mast cells in rat thymus in the control (*a*, *e*), after vilon injection (*b*), on day 8 after γ-irradiation (*c*, *f*), and after vilon treatment+γ-irradiation (*d*, *g*). *e-g*) selective toluidine blue staining of mast cells at pH 0.5, ×175. Here and in Figs. 2, 3: *a-d*) immunohistochemical reaction of cell nuclei with antibodies to PCNA. Avidin-biotin-peroxidase method, diaminobenzidine, ×175.

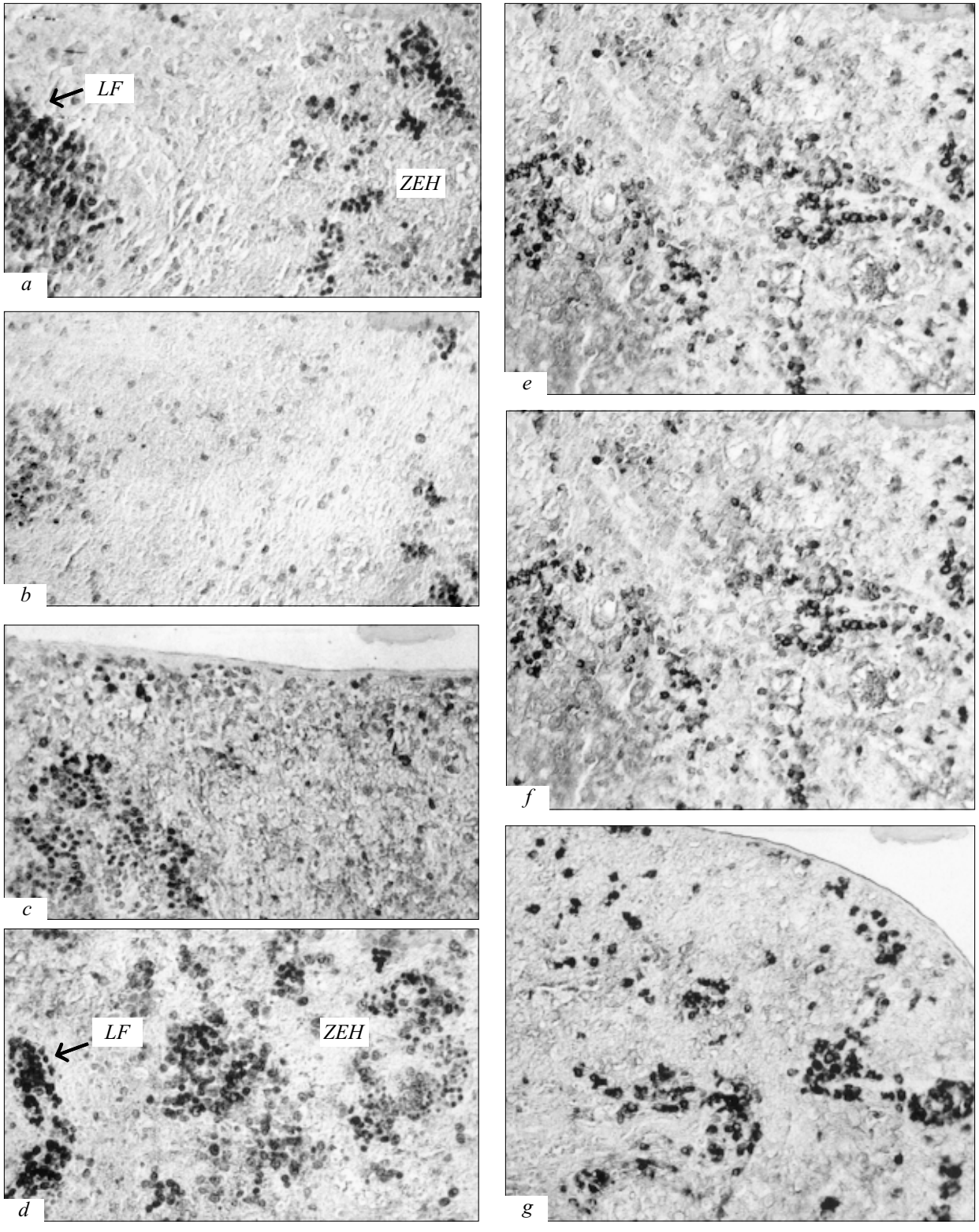

Fig. 2. Proliferative activity and immunoglobulin-producing cells in rat spleen in the control (*a*, *e*), after epithalon treatment (*b*), on day 8 after γ-irradiation (*c*, *f*), and after vilon+γ-irradiation (*d*, *g*). *e-g*) immunoglobulin-containing cells. Biotin-streptavidin-peroxidase method, diaminobenzidine, ×175. LF: lymphoid follicles; ZEH: zones of extramedullary hemopoiesis.

TABLE 2. Quantitative Characteristics of Rat Duodenum (*M*±*m*)

Parameter	Control (*n*=6)	Vilon (*n*=3)	Epithalon (*n*=3)	γ-Irradiation		
				without correction (*n*=3/12*)	+vilon (*n*=5/12*)	+epithalon (*n*=1/6*)
I_{PCNA}, %	44.8±0.2	48.2±1.4^{+}	33.3±4.2^{+}	46.5±0.7^{+}	49.8±0.7^{+o}	19.2
MI, %	2.9±0.1	4.4±0.3^{+}	2.9±0.2	4.2±0.4^{+}	4.7±0.1^{+}	2.5
Density of serotonin-positive cells, %	0.64±0.02	0.67±0.06	0.57±0.07	0.43±0.08^{+}	0.71±0.09	0.30
Number of EC per mm^2	162±4	146±17	136±17	130±12^{+}	147±18	65
Optical density of EC staining, arb. units	0.205±0.002	0.193±0.008	0.182±0.014^{+}	0.209±0.005	0.202±0.012	0.195
Number of MC per mm^2	169±23	163±33	146±19	18±2^{+}	24±3^{+}	14

during this period (Fig. 3, *c*). Histological analysis also indicates that the structure of epithelial lining started to normalize. However the count of EC remained below the normal (Fig. 3, *g*). The content of MC in the lamina propria decreased almost 10-fold. This agrees with the conclusion about high sensitivity of mucosal MC to ionizing radiation and their slow recovery after irradiation in sublethal doses [11].

Vilon promoted recovery in organs of irradiated animals. Differentiation into cortical and medullary layer was clearly seen in thymic lobules (Fig. 1, *d*). Numerical density of cells in the cortical zone was lower than in irradiated animals not treated with vilon, but I_{PCNA} reached 55% due to high content of PCNA-positive nuclei. In the spleen of rats treated with vilon, proliferative activity in lymphoid follicles and in zones of extramedullary hemopoietic increased 1.6- and 4-fold, respectively, in comparison with those in untreated animals. Moreover, numerical density of Ig-containing cells increased 1.7-fold (Fig. 2, *g*).

Vilon normalized histological picture of intestinal crypts in irradiated animals (Fig. 3, *d*). I_{PCNA} reached almost 50% and MI 4.7%. Numerical density of EC (Fig. 3, *h*) returned to control values. It seems that survival of 5 animals by the control term in this group and death of only 3 rats on days 4-5 during the enteric phase was justified. Analysis of the data showed just a negligible increase in the count of MC in the duodenal mucosa and their pronounced hyperplasia in the thymus (Fig. 1, *g*).

At present MC are believed to play an important role in the maintenance of tissue homeostasis. MC participate in immune reactions and regulation of local blood flow, regulate the content of free monoamines and promote recovery of damaged connective tissue, contribute to the development of endothelium and regulation of electrolyte transport in the intestine [8, 12]. We can therefore consider that the function of the intestinal mucosa cannot be considered fully restored by this term even after vilon treatment. A different picture was observed in the thymus. MC in the intestinal mucosa and connective tissue of lymphoid tissues phenotypically belong to different mastocyte populations, and phenotypical differentiation is controlled by local microenvironmental factors and lymphokines produced by T lymphocytes [4]. In turn, mRNA responsible for the synthesis of a spectrum of multifunctional cytokines are expressed in differentiating MC [4,8]. These cytokines regulate proliferation and functioning of MC and other hemo- and lymphopoietic cells by the autocrine or paracrine pathway. Hence, hyperregeneration of MC and thymocytes in irradiated animals treated with vilon can be explained by their mutual potentiating effects. Moreover, this effect suggests that vilon stimulate proliferative activity of T lymphocyte and MC precursors — bone marrow stem cells survived after γ-irradiation.

It was impossible to carry out a representative quantitative analysis in the epithalon group, because only 1 rats survived by day 8. We should just mention that after epithalon injections 4 irradiated rats died in the enteric phase and the majority of the studied parameters in the survivor did not virtually differ from those in untreated irradiated animals.

Hence, our complex studies indicate that vilon and epithalon modify the course of physiological processes in the studied organs via different mechanisms. Vilon significantly stimulated proliferative activity of T lymphocytes and enhanced proliferative potential of intestinal stem cells. It seems that this effect promotes postradiation recovery of critical organs. The most objective effect of epithalon is deceleration of metabolic processes in the duodenal mucosa and suppression of hemopoiesis and lymphopoiesis in the spleen.

Data on the inhibitory effect of epithalon on cell proliferation indicate that this peptide should be tried as a potential antitumor agent.

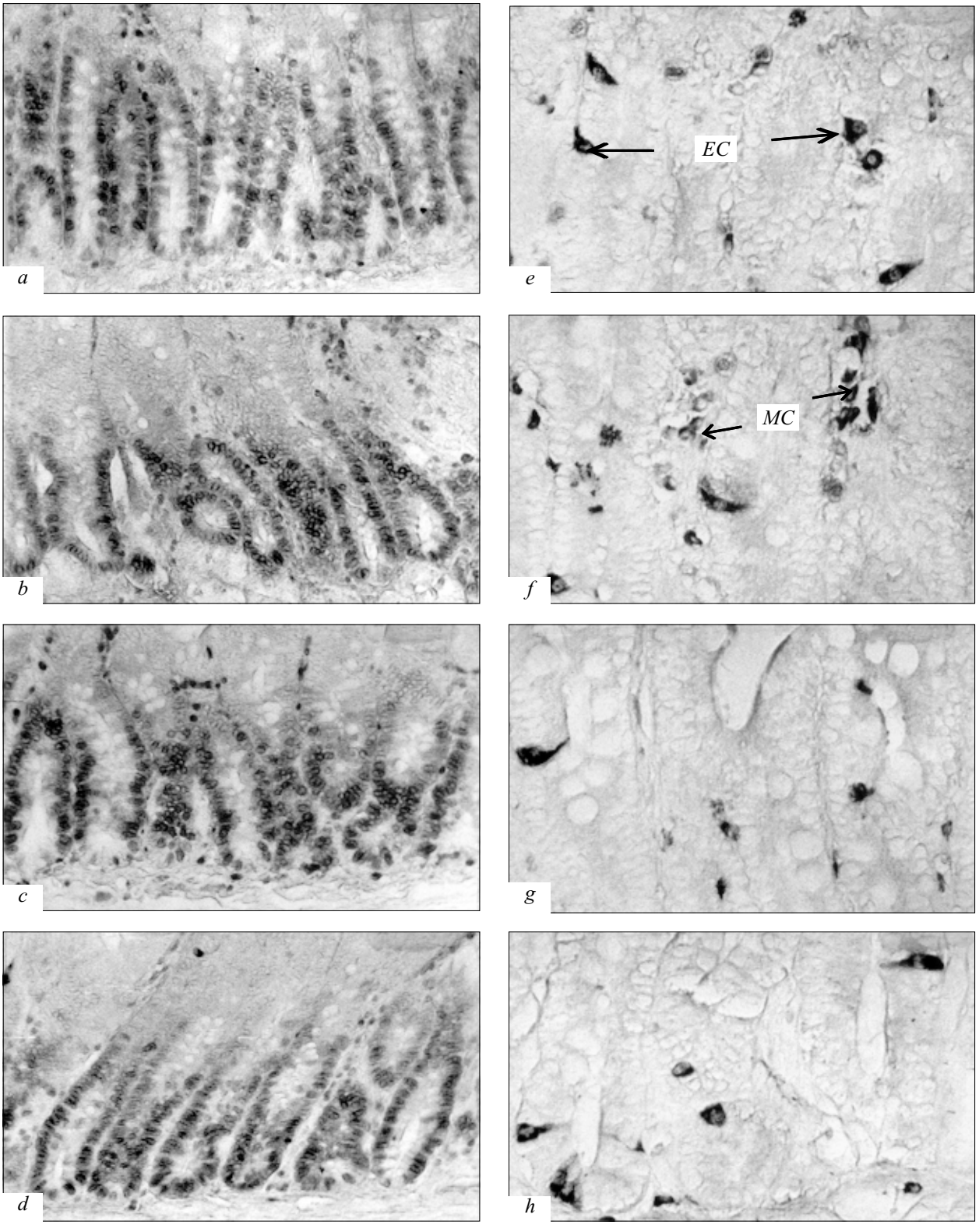

Fig. 3. Proliferative activity and serotonin-containing cells in rat duodenum in the control (*a*, *e*), after treatment with epithalon (*b*, *f*), on day 8 after γ-irradiation (*c*, *g*), and after treatment with vilon following γ-irradiation (*d*, *h*). *e*-*h*) immunohistochemical reaction to serotonin. Biotin-streptavidin-peroxidase method, diaminobenzidine, ×350. EC: enterochromaffine cells; MC: mast cells.

REFERENCES

1. A. A. Ivanov, N. N. Klemparskaya, G. A. Shal'nova, *et al., Antitumor Effects of Immunoglobulins* [in Russian], Moscow (1990).
2. V. S. Smirnov, V. Kh. Khavinson, G. M. Yakovlev, and V. S. Novikov, *Correction of Radiation Immunodeficiencies* [in Russian], St. Petersburg (1992).
3. A. Dubois and R. I. Walker, *Gastroenterology*, **95**, No. 2, 500-507 (1988).
4. S. J. Galli, *Lab. Invest.*, **62**, No. 1, 5-33 (1990).
5. I. M. Kvetnoy, V. V. Yuzhakov, A. O. Molotkov, *et al., Scanning Microsc.*, **10**, No. 1, 261-278 (1996).
6. B. R. Leigh, W. Khan, S. L. Hancock, and S. J. Knox, *Radiat. Res.*, **142**, No. 1, 12-15 (1995).
7. W. K. MacNaughton, K. E. Leach, L. Prud'homme-Lalonde, and K. A. Sharkey, *Gastroenterology*, **106**, No. 2, 324-335 (1994).
8. A. Moller, J. Grabbe, and B. M. Czarnetzki, *Skin Pharmacol.*, **4**, Suppl. 1, 56-63 (1991).
9. V. G. Morozov and V. Kh. Khavinson, *Int. J. Immunopharmacol.*, **19**, No. 9-10, 501-505 (1997).
10. C. S. Potten, G. Owen, D. Hewitt, *et al., Gut*, **36**, No. 6, 864-873 (1995).
11. D. M. Sedgwick and A. Ferguson, *Int. J. Rad. Biol.*, **65**, No. 4, 483-495 (1994).
12. S. I. Wasserman, *J. Allergy Clin. Immunol.*, **86**, No. 4, 590-593 (1990).
13. S. G. Wu and T. Miyamoto, *Radiat. Res.*, **123**, No. 1, 112-115 (1990).