Peptidegic Stimulation of Differentiation of Pineal Immune Cells

N. S. Linkova[1], V. Kh. Khavinson[1,2], N. I. Chalisova[1,2], A. S. Katanugina[2], and E. A. Koncevaya[1]

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We studied cell composition of the pineal lymphoid tissue and the effect of peptides on its differentiation and proliferation capacity. It was shown that the lymphoid component of the pineal gland in organotypic culture is primarily presented by low-differentiated $CD5^+$-lymphocytes, while mature T and B cells are less abundant. Dipeptide vilon stimulates differentiation of precursors into T-helpers, cytotoxic T lymphocytes, and B cells, while tetrapeptide epithalon stimulated their differentiation towards B cells. Tripeptide vesugen had no effect on differentiation capacity of immune cells of the pineal gland, but enhanced their proliferation potential. Thus, dipeptide vilon acts as an inductor of differentiation of pineal immune cells, which can play an important compensatory role in age-related atrophy of the thymus, the central organ of the immune system.

Key Words: *differentiation; lymphocytes; short peptides; pineal cell culture*

The neuroimmunoendocrine system and its central organs the thymus and pineal gland are important regulators of homeostasis [1,2,6]. Since the pineal gland and thymus are the organs of the integrated neuroimmunoendocrine system, similar processes and signaling interactions in these organs attracted much recent attention [5,6]. For instance, secretion of neuropeptide CGRP (calcitonin gene related peptide), transcription factor pCREB, and matrix metalloproteinases MMP2 and MMP9, specific proteins involved into remodeling of the extracellular matrix, was detected in both the pineal gland and thymus [6]. The close relationship between the pineal gland and thymus is confirmed by the data that the lymphoid tissue occupies about 20% of the thymus area; it is presented by lymphocytes secreting cytokines similar to those expressed in the thymus [3,6]. Moreover, MMP2 detected in the pineal gland is primarily secreted by immune cells. During age-related involution of the thymus, its functions are delegated to the lymphoid tissue in other organs, *e.g.* pineal gland [4]. The lymphoid component of the pineal gland is presented by T and B cells, and low-differentiated lymphocyte precursors (predominant cell type), which can produce an additional pool of immune cells upon induction of differentiation.

Various signal mechanisms are involved in the processes of cell differentiation, the most important of them are cytokines and cytomedins [8,10,11]. On the basis of the analysis of amino acid sequences of cytomedins isolated from different tissues, short peptides containing the most prevalent amino acids were synthesized at the St. Petersburg Institute of Bioregulation and Gerontology [9,12].

We previously showed that some short peptides stimulate differentiation of $CD34^+$ BM stem cells from human embryo into $CD14^+$ cells (myelocytes), $CD3^+$ cells (T cell precursors), $CD4^+$ cells (T helpers), and $CD8^+$ cells (Cytotoxic T cells), while peptide vesugen promotes differentiation of $CD4^+CD8^+$ thymocytes into T helpers [4]. Thus, stimulation of cell differentiation in the thymus and BM, the central organs of

[1]St. Petersburg Institute of Bioregulation and Gerontology, Northwestern Division of the Russian Academy of Medical Sciences; [2]I. P. Pavlov Institute of Physiology, Russian Academy of Sciences, St. Petersburg, Russia. ***Address for correspondence:*** miayy@yandex.ru. N. S. Linkova

immunogenesis, under the effect of short peptides was demonstrated, but the effect of these peptides on the lymphoid component of peripheral sources of lymphopoiesis, *e.g.* pineal gland, is little studied.

In light of this, we studied the effect of some short peptides on differentiation of pineal lymphoid cells in organotypic culture.

MATERIALS AND METHODS

The pineal glands were isolated from 2-3-month-old male Wistar rats; the animals were decapitated with a guillotine. The pineal gland isolated using eye surgical instruments were placed into a sterile Petri dish and divided into explants (~1 mm^3 fragments). The explants were transferred into a Petri dish (10 per dish) with collagen coating (35×2.5 mm, Jet Biofil) and cultured in 3 ml nutrient medium consisting of Hanks saline (45%), Eagle medium (45%), and fetal calf serum (10%) and supplemented with glucose (10 mg/ml) and gentamicin (0.5 mg/ml). The explants (n=40) were divided into 4 groups and incubated with saline (control) or one of the test peptides (experimental groups): epithalon (Ala-Glu-Asp-Gly), vesugen (Lys-Glu-Asp), or vilon (Lys-Glu) in a concentration of 10 ng/ml. The explants were cultured in a CO_2-incubator (5% CO_2) at 36.7°C. The duration of culturing was 3 days, because this period corresponds to the formation of the growth zone consisting of proliferating and migrating pinealocytes, fibroblasts, and immune cells [7].

For immunocytochemical analysis of the explant growth zone, the cultures were fixed with 95% ethanol. Immunocytochemical reaction with antibodies to markers of low-differentiated lymphocytes CD5 (1:30, Novocastra), T-helpers CD4 (ready-to-use, Novocastra), cytotoxic T cells CD8 (ready-to-use, Novocastra), and B cells CD20 (1:30, Novocastra) was performed using a standard single-stage protocol with high-temperature antigen demasking in citrate buffer (pH 6.0). Biotynilated antimouse and antirabbit immunoglobulins (a universal kit) were used as secondary antibodies. The reaction was visualized using a complex of avidin with biotinylated horseradish peroxidase and diaminobenzidine (ABC-kit, Dako).

Morphometry was performed using a computer-assisted microimage analysis system consisting of Nikon Eclipse E400 microscope, Nikon DXM1200 digital camera, Intel Pentium 4-based computer, and VideotestMorphology 5.0 software. In each case, at least 5 fields of view were analyzed at ×100. The area of marker expression was determined as the ratio of the area occupied by immunopositive cells to the total area of cells in the field of view. The area of expression is a widely used morphometric parameter characterizing the number of cells expressing the studied marker. Significance of differences was evaluated using the Student test.

RESULTS

In control pineal cultures, the distribution of different subpopulations of immune cells CD5:CD20:CD4:CD8 was 58:2:1:1, which attested to considerable predominance of non-differentiated lymphocytes over mature cell populations and confirmed our previous data obtained on human autopsy material.

Differentiation of immune cells in the organotypic pineal culture depended on the structure of the test peptides.

Vilon and epithalon reduced the expression of CD5 marker by 5 and 12 times, respectively, in comparison with the control, whereas vesugen had no effect on this parameter (Fig. 1, *a*). Thus, two of the three test peptides, vilon and epithalon, can be a candidate for differentiation inductors. All the test peptides increased the expression of CD20 marker in the pineal cell culture: by 25 (vilon), 32.5 (vesugen), and 6 (epithalon) times in comparison with the control (Fig. 1, *b*). The area of expression of T-helper marker CD4 did not change under the effect of epithalon, but increased after addition of vilon and vesugen to the pineal cell culture by 5.2 and 15 times, respectively (Fig. 1, *c*). Expression of CD8 marker (cytotoxic T cells) was not modulated by vesugen and epithalon, but was stimulated by vilon (by 11 times in comparison with the control; Fig. 1, *d*).

Comparing the effect of short peptides on the expression of different markers of immune cells in the pineal gland, we can hypothesize their effects on differentiation of lymphocyte precursors.

Epithalon reduced the number of low-differentiated $CD5^+$ cells, enhanced the expression of B-cell marker CD20, and had no effect on mature $CD4^+CD8^+$ T cells. It seems quite possible that epithalon can stimulate differentiation of lymphocyte precursors only towards B cells.

Vesugen had no effect of lymphocyte precursors, but stimulated the expression of CD4 and CD20 markers. Thus, vesugen had no effect on differentiation of lymphocyte precursors in the pineal gland, but enhanced proliferative activity of mature T helpers and B cells. These findings agree with previous data on stimulatory effect of this tripeptide on proliferation of various cell populations.

Vilon is the most promising inductor of differentiation of immune cells in the pineal gland. It considerably reduced the expression of non-differentiated cell marker and stimulated the expression of all studied mature cells (cytotoxic T cells, T helpers, and B cells) and its effect in all cases many times surpassed the re-

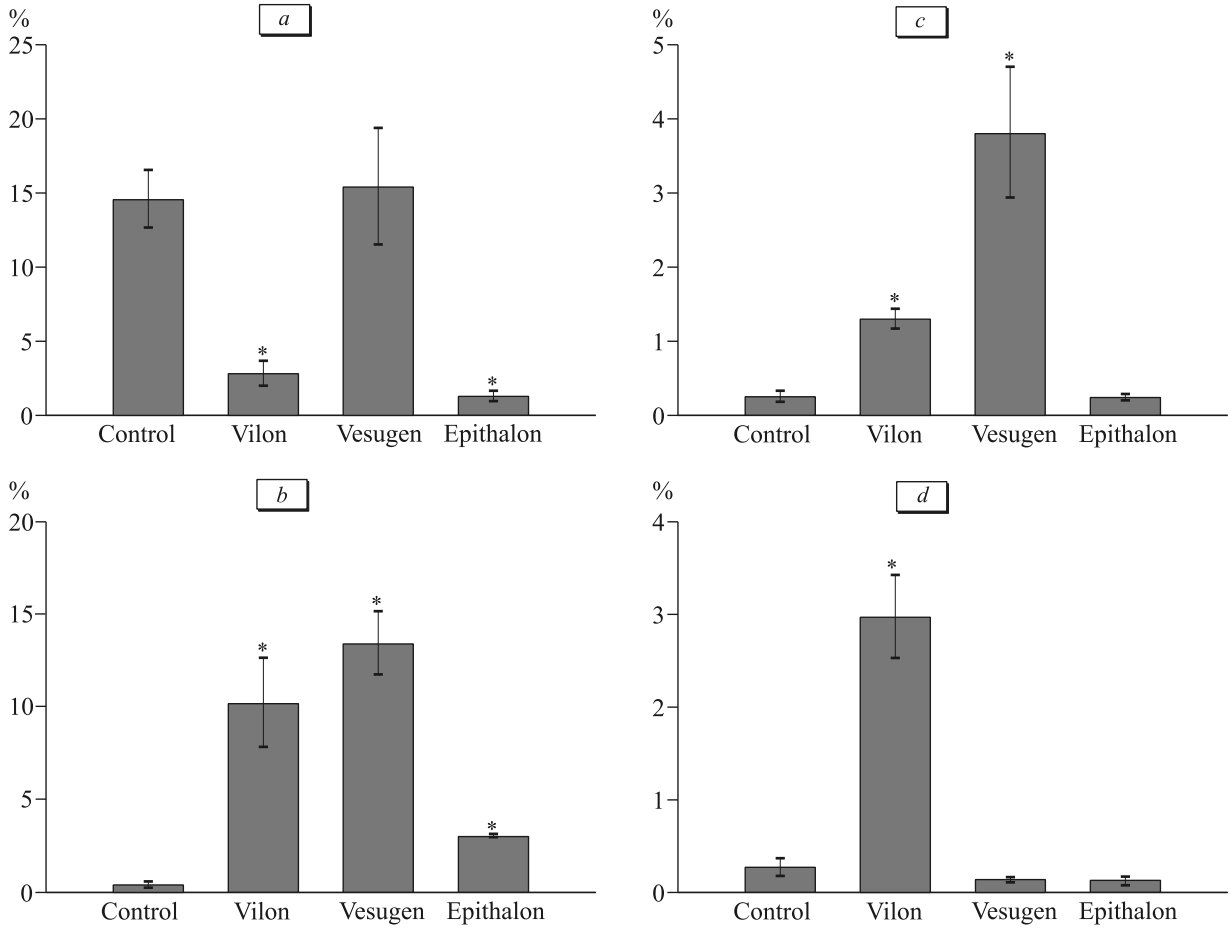

Fig. 1. Effect of short peptides on the expression of immune cell markers in the lymphoid component of the pineal gland. *a*) low-differentiated $CD5^+$ lymphocytes; *b*) $CD20^+$ B lymphocytes; *c*) $CD4^+$ T helpers; *d*) $CD8^+$ cytotoxic T lymphocytes. Ordinate: area of marker expression and immune cells. Here and in Fig. 2: $*p<0.05$ in comparison with the control.

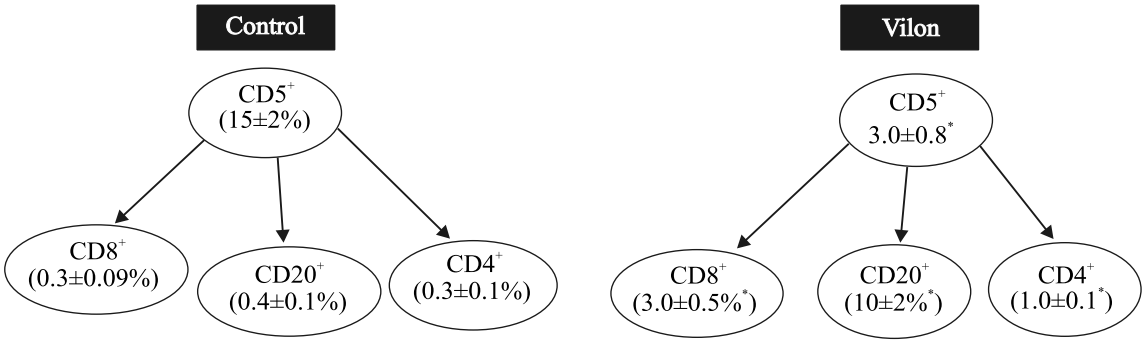

Fig. 2. Dipeptide vilon stimulates differentiation of immune cells. Areas of marker expression: CD5: low-differentiated lymphocytes; CD20: B lymphocytes; CD4: T helpers; CD8: cytotoxic T lymphocytes.

sults obtained in control cultures (Fig. 2). Thus, vilon not only acts as inductor of differentiation of pineal immune cells, but also exhibits a wide spectrum of differentiation pathways. This pronounced effect of vilon on differentiation of immune cells of the lymphoid component of the pineal gland can be explained by the fact that this dipeptide is synthesized on the basis of cytomedin thymalin isolated from the thymus and exhibiting pronounced immunomodulatory properties.

Our findings suggest that vilon induces differentiation of the pineal lymphoid tissue towards T and B cells, epithalon stimulates its differentiation only towards B cells, while vesugen has no effect on differentiation, but stimulates proliferation of T helpers and B cells.

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