Effect of Amino Acids on Expression of Signal Molecules in Organotypic Culture of the Spleen

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Opposite effects of amino acids on proliferation and differentiation of immune cells in organotypic culture of the spleen were demonstrated. It was found that methionine stimulates differentiation of $CD5^+$ lymphocyte precursors into T-helpers, cytotoxic T lymphocytes, and B cells and induces proliferation of $CD68^+$-macrophages. Glutamic acid and arginine induce proliferation of B cells, while histidine and leucine promote differentiation of precursors towards cytotoxic T cells. The opposite effects of amino acids on immune cells of the spleen are related to different hydrophobicity of their side chains determining the type of intermolecular interaction.

Key Words: *amino acids; proliferation; differentiation; immune cells; spleen*

The effects of encoded amino acids on cell processes attracted much attention over the last two decades. The data have been accumulated that amino acids not only serve as the plastic material for protein molecules, but also can modulate the expression of target genes and regulate homeostasis by acting as signal molecules. Thus, evaluation of the parameters of specific and unspecific resistance showed that lysine, arginine, and glutamic and aspartic acids, and tryptophan exhibit different immunostimulating, phagocytosis-stimulating, and detoxification properties. Experiments on mice showed that lysine and arginine after subcutaneous injection stimulate phagocytosis, but did not protect from toxic substances; lysine did not affect the immune response, but arginine suppressed it [1]. Methionine, tyrosine, and phenylalanine deprivation produces an inhibitory effect and induces G0/G1 cell cycle arrest in cultured androgen-independent prostate cancer PC3 and DU145 cells. Methionine deprivation enhances apoptosis in PC3 cells and tyrosine and phenylalanine deprivation stimulates it in DU145 cells. Methionine, tyrosine, and phenylalanine deprivation inhibits invasion of both cell lines, but glutamine deprivation inhibits only DU145 invasion, *i.e.* inhibition of invasion did not depend on apoptosis induction [12]. Of amino acids with branched side chains (valine, leucine, and isoleucine), only leucine in a concentration of 10^{-5}-10^{-3} M activated DNA synthesis and proliferation in cultured rat hepatocytes [13]. There are data on the effect of arginine on cell proliferation and apoptosis processes. Addition of arginase (reducing arginine concentration due to enzymatic degradation) to the culture of normal cells induces G0/G1 cell cycle arrest, but the cells recovered after 1 week. However, in a culture of malignant cells this enzyme induced massive cell death over 3-5 days and less than 0.01% cells survived after addition of arginine [15]. Arginine produces an apoptosis-inducing effect on cultured rat postnatal retinal cells (in the central retina of 5-day-old rats and in the peripheral retina in 3-5-day-old rats) [12]. H_2O_2 induced intensive apoptosis of endothelial cells cultured in the absence of arginine; addition of 60 μM arginine (normal blood level for humans and rats) improved cell survival by 50% and addition of 200 μM arginine and more produced even

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more pronounced effect [17]. Experiments on organotypic cultures showed that hydrophilic amino acids with charged side chains stimulate cell proliferation in various cell cultures, including splenic lymphoid cells [7-9]. There are data that some amino acids can activate mTORC1 (mammalian target of rapamycin, complex 1) and thereby control transcription (including initiation and elongation factors) and translation processes [14].

Processes of cell proliferation and differentiation involve a number of signal mechanisms, including those realized via cytokines and cytomedins [2,3,5, 6,10]. In was demonstrated that short peptides Glu-Asp-Ala and Lys-Glu-Asp stimulate proliferation and differentiation of BM SC and T cells [9]. In light of this we can hypothesize the involvement of encoded amino acids into cell differentiation processes. Here we studied the effect of some hydrophobic and hydrophilic amino acids on proliferation and differentiation of lymphoid cells of the spleen in organotypic culture.

MATERIALS AND METHODS

The spleen was isolated from 3-month-old male Wistar rats; the animals were decapitated with a guillotine. The spleen was placed in sterile Petri dish and cut into explants (1 mm^3 fragments). A total of 850 explants were examined. The explants were transferred into a Petri dish with collagen coating (35×2.5 mm, Jet Biofil; 10-12 explants per dish) 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 medium with or without the test amino acids in effective concentrations of 0.05 ng/ml was added to Petri dishes with experimental and control explants, respectively. The following amino acids were used (Sigma): glutamic acid (Glu), aspartic acid (Asp), lysine (Lys), arginine (Arg), histidine (His), valine (Val), methionine (Met), and leucine (Leu). 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 (Fig. 1) [7].

For immunocytochemical analysis of the explant growth zone, the cultures were fixed with 96% ethanol. The cells were permeabilized with 0.5% triton X-100 and then immunocytochemical reaction with antibodies to low-differentiated lymphocyte marker CD5 (1:30; Novocastra), T-helper marker CD4 (ready-to-use; Novocastra), cytotoxic T cell marker CD8 (ready-to-use; Novocastra), B cell marker CD20 (1:30, Novocastra), and macrophage marker CD68 (1:30; Novocastra) was performed using a standard single-stage protocol with high-temperature antigen unmasking in citrate buffer (pH 6.0). Biotinylated antimouse immunoglobulins (a universal kit) were used as secondary antibodies. The reaction was visualized using a complex of avidin-biotin-horseradish peroxidase complex with 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. Five fields of view were assayed in each case. The data were processed statistically using Statistica 7.0 software.

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. Significance of differences was evaluated using the Student *t* test.

RESULTS

Amino acids produced different effects on proliferation and differentiation of immune cells in organotypic culture. Hydrophilic amino acids with negatively

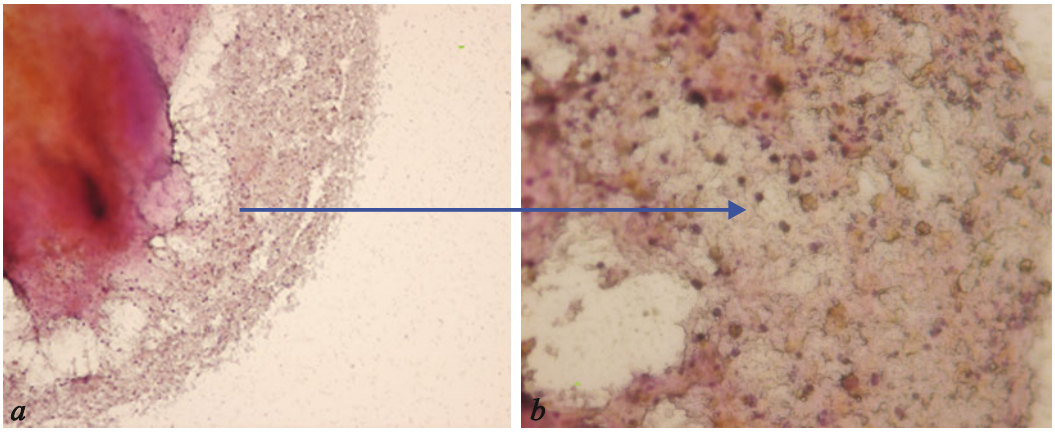

Fig. 1. Growth zone (cell monolayer) in organotypic culture of the spleen. Hematoxylin and eosin staining. *a*) ×100, *b*) ×400.

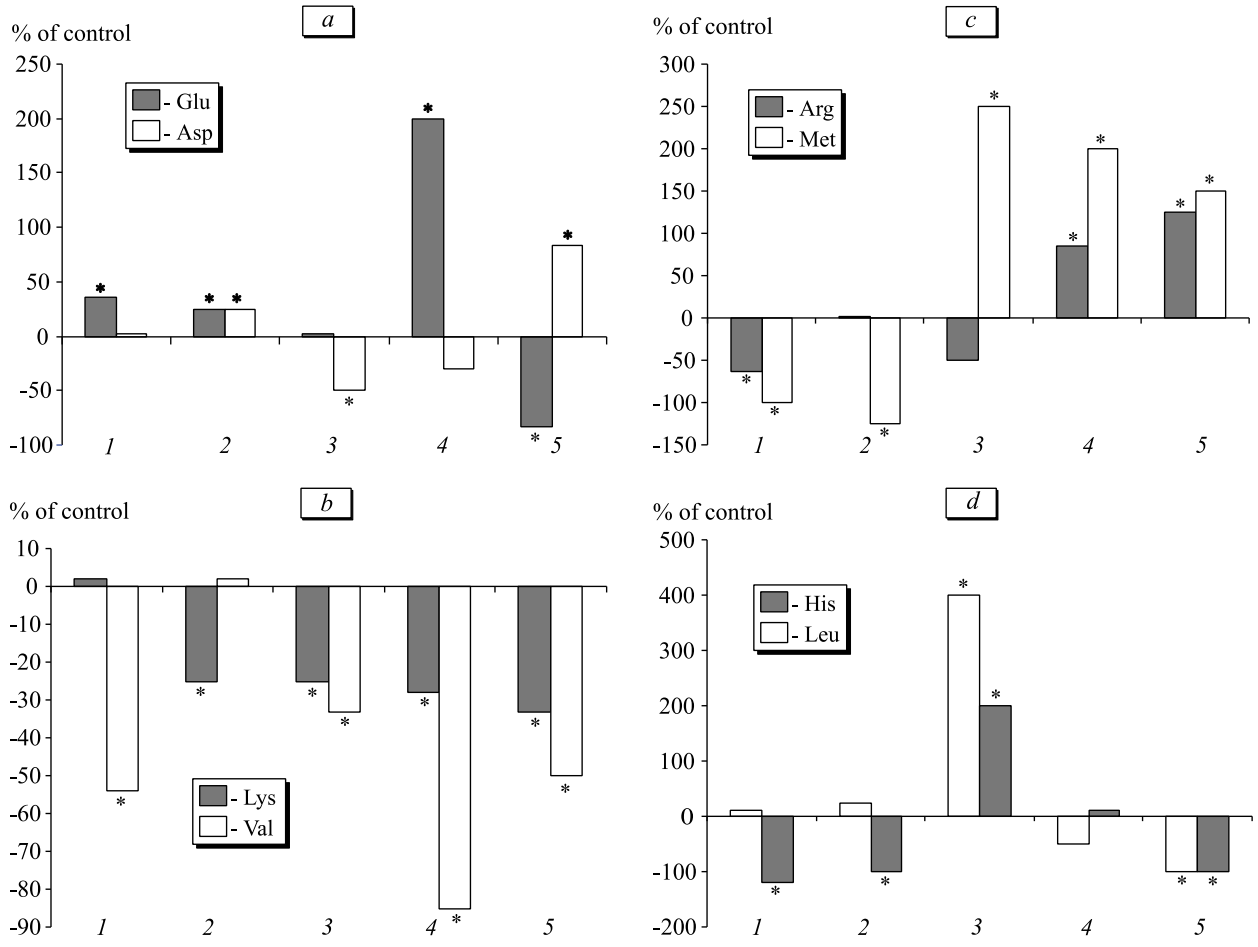

Fig. 2. Effect of amino acids on expression of signal molecules in organotypic culture of the spleen. *a*) glutamic (Glu) and aspartic acids (Asp), *b*) lysine (Lys) and valine (Val), *c*) arginine (Arg) and methionine (Met), *d*) histidine (His) and leucine (Leu). *1*) CD4, *2*) CD5, *3*) CD8, *4*) CD20, *5*) CD68. *$p<0.05$ in comparison with the control (without amino acids – zero line).

changed side chains (glutamic and aspartic acids) had no appreciable effects on expression of CD4, CD5, and CD8 markers and produced opposite effects on CD20 and CD68 expression (Fig. 2, *a*). Aspartic acid 2-fold reduced CD20 expression in comparison with the control, while glutamic acid increased the synthesis of this membrane glycoprotein by 20 times. At the same time, aspartic acid 6-fold increased CD68 expression, while glutamic acid reduced it by 7 times in comparison with the control. Hence, aspartic acid stimulated proliferation of resting macrophages, while glutamic acid reduced the number of $CD68^+$-cells.

Addition of lysine, a hydrophilic amino acid with positively charged side chain, did not affect CD4 expression and reduced the expression of the rest markers (CD5, CD8, CD20, and CD68) by 2.5-3 times in comparison with the control (Fig. 2, *b*). Similar changes were produced by hydrophibic amino acid valine (Fig. 2, *b*), but in this case expression of CD5 marker remained at the control level and expression of other markers decreased by 2-5 times, which attested to reduced differentiation capacities of lymphocyte precursors in the spleen. Another hydrophilic amino acid with positively charged side chain (arginine) enhanced expression of CD20 and CD68 by 20-22 times in comparison with the control. Similar effects were produced by methionine: it increased CD20 and CD68 expression by 8-10 times in comparison with the control (Fig. 2, *c*). Moreover, methionine 30-fold enhanced CD8 expression in comparison with the control and 9-10-fold reduced CD4 and CD5 expression. Hence, methionine induced differentiation of lymphocyte precursors towards T-suppressors, but not towards T-helpers and B cells. Histidine, an amino acid with positively charged side chain, 40-fold increased expression of CD8 marker in comparison with the control and reduced the expression of CD20 and CD68 by 5 and 10 times. Similar effects were produced by leucine: it 20-fold increased expression of CD8 and 7-fold reduced expression of CD68 (Fig. 2, *d*).

Thus, we revealed differently directed effects of amino acids on proliferation and differentiation of im-

mune cells in organotypic culture of the spleen. Of all physicochemical properties of amino acids, side chain hydrophobicity is most important for intermolecular interactions. Glutamic acid (with negatively charged side chain) and arginine (with positively charged side chain) enhanced proliferative activity of mature B cells. These findings agree with previous data on stimulatory effect of these amino acids on proliferation of various cell populations, including lymphoid cells [7-9]. Histidine induced differentiation of precursor cells into cytotoxic T cells. Methionine was the most promising inductor of differentiation of immune cells: it reduced expression of undifferentiated lymphocyte marker CD5 and simultaneously stimulated expression of markers of mature cells: both subpopulations of T cells and B cells. Proliferation of $CD68^{+}$-macrophages can be stimulated by aspartic acid, arginine, and methionine.

These findings provide the basis for creation of bioregulatory peptides capable of stimulating proliferation and differentiation of immune system cells.

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