METHODS

A Method of Creating a Cell Monolayer Based on Organotypic Culture for Screening of Physiologically Active Substances

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We developed a method of culturing and phenotyping of a monolayer of cells of the retinal tissue, thymus and spleen on the basis of organotypic culture. All characteristic types of neurons and fibroblasts were found in their microenvironment in the retinal cell monolayer. Lymphocytes, macrophages, and fibroblasts were verified in the monolayer of thymus and spleen cells. Histological staining, immunocytochemistry, and electron microscopy demonstrated the possibility of assessing the differentiation degree and functional activity of the cell monolayer. The developed technique preserves cell-cell interactions and a variety of cell types characteristic of the examined organ in the monolayer. This opens up new prospects for its application in basic research and in screening of different physiologically active substances.

Key Words: *monolayer; microenvironment; organotypic culture; screening physiologically active substances*

The method of cell culture is widely used in molecular biology and transplantology to study the dynamics of growth, morphology, and expression of various signaling molecules and testing biological activity of substances, including peptides [2,6,8,9]. Dissociated cell cultures are most widely used. Some of them (HeLa cells, mesenchymal stem cells of humans and animals, lymphoblast-like immortalized cell lines) are continuously maintained in cell culture banks [5].

However, this method has several disadvantages. Dissociated culture usually contains one or seldom two types of cells, *i.e.* the cells in this model lack natural microenvironment typical of living tissue [1,10]. Cell-cell interactions are an important regulatory system of local homeostasis in the tissue inducing cell proliferation and differentiation. The absence of these interactions hampers extrapolation of results obtained in dissociated cultures to the processes occurring in the living tissues. In addition, cultured cells deprived of their natural microenvironment often change their phenotype and lose some properties typical of primary culture. Many types of cells, especially nerve cells, have low proliferative potential. They do not grow well in dissociated cultures, and selection of optimal conditions for their culturing (type of culture medium, supporting cellular elements simulating the microenvironment, combination of growth factors) is a difficult task [11,12]. Immortalized cell cultures are widely used the solution of this problem. They partly retain the properties of native cells, but are characterized by accelerated proliferation because of their similarity to cancer subpopulations. The use

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of immortalized cells for testing biological activity of drugs is limited, because their similarity to cancer cells can significantly affect the results. At the same time, the maintenance of dissociated culture for the experiment takes at least 3 weeks and requires considerable financial and labor costs.

Organotypic cultures have several advantages over dissociated cell lines. First, organotypic culture comprises different cell types specific to the organ from which it was obtained, *i.e*. it retains tissue-specific microenvironment and maximally approached the natural conditions of cell functioning. Moreover, culturing of organotypic cultures for the experiment takes from 3 to 5 days, which significantly reduces the time and costs of the experiment.

It has been established that almost all types of tissues including nervous tissue (*e.g.* retinal cells, pineal gland, and subcortical structures) can be grown in organotypic culture. However, the use of a monolayer of cells representing a growth zone of the organotypic culture was not widely used due to the fact that the properties of these cells are not sufficiently characterized [3,4].

Here we studied the morphology and molecular properties of cell monolayer in the growth zone of organotypic culture to assess the feasibility of its application to test the biological activity of drugs of different nature.

MATERIALS AND METHODS

Spleen and thymus were obtained from 2-3-month-old male Wistar rats grown in the nursery of I. P. Pavlov Institute of Physiology RAS (St. Petersburg). The retina was isolated in 10-day-old chick embryos provided by Institute of Influenza, Ministry of Health and Social Development of the Russian Federation (St. Petersburg). All the organs isolated from animals with instruments for eye surgery were placed in sterile Petri dishes and divided into explants (fragments of about 1 mm^3), transferred to collagen-coated Petri dishes (35×2.5 mm, Jet Biofil, 10 fragments per dish), and cultured in 3 ml culture medium consisting of 45% Hanks solution, 45% of Eagle medium, 10% of fetal bovine serum, 10 mg/ml glucose, and 0.5 mg/ml gentamicin. All explants were cultured in a CO_2 incubator at 36.7°C in a medium containing 5% CO_2. All organs were cultured for 5 days. After 2 days of culturing, two zones were formed in the tissue explants: denser central (multilayered) and peripheral zone (cell monolayer) as a characteristic halo around the explant. On days 3-5 of culturing after forming the cell monolayer, its area was increased.

For immunocytochemical study, the growth zones of the explants in all cultures of all organs were fixed with 96% ethyl alcohol cooled to -20°C. For histological study of the organotypic cultures of animal organs aimed at describing the cell morphology in the growth zone, they were stained with hematoxylin and eosin.

In spleen and thymus cultures, immunocytochemical reaction was performed with antibodies against the markers of poorly differentiated lymphocytes CD5 (1:30, Novocastra), T helpers CD4 (ready to use, Novocastra), cytotoxic T lymphocytes CD8 (ready to use, Novocastra), B lymphocytes CD20 (1:30, Novocastra), and macrophages CD68 (1:30, Novocastra) using a standard single-step protocol with high temperature antigen unmasking in citrate buffer, pH 6.0. The differentiation and functional activity of retinal cells was investigated immunocytochemically using markers of Brn3, Marth5 (differentiation of ganglion cells), Chx10, Vsx1 (differentiation of bipolar cells), Pax6 (the initial stages of differentiation of retinal neurons), Math1, Prox1 (differentiation of amacrine cells), TTR (transthyretin, differentiation of pigment epithelium; all 1:50, Dako) and antibodies against serotonin, melatonin, and calretinin (ready-to-use, Dako).

Permeabilized in 0.5% Triton X-100 was carried out. Universal set containing biotinylated anti-mouse immunoglobulins was used as secondary antibodies. Reaction sites were visualized with avidin complex with the biotinylated horseradish peroxidase and diaminobenzidine (ABC-kit, Dako).

The data were morphometrically evaluated using a computer-assisted microscopic image analysis system consisting a Nikon Eclipse E400 microscope, a Nikon DXM1200 digital camera, a computer based on Intel Pentium 4, and VidiotestMorphology 5.0 software. In each case, five fields of view were examined at a magnification of 100×.

For electron microscopy, paraffin sections of spleen organotypic culture were prepared. The explants were fixed in acid Bouin's fluid for 2 h. The material was then washed in 70% ethanol, dehydrated in alcohols of increasing concentration, passed through chloroform, and embedded in purified homogenized paraffin for histological studies supplemented with 5% wax. The explants were immersed in cold 2.5% glutaraldehyde solution (Sigma) for 2 h. Pre-fixed fragments were washed with 0.1 M phosphate buffer with calcium chloride. Then the tissue blocks were additionally fixed with OsO_4 (SMT Geraetehandel GmbH) for 2 h and again washed with phosphate buffer. The specimen was further dehydrated in alcohols, absolute acetone, and embedded in complete Epon mixture (Epoxy-Einbettungsmittel-Kit, SMT Geraetehandel GmbH) according the protocol of the embedding kit. Ultrastructural studies were performed using a JEOL JEM-100S electron microscope with ultrathin sections cut on LKB-7A ultramicrotome and contrasted with

uranyl acetate and lead citrate. Cell type was identified on serial semithin and ultrathin sections. Semithin sections were stained with methylene blue-azure II-basic fuchsin by the method of Humphrey and Pittman [7].

RESULTS

Histological staining of the growth zone (monolayer) in the organotypic cultures of embryonic chick retinal cells revealed elongated and triangular cells; most of them had neuron-like morphology. The cells contacted with each other forming a network (Fig. 1, *a*-*c*). Most of them had abundant cytoplasm, the nucleus with one or more large nucleoli was clearly seen (Fig. 1, *d*). The cells with two or more processes differing in thickness were detected. Small part of cells was spindle-shaped and looked like fibroblasts (Fig. 1, *c*). There were also round darkened cells resembling pigment epithelium (Fig. 1, *b*). Some cells with light cytoplasm were round. In some cells, degraded thinning processes were seen. Their nuclei were fragmented with signs of karyorhexis, which indicated the presence of dying neurons in culture (Fig. 1, *b*, *d*). The results of histological examination indicating the presence of nerve cells in retinal culture were confirmed by immunocytochemistry, which enabled us to identify different types of retinal cells. The expression of the following markers was immunocytochemically verifed: Brn3, Marth5, Chx10, Vsx1, Pax6, Math1, Prox1, TTR, serotonin, melatonin, and calretinin (markers of functional activity of retinal cells; Fig. 2).

Histological staining of the growth zone of the thymus explants revealed many cells with large well discernible nuclei of uniform color, which occupied most of the cell and looked like lymphocytes. Among the lymphocyte-like cells, single larger cells with eosinophilic cytoplasm resembling macrophages were recorded. For precise evaluation of the type of immune spleen cells, immunocytochemical study was carried out with the markers to CD20, CD5, CD4, CD8, and CD68. Immunocytochemical staining was positive for all studied markers. Generalizing these results with those of morphological survey we can conclude that the growth zone of the explants of the thymus and spleen is pre-

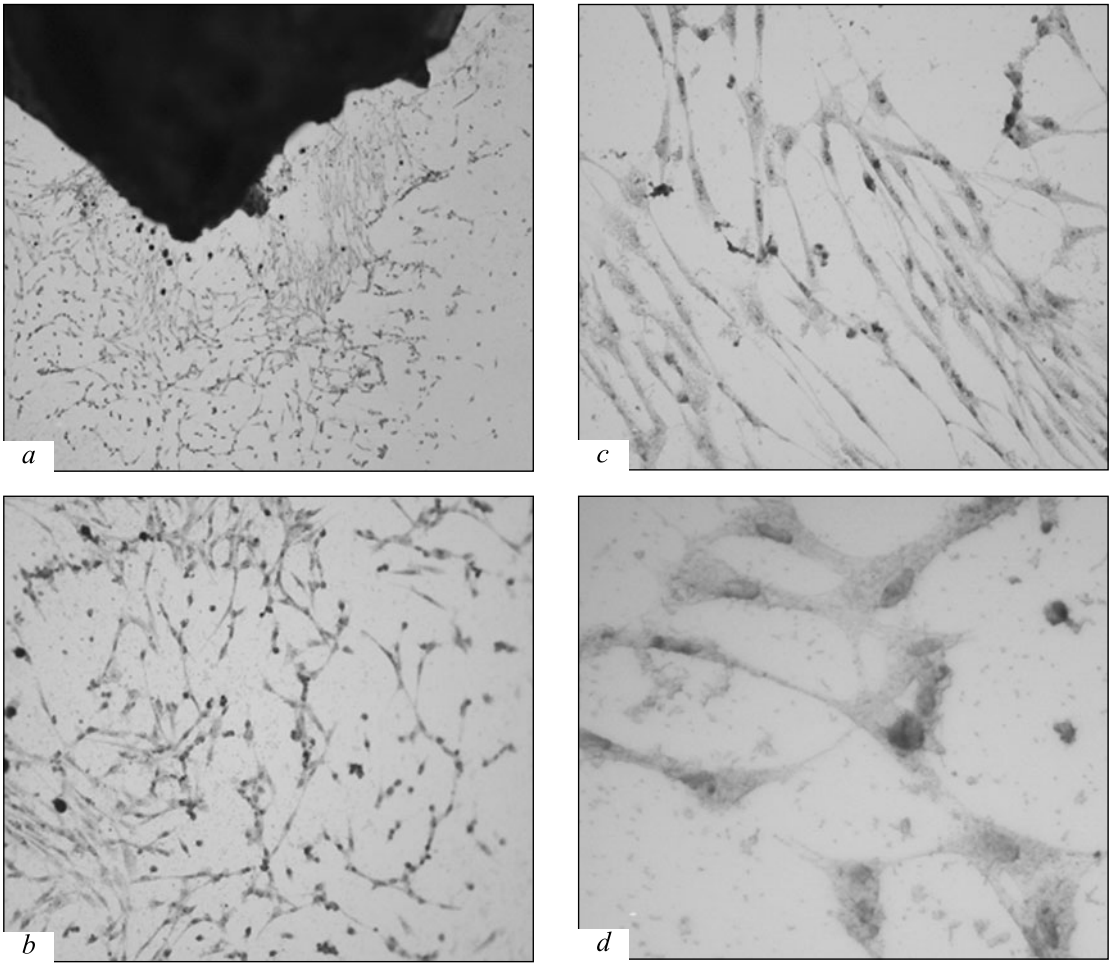

Fig. 1. Organotypic culture of retinal cells. Staining with hematoxylin and eosin. Magnification: *a*) ×40, *b*) ×100, *c*) ×200, *d*) ×400.

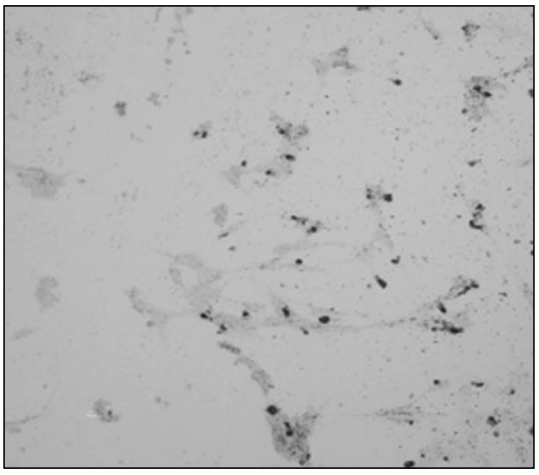

Fig. 2. Organotypic culture of retinal cells: immunocytochemical study with antibodies to the marker of calretinin (×200).

sented by cytotoxic T cells, T-helpers, B cells, undifferentiated lymphocytes, and macrophages. In the thymus, staining for T cell markers CD4 and CD8 was most intense, whereas the maximum expression in the spleen was characteristic of CD20, the B lymphocyte marke.

Electron-microscopic study on paraffin sections revealed a thin capsule presented by 1 or 2 layers of spindle-shaped fibroblasts in the border growth zone of the explant and forming a monolayer of spleen cells. The nuclei of fibroblasts had an elongated oval shape; well-developed rough endoplasmic reticulum and lysosome-like granules were determined in the cytoplasm. Among fibroblasts of the capsule, macrophages with characteristic polymorphic granules were often found. The surface of the capsule was covered with mesothelium, simple flat epithelium. Continuous mesothelial layer was absent. The mesothelial cells often separated from each other, acquire a round shape, and apparently detached from the basement membrane. As a result, large gaps were formed in place of desquamated cells, through which some of the cells migrated out of the explant. Macrophages, fibroblasts, and lymphoblasts were recorded among the migrating cells (Fig. 3, *a*). Single cells or groups of cells showing cytolysis, necrosis and with morphologic signs of apoptosis were identified just beneath the capsule. Reticular cells of red pulp formed a dense network filled with macrophages and other stromal cell elements. The most intensive migration from the explants and proliferation of cells forming a monolayer was observed in the zone of disintegrating capsule (Fig. 3).

Thus, in the monolayer comprising the growth zone of organotypic culture of retinal cells, histological and immunocytochemical survey found different types of differentiating and mature (functionally active) retinal neurons combined with fibroblasts and glial elements. In the monolayer formed during organotypic culturing of the thymus and spleen, T and B lymphocytes, poorly differentiated cells, macrophages and fibroblasts were immunocytochemically verified.

Histological, immunocytochemical, and electron microscopic studies of the cells monolayer, which formed around the explant of organotypic culture of nervous (retina) and immune (spleen, thymus) tissues, indicate that the resulting monolayer retained the variety of cells of the tissue, from which it was received. Thus, staining with hematoxylin and eosin as well as immunocytochemical survey revealed various types of poorly differentiated and mature neurons in the monolayer of retinal cells; in the thymus and spleen, major subpopulations of intrinsic immune cells were detected. In organotypic culturing of the monolayer in the retina, thymus, and spleen, a small number of fibroblast-like cells playing the role of the microenvironment was revealed.

Electron microscopy of spleen explants showed that the capsule of the central explant was destroyed when cultured for more than 2 days, and it allowes the cells to migrate and form a monolayer representing a growth zone of tissue-specific cultured cells.

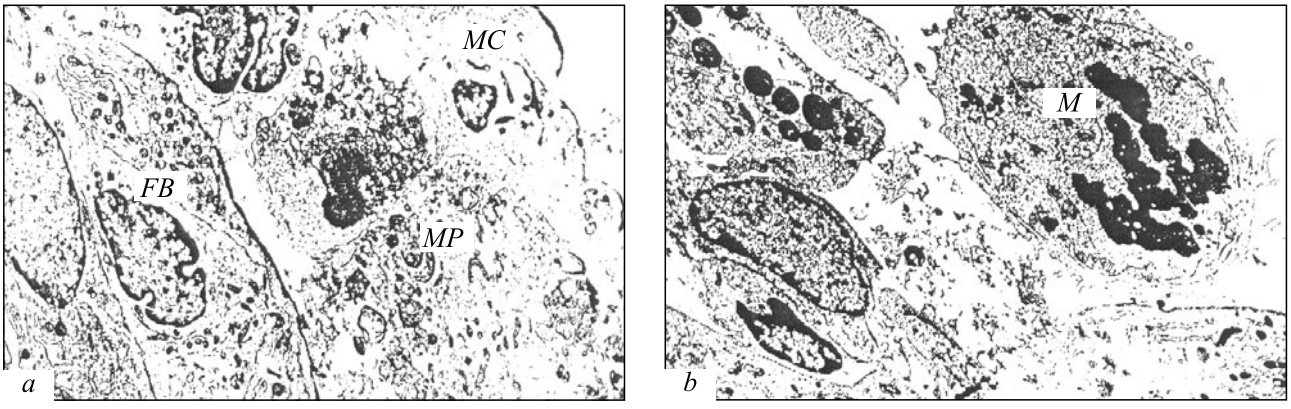

Fig. 3. The ultrastructural organization of the capsule and the subcapsular zone of spleen explant of 3-day-old rat: electron microscopy. Magnification: *a*) ×8200, *b*) ×8750. *MC*, mesothelial cells; *MP*, macrophages; *FB*, fibroblasts; *M*, mitosis.

Thus, the monolayer of the cells of various types along with elements of their natural microenvironment produced on the basis of organotypic culture characteristic of this organ is an adequate model to study the processes of proliferation, differentiation and apoptosis in the organs of the neuro-immune-endocrine system. It may be used both for in basic research of intercellular interactions and for testing the biological activity of the peptide and non-protein compounds.

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