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Monoclonal Antibodies to P Epitope of Chordin Detect Type II Astrocytes in Glial Cultures

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The expression of chordin P-epitope in dissociated cell culture of rat brain is studied by indirect immunofluorescence. Specific fluorescence is confined to compact bipolar and stellate cells on a layer of P-negative spread cells. Granular cells of the cerebellum, spinal neurons, neurons, and Schwann cells are P-negative. Double staining with monoclonal and polyclonal antibodies to glial fibrillar acid protein (an astrocyte marker) shows that this protein is expressed by P-positive stellate cells and P-negative cells of the underlying monolayer. Thus, monoclonal antibodies to P-epitope detect type II astrocytes.

Key Words: *neuroglia development; chordin P-epitope; CNS culture; astrocytes*

In 1984, a new acid glycoprotein was isolated from the sturgeon chord, characterized, and termed chordin [5,12]. Monoclonal antibodies (MAb) to the repeated site of chordin molecule (P-epitope) were obtained [13]. Further studies showed that P-epitope is also present in the nervous system of all vertebrates. Therefore, the proteins of the central nervous system (CNS) carrying P-epitope were called neurochordins [12]. Immunocytochemically detected expression of P-epitope in other organs and tissues was either not typical of all systematic groups or was transitory, occurring only during some stages of ontogenesis [1]. P-epitope was found in almost all parts of the brain and spinal cord of sterlet and triton larvae and chicken and human embryos. Its expression was higher in the white matter and nerve fiber bundles [1,2].

Tissue distribution of P-epitope in vertebrates suggests its potential significance as a neurospecific marker. The specific cellular localization of proteins carrying P-epitope so far remains unclear due to the complex structure of the nervous tissue. This study is an attempt to localize neurochordin with the use of tissue and cell cultures as simple systems. For this purpose primary dissociated glial cultures of the forebrain, cerebellum, and spine as well as organotypical cultures of the sympathetic and spinal ganglia were studied by immunocytochemical methods.

MATERIALS AND METHODS

Dissociated glial cultures were prepared as described elsewhere [10]. Cerebral hemispheres were isolated under sterile conditions from newborn (days 1-2) rats and put in serum-free culture medium to remove the membranes. Then the brain was minced and suspended by pipetting. The resultant suspension was successively filtered (230 and 140 μ pore diameter). The filtrate was layered onto slides covered with 0.01% polylysine. Primary cultures were grown in Eagle's medium with and without 2 mM glutamine, 20% fetal calf serum, 50 U/ml penicillin, and 50 μg/

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ml streptomycin. During subculturing, the serum concentration was reduced to 10%.

For preparing dissociated cultures of cerebellar granular cells 5-day rat pups were used, 14-day-old rat embryos were used to obtain cultures of the spine, and newborn rats were used for cultures of the peripheral ganglia. Further treatment of tissues and culturing procedures were described elsewhere [4]. Cerebellar cells were cultured in medium consisting of synthetic Eagle's MEM medium (70%), Simms' solution (20%), human placental serum (5%), fetal calf serum (5%), glucose (600 mg/liter), insulin (0.1 U/ml), and 10^{-2} M organic HEPES buffer. Medium for culturing dissociated spinal cells consisted of Eagle's MEM (50 ml), fetal calf serum (30 ml), Hanks' solution (11.5 ml), 5 ml of 20% glucose solution, 0.2 U/ml insulin, penicillin, and streptomycin. For organotypical cultures of the peripheral ganglia, cervical sympathetic ganglion and spinal ganglion associated with it were isolated, placed on collagen-coated slides, and cultured in the same medium as the spine.

The expression of specific antigens was detected by indirect immunofluorescence. Monoclonal antibodies to P-epitope of neurochordin diluted 1:50 and polyclonal antibodies (PAb) to glial fibrillar acid protein (GFAP) (1:50) were used [3]. Calbiochem fluorescein-conjugated antibodies to murine and rabbit immunoglobulins diluted 1:25 and 1:50, respectively, or Sigma antibodies (1:100 and 1:50, respectively), and Sigma rhodamine-conjugated antibodies to rabbit immunoglobulins diluted 1:80 were used as secondary antibodies. The antibodies were diluted in buffered saline, pH 7.4, with 1% bovine serum albumin and 0.01% sodium azide. Cells were fixed in cooled acetone for 5 min and processed by conventional methods.

RESULTS

In dissociated cultures of the rat brain, indirect immunofluorescence revealed specific binding of anti-P-epitope MAb to a) uni- and bipolar cell elements with nonramified or slightly ramified processes of various length (Fig. 1, *a*, *b*); b) multipolar cells with slight and medium degree of branching of the straight processes (Fig. 1, *c*, *d*); c) multipolar stellate cells with eccentrically localized nuclei and numerous highly ramified processes of various length (Fig. 1, *e*).

The expression of the neurochordin P-epitope was detected on nonfixed live cells, which confirms its localization on the cell surface.

The fibroblast-like cells forming a monolayer and individual macrophage-like cells were not fluorescent.

Localization on the surface of a layer formed by P-negative cells or at least binding to these cells is a common feature of all listed immunopositive cells. If the cell layer was not formed in short-lived dissociated cultures, P-positive cells formed a network with their processes. Sometimes accumulations of such cells were seen.

Some glial cultures contained P-positive cells with highly ramified processes whose terminal and sometimes lateral portions ramified into numerous flattened lamellar sites.

Treatment of glial cultures with MAb to the neurochordin P-epitope and PAb to GFAP followed by detection with fluorescein-conjugated antibodies to mouse immunoglobulins and with rhodamine-conjugated antibodies to rabbit immunoglobulins, respectively, revealed a simultaneous expression in the glial cultures of P-epitope and GFAP by individual multipolar stellate cells (Fig. 1, *e*, *f*). This proves their astrocytic origin. P-positive cells of this type were detected on the surface of the cell layer (Fig. 2, *a*). On the other hand, anti-GFAP antibodies bound not only to stellate cells, but to also fibroblast-like cells of the underlying monolayer as well, staining red virtually the whole layer (Fig. 2, *b*). This indicates the predominance of another type of GFAP-positive P-negative astrocytes.

Bright specific fluorescence of P-epitope was observed in cell aggregations and glioneurite bundles of dissociated cultures of the cerebellum and spinal cord, but it was rather difficult to discern individual cells. At the same time, P-positive glial cells were well seen in nonformed young (2-day) cultures and loose sites of later (6-day) cultures. The same morphological types as in glial cultures were seen, but the cells were more diverse. Presumably, this was due to the predominance of nerve cells in the culture, though spinal neurons and cerebellar granular cells outside the aggregation were P-negative. P-epitope was not expressed in neurons and Schwann cells of organotypical cultures of the peripheral nerve ganglia.

Using antibodies to GFAP (an astrocyte marker), galactocerebroside (oligodendrogliocyte marker), and antibodies A2B5 to immature elements of nerve tissue, we demonstrated that in a culture of the rat optic nerve oligodendrogliocyte (O) and the so-called type II astrocytes (2A), which correspond to fibrillar astrocytes by some signs, have a common pathway of development [11,14,15]. Both have a common precursor (O2A cells), which is transformed into O or 2A, depending on culturing conditions. Typical morphological features in the development of these cells were revealed: from uni- and bipolar O2A precursors via multipolar cells with straight

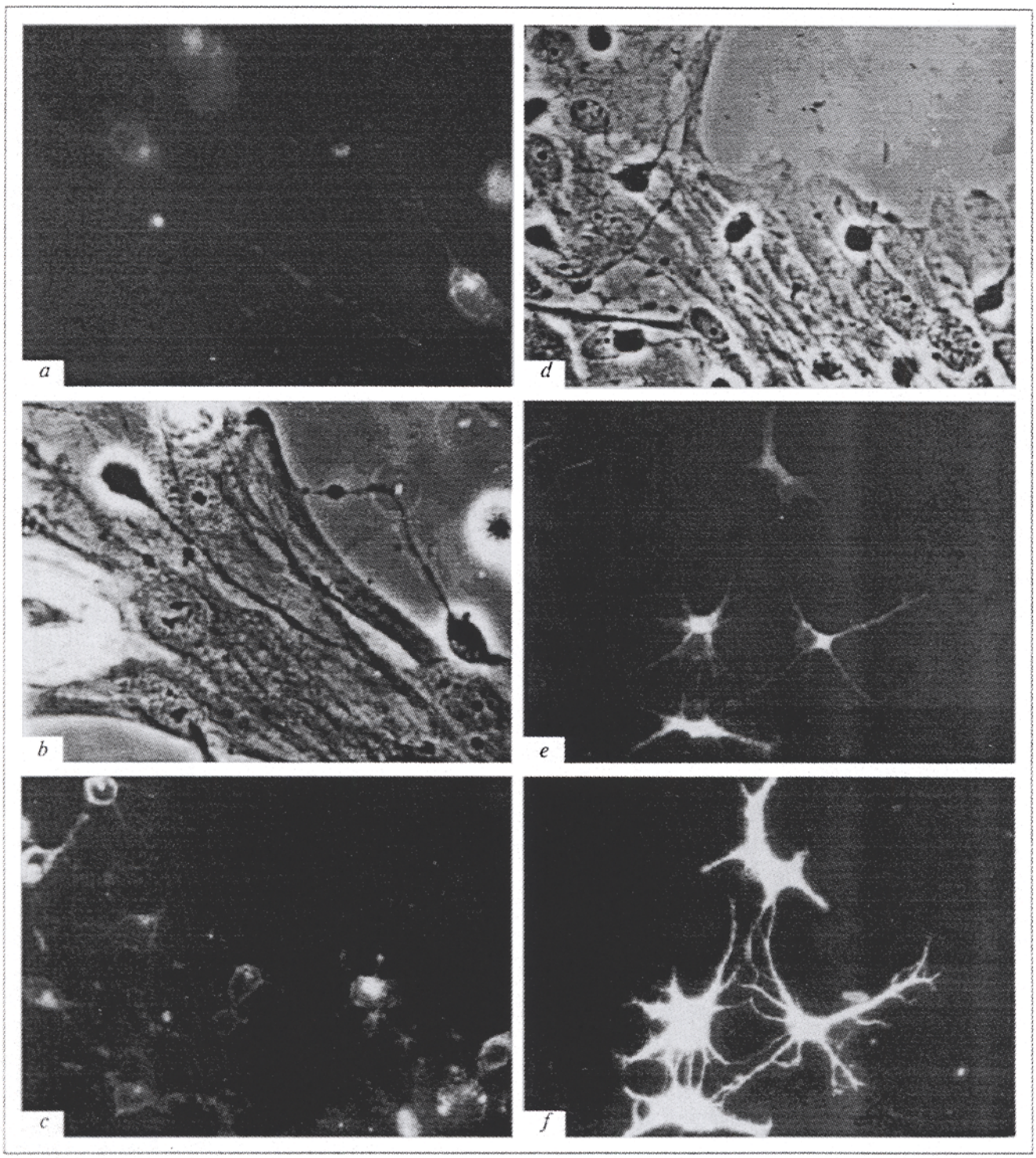

Fig. 1. Indirect immunofluorescence in a dissociated glial culture of the rat brain. *a*) P-positive uni- and bipolar cells on the surface of P-negative cell layer, day 9 of culturing, ×210; *b*) the same, phase contrast; *c*) P-positive multipolar cells with slightly ramified processes, day 19 of culturing, ×160; *d*) the same, phase contrast; *e*, *f*) double immunofluorescent staining of multipolar cells with monoclonal antibodies to P-epitope (*e*: green fluorescence of fluorescein-conjugated antibodies to mouse immunoglobulin) and with polyclonal antibodies to glial fibrillar acid protein (*f*: red fluorescence of rhodamine-conjugated antibodies to rabbit immunoglobulin), day 34, ×400.

short poorly ramified processes to mature stellate elements with well-developed ramified processes. Simultaneously, a specific pathway of the development of type 1 astrocytes (protoplasmic, 1A) was shown; these cells more actively propagate in the culture and form an underlying cell layer. Later, these conclusions, i.e.,

the existence of O2A precursors, were confirmed for other brain structures [6-9].

Anti-P-epitope MAb detected cells whose shape in a dissociated glial culture of the rat brain varied from uni- and bipolar to stellate. They rarely spread, did not form a confluent layer, and were located predominantly on the surface of a cell layer formed by P-negative cells. These P-positive cells were morphologically and phenotypically similar to the cells of O2A lineage. The expression of GFAP in stellate P-positive cells indicates that at least part of P-positive cells belong to A2 type. However, the expression of P-epitope in mono- and bipolar elements suggests that it is present on the surface of O2A precursors and, probably, on oligodendrogliocyte. Further studies are required to find out whether P-epitope is not expressed by nerve cells, which was revealed for cerebellar granular cells and spinal neurons, since the phenotype of these cells in the CNS varies considerably.

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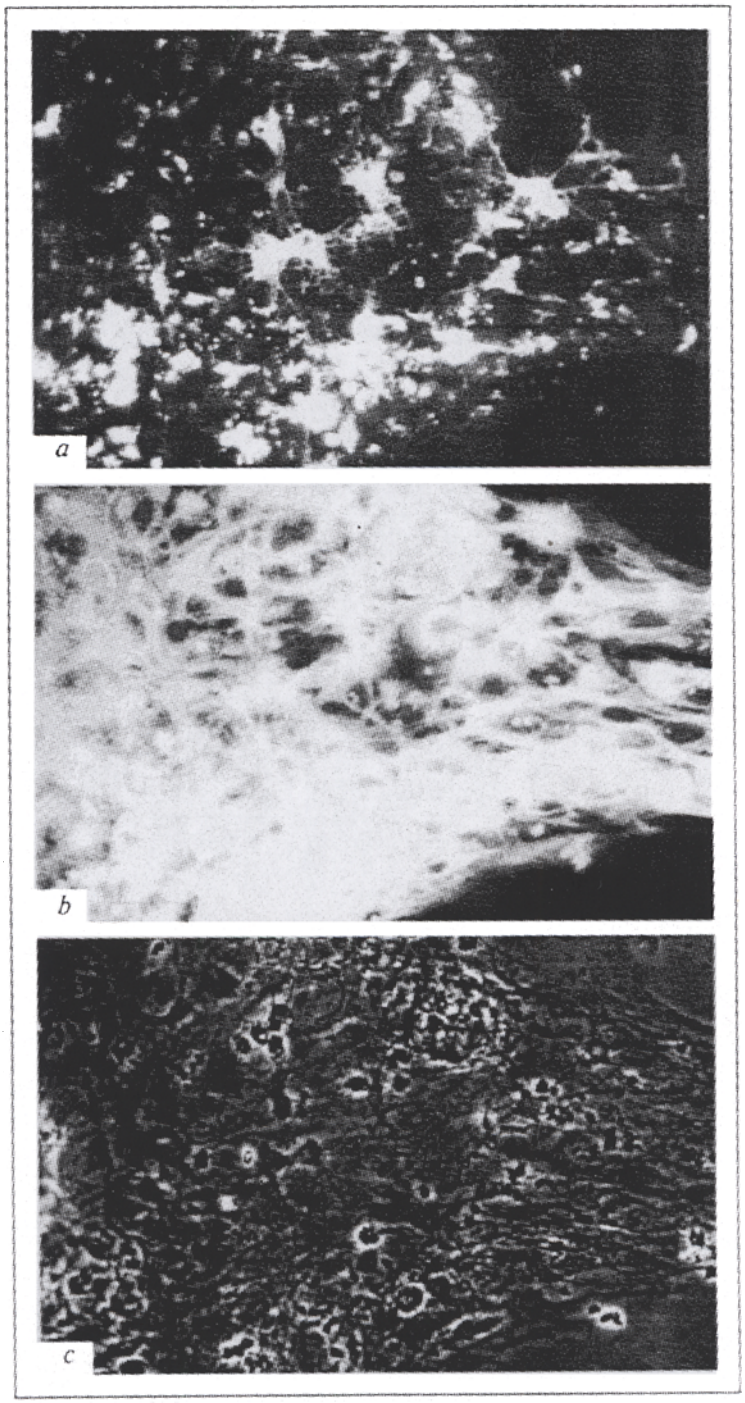

Fig. 2. Double immunofluorescent staining of glial culture, ×100. *a*) green fluorescence of P-positive elements on the surface of P-negative layer; *b*) red fluorescence of cells positive to glial fibrillar acid protein; *c*) the same, phase contrast, day 34.