Cellular Localization of GM1-Ganglioside with Biotinylated Choleragen and Avidin Peroxidase in Primary Cultured Cells from Rat Brain

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A new technique capable of demonstrating the presence and cellular localization of the ganglioside GM1 in primary cultured cells from the brains of newborn rats is described. The method is based on the highly specific binding of biotinylated choleragen to ganglioside GM1, and takes advantage of the high affinity of avidin for biotin. Thus, the biotinylated choleragen–ganglioside GM1 complex can be visualized by the use of avidin peroxidase. The results of this nonimmunologic method indicate that the concentration of ganglioside GM1 is much lower in culture astroglial cells than in neurons and oligodendroglial cells.

KEY WORDS. Avidin peroxidase; Biotinylated choleragen; Ganglioside GM1; Cell primary culture; Glial cells; Choleragen; Neurons.

Introduction

Affinity cytochemistry utilizing the biotin–avidin complex for the visualization of reactive sites has several advantages over the more classical immunocytochemical procedures (Berman and Basch, 1980; Guesdon, Ternyck, and Avrameas, 1979). Avidin has a very high affinity for biotin (Finn et al., 1980), and the interaction is almost irreversible. Thus, the laborious procedures involved in antibody preparation can be avoided. Additionally, the availability of numerous biotinylation reagents for coupling, as well as different aviden conjugates (avidin–fluorescein isothiocyanate, avidin–rhodamine, and avidin–enzyme complexes) provide the means for using a method that is at once more simple, versatile, specific, and sensitive than most immunocytochemical methods.

Recently, Holtzman et al. (1982) and Axelrod (1980) reported the utilization of complexes of biotinyl–toxin, acetylcholine receptor, and avidin for the electron-microscopic and fluorescent visualization of the acetylcholine (nicotinic) receptor. The present report adapts the biotin–avidin system for the visualization of ganglioside GM1 in cells derived from primary cultures of newborn rat brain cells. Although anti-ganglioside antibodies can be used for the cellular localization of gangliosides, the polyclonal antibodies ordinarily used do not show absolute specificity for specific gangliosides. This is because many gangliosides show structural similarities that cannot be distinguished by polyclonal-antibody preparations. Ganglioside GM1 is a receptor that shows a high affinity and specificity for the toxin choleragen (Cuatrecasas, 1973; Fishman and Brady, 1976; Moss et al., 1976; Hansson, Holmgren, and Svennerholm, 1977).

Materials and Methods

Reagents. Eagle’s minimum essential medium (Eagle, 1955) without NaHCO3 or glutamine (MEM); fetal bovine serum (FBS); Dulbecco’s phosphate-buffered saline (PBS); kanamycin (10,000 μg/mL); and Trypan blue stain (0.4%) were all obtained commercially (Grand Island Biological Co., Long Island, NY). Cholera toxin (B subunit), biotin, avidin–peroxidase in the form of the enzyme-labeled powder, 3,3’-diaminobenzidine (DAB), and Mayer’s hematoxylin solution were also purchased commercially (Sigma Chemical Co., St. Louis, MO). A collector tissue sieve was obtained from Bellco Glass Inc. (Vineland, NJ). Hydrogen peroxide (50%) was obtained from Fisher Scientific (St. Louis, MO).

Primary culture conditions. Pregnant rats (Sprague-Dawley strain) were obtained from Taconic Farms (Germantown, NY). Newborn rats were used for the cell-culture studies. The cerebral hemispheres were dissected out and placed in MEM containing 10% FBS. The tissue was then put into a pan and triturated into a paste using a pestle, after which the paste was extruded through a stainless steel mesh (180-μm opening size, 40-mesh screen). This was repeated using a mesh with a smaller opening size (190-μm opening size 80-mesh screen). The cells were extruded into an MEM-supplemented tissue-culture dish.
Figure 1. (a) Seven-day-old primary culture of cells prepared from newborn rat brain. The biotin-avidin complex provided a dense brown stain that served to identify the small oligodendroglial cells, most of which are concentrated in the upper-left-hand quadrant of the photo. The unstained bodies are the nuclei of the barely perceptible astroglial cells. (b) Controls consisting of cells that had been treated with non-biotinylated choleragen. Oligodendroglial cells are not visible. The numerous nuclei are enclosed within the barely perceptible astroglial-cell bodies. Original magnification × 400; bar = 25 μm.

Any cells remaining in the mesh were rinsed into the culture dish with 10 ml of MEM (containing 10% FBS, 0.045% sodium bicarbonate, and 100 μg/ml of kanamycin). The cell suspension was then centrifuged at 500 × g for 5 min. The supernatant was discarded and the cell pellets were resuspended, dispersed by pipetting, and recentrifuged as described. After centrifugation, each cell pellet was resuspended in MEM which contained 10% FBS. The mixture was then again centrifuged at 500 × g for 5 min. After this the cells were counted and plated in 2 ml of MEM (containing 10% FBS, 0.045% sodium bicarbonate, and 200 μg/ml kanamycin) at a density of 10⁵ viable cells per 93 × 16-mm plastic Leighton tube (Costar, Cambridge, MA). Cell viability was determined by Trypan blue exclusion. All cell
cultures were grown in an incorporated controlled environment incubator at 37°C in an atmosphere of 95% air and 5% CO₂. The medium was renewed every fourth day of culture.

Treatment of cells with biotin-choleragen and avidin-peroxidase complexes. In most experiments, 93-mm plastic Leighton tubes with 5-cm² plastic coverslips were incubated for 7 days or 21 days at 37°C. The coverslips were then washed five times with PBS and the culture was fixed with a freshly prepared 2% solution of paraformaldehyde in PBS for 15 min at 4°C, washed twice with PBS for 5 min, and immersed for 10 min in methanol (−20°C). After rinsing three times with PBS, the coverslips were treated with biotin-choleragen at 5 μg/ml in PBS at 4°C overnight. Controls were treated with choleragen only rather than biotin-choleragen.

Choleragen was biotinylated by the modified method of Bayer, Skutelsky, and Wilchek (1979). Briefly, 2 mg of the choleragen B subunit of 1 ml of a solution of 50 mM Tris chloride, 0.2 M NaCl, and 1 mM Na₂EDTA at pH 7.5 was biotinylated with 30 μl of a solution of biotinyl-N-hydroxysuccinimide ester (5.5 mg/ml) in dimethyl formamide. The solution was kept at room temperature for 4 hr and dialyzed against Dulbecco's phosphate buffered saline for 3 days at 4°C, with three buffer changes.

After several washings (at least five times for 10 min at 37°C) with PBS containing bovine serum albumin (BSA fraction V, Sigma Chemical Co.) at 2 mg/ml, the cultured cells were treated with avidin-peroxidase (approximately 0.8 mole of peroxidase per mole of avidin) at 5 μg/ml in PBS at 37°C. After several more washings with PBS/albumin, the reaction was developed with hydrogen peroxide as substrate and DAB as electron donor, yielding a brown, insoluble product.

Figure 2. A neuron that was positively stained using the biotinylated choleraein/avidin-peroxide procedure. A composite photo is provided in order to demonstrate the termination of the positively stained processes. Original magnification ×400; bar = 25 μm.
The staining of 7-day cultures of newborn rat brain cells for ganglioside GM1 using the biotinylated cholera antigen-din-biotin complex was examined with a Leitz (Wetzlar) microscope. Results and Discussion

The staining of 7-day cultures of newborn rat brain cells for ganglioside GM1 using the biotinylated cholera antigen-din-biotin complex was examined with a Leitz (Wetzlar) microscope. The staining of 7-day cultures of newborn rat brain cells for ganglioside GM1 using the biotinylated cholera antigen-din-biotin complex was examined with a Leitz (Wetzlar) microscope. The small, positively stained oligodendroglial cells have spider-like processes and a high nucleus-to-cytoplasm cross-sectional-area ratio. It is noteworthy that the peroxidase-labeled brown particles appeared over the body, nucleus, and processes of these cells. Presumably, the entire surface of the cell was stained. Controls on which nonbiotinylated choleraugest was used prior to treatment with avidin-peroxidase did not exhibit staining of these oligodendroglial cells (Figure 1b). It was necessary to bind the endogenous ganglioside GM1 with biotinylated cholera antigen before the avidin-peroxidase reaction product could be observed.

Astrocytes were not stained by this method—a result that agreed with that obtained with anti-ganglioside GM1 antisera (Asou and Brunngraber, 1983) and with an assay of cholera-toxin binding sites in monolayer cultures of astroglial cells (Willinger and Schachner, 1980). Figures 1a and 1b both show the unstained large nuclei of astroglial cells. The outlines of the astroglial cell bodies are barely perceptible. The morphology of the astroglia is variable, but they generally appear to be smaller than neurons and considerably larger than oligodendroglia. Astrocytes appear as large, flat, stellate and spindle-shaped cells. They contain large nuclei and fibrous processes. These cells are morphologically identified by the immunohistochemical visualization of glial fibrillary acidic protein (Asou, Brunngraber, and Delpech, 1983; Asou and Brunngraber, 1983), which appears in the cytoplasm surrounding the nucleus. The concentration of ganglioside GM1, estimated by extraction and chemical assay, was found to be much lower in astroglial cells derived from astrocytomas than in cultured neuroblast cells (Robert et al., 1973; Mandel et al., 1980), and purified astroglial preparations derived from primary cultures were found to be devoid of ganglioside GM1 (Asou and Brunngraber, 1983).

The cell bodies and processes of neurons showed a dense stain in newborn rat brain cells cultured for 21 days (Figure 2). This finding is consistent with that of Raff et al. (1979), who used a cholera toxin-antitoxin staining reaction, and with the results of studies employing anti-ganglioside GM1 antisera in this laboratory.

Biotinylated cholera antigen, useful in the present experiment, in which all observations were made with light microscopy, can be expected to be useful in electron or florescent microscopy as well, since avidin derivatives with electron-dense or florescent markers are now commercially available. The present study suggests the possibility of biotinylating other toxins known to bind specific gangliosides, so that they may subsequently be visualized with suitable markers conjugated to avidin.

The experiments reported here do not reveal whether the binding specificity of cholera toxin is subtly altered as a consequence of coupling with biotin. However, the product obtained by the biotinylation of bungarotoxin (BTX) was shown to be capable of binding irreversibly to the acetylcholine receptor (Axelrod, 1980). Anti-ganglioside GM1 peroxidase-antiperoxidase procedures (with controls consisting of antisera from which the anti-ganglioside antibody had been removed by absorption with ganglioside GM1) have provided identical staining reactions and an identical staining distribution among various neuronal cell types to those obtained with the method outlined in this report (Asou and Brunngraber, 1983).

Since avidin is multivalent (Holtzmann et al., 1982), the binding sites for biotinylated cholera may become clustered upon avidin binding, as a result of cross-linking. Electron-microscopic examination will be required to settle this point.

Literature Cited

Axelrod D (1980): Crosslinkage and visualization of acetylcholine receptors on myotubes with biotinylated α-bungarotoxin and fluorescent avidin. Proc Natl Acad Sci USA 77:4823
