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Cell type-specific expression of ganglioside antigens in the central nervous system

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Abstract: We established an improved method for the generation of mouse monoclonal antibodies (MAbs) to gangliosides by immunizing mice with purified gangliosides. Using this method, we generated and characterized a large number of the MAbs specific for gangliosides. These MAbs enabled us to examine the distribution of ganglioside in the brain. Immunohisto- and immunocyto-chemical studies suggested that there is a cell type-specific expression of gangliosides in the central nervous system.

INTRODUCTION

Gangliosides (a), sialic acid-containing glycosphingolipids, are normal membrane constituents and are highly expressed in the vertebrate central nervous system (1). Owing to their topological localization on the outer surface of neural plasma membranes and their unique chemical structure, gangliosides have been implicated in a variety of phenomena involving cell-cell recognition, neurite outgrowth, synaptogenesis, transmembrane signalling, and cell growth and differentiation (2-5). An understanding of the cellular localization of gangliosides in the brain could provide insight into the possible function of these molecules. In the past decade, cholera and tetanus toxins, and several polyclonal and monoclonal antibodies (MAbs) reacting with gangliosides have been used as probes for detecting gangliosides in neurons and glia (6, 7). It was, however, difficult to generate MAbs specific for individual gangliosides. We recently established an improved method for the generation of mouse MAbs to gangliosides by immunizing mice with purified gangliosides (8-15). These MAbs enabled us to examine the distribution of ganglioside in the central nervous system. First, we examined the distribution of gangliosides in adult rat brain, including cerebrum, cerebellum, hippocampus and a spinal cord by an immunohistochemical technique. The study revealed the differential distribution patterns of gangliosides in the brain regions (16, 17). Next, we studied the distribution of gangliosides during the development of postnatal rat cerebellar cortex by the same technique, since the cerebellar cortex is a good system to study various events at the cellular and molecular levels. Our findings demonstrated that the expression of each ganglioside changes dramatically during the development (18). Subsequently, we attempted to determine the expression of gangliosides in primary cultures of the rat cerebellum by an immunofluorescence technique. The study also revealed a cell type-specific expression of gangliosides in primary cultures of the rat cerebellum (19). Thus, our studies suggested that the expression of each ganglioside may be restricted to subsets of neuronal groups and their surrounding neuropil. At present, we are working on the molecular cloning of proteins involved in ganglioside biosynthesis and their expression, such as glycosyltransferases and related factors, using a direct expression cloning method with MAbs, and on the purification and characterization of the binding protein of gangliosides concentrated in the synaptic regions.

IMMUNOGENICITY OF GANGLIOSIDES IN MICE

Note a: Sialic acid moiety of the ganglioside is NeuAc unless otherwise noted; gangliosides are named according to the nomenclature of Svennerholm (27). Otherwise, the nomenclature used follows the IUPAC-IUB recommendation (28).

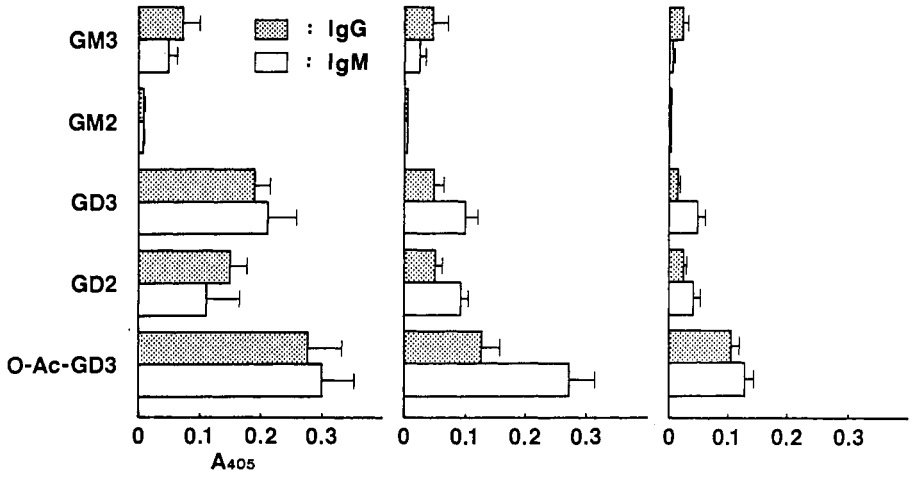

Fig. 1. Antibody reactivity of mouse sera after immunization with gangliosides isolated from human melanoma cells. Ten different strains of inbred mice were immunized with each ganglioside (100 μg, total amount per mouse) coated on acid-treated *S. minnesota* (250 μg, total amount per mouse) intraveneously five times on day 0, 4, 7, 11 and 21. Sera were obtained after 3 days from the last injection. Titers of the sera were determined by ELISA. All of the gangliosides were prepared from M14 cells and have NeuAc as their sialic acid moiety. Each column and horizontal bar presents the mean and standard deviation of triplicate determinations of five mice. Group 1 (left panel), C3H/HeN and A/J; 2 (center panel), Balb/c, C57BL/6, DBA/2 and nu/nuBalb/c; 3 (right panel), C3H/HeJ, AKR/N, CBA/N and NZB/N. Antibody reactivity of C3H/HeN, Balb/c and C3H/HeJ is shown as a representative of group 1, 2 and 3, respectively. (8)

In the past decade, MAbs to gangliosides have been established by immunizing Balb/c or C57BL/6 mice with neuroectoderm-derived tumor cell. To develop an improved method for generating murine MAbs to gangliosides with high efficiency, we studied antibody responses to various gangliosides in 10 strains of inbred mice. Twelve gangliosides having NeuAc as their sialic acid moiety, four gangliosides having NeuGc and four asialo-gangliosides were injected intravenenously adsorbed to *Salmonella minnesota*. The antibody titers of the mice sera were determined by an enzyme-linked immunosorbent assay and an immune adherence assay. Antibody responses were found to depend not only on the ganglioside used as an immunogen but also on the mouse strain. Gangliosides having a trisaccharide sequence (NeuAcα2-8NeuAcα2-3Gal-) such as GD3, GD2, GD1b, GT1a and GQ1b, in particular *O*-Ac-GD3 induced high-titer antibody responses, whereas those having a disaccharide sequence (NeuAcα2-3Gal-) such as GM4, GM3, GM2, GM1, GD1a and GT1b induced low-titer antibody responses. On the other hand, gangliosides with NeuGc developed minimum titers. In contrast, asialo-gangliosides induced much higher responses than the corresponding gangliosides. The differences in ceramide portions of these gangliosides did not appear to be involved in inducing antibody responses. Mice could be divided into three groups according to the maganitude of their antibody responses: Group 1, those that produce the highest antibody responses (C3H/HeN and A/J); Group 2, those that demonstrate moderate antibody titers (Balb/c, C57BL/6, DBA/2 and nu/nu Balb/c); and Group 3, those that make minimum responses (AKR/N, C3H/HeJ, CBA/N and NZB/N) (Fig. 1). The pattern of reactivity to the various gangliosides was similar in all the strains tested. These results suggest that C3H/HeN and A/J mice may be more suitable than other strains of mice for raising MAbs to gangliosides (8).

GENERATION AND CHARACTERIZATION OF MABS TO GANGLIOSIDES

Using the method mentioned above, we generated a large number of the MAbs speicific for gangliosides. They include MAbs to a-series, b-series, NeuGc-containing ganglioisdes, ganglioside lactones and de-N-acetyl-ganglioisdes. The production and characterization of these MAbs has been described previously

TABLE 1. Monoclonal Antibodies to Gangliosides Established in Our Laboratory

Ganglioside[1]	Designation	Class of Ig	Reference
a-series			
GM3	GMR6	IgM (k)	10
GM2	GMB28	IgM (k)	10
GM1	GMB16	IgM (k)	10
GD1a	GMR17	IgM (k)	10
GT1a	GMR11	IgM (k)	10
b-series			
GD3	GMR19	IgM (k)	9
GD2	GMR7	IgM (k)	9
GD1b	GGR12	IgG2b (k)	9
GT1b	GMR5	IgM (k)	9
GQ1b	GMR13	IgM (k)	9
Miscellaneous			
GM1b	GGR51	IgG2a (k)	17
GQ1bα	GGR41	IgG2a (k)	13
GM4	AMR10	IgM (k)	12
IV^3NeuAcα-nLc4Cer	NGR54	IgG3 (k)	17
IV^6NeuAcα-nLc4Cer	NGR53	IgG3 (k)	17
O-Ac-disialoganglioside	GMR2	IgM (k)	9
NeuGc-containing			
GM3 (NeuGc)	GMR8	IgM (k)	11
GM2 (NeuGc)	GMR14	IgM (k)	11
GD3 (NeuGc-NeuGc-)	GMR3	IgM (k)	11
De-N-acetyl-			
GM3	SMR36	IgM (k)	15
GD3	SGR37	IgG3 (k)	15
Lactone			
GM1	AMR38	IgM (k)	14
GD1a	AMR40	IgM (k)	14
GD3	AMR19	IgM (k)	14

[1]Sialic acid moiety of ganglioside is *N*-acetylneuraminic acid (NeuAc) unless otherwise noted.

(9-15). Briefly, all of the MAbs except MAb GGR41 (anti-GQ1bα) were generated by immunizing C3H/HeN mice with purified gangliosides adsorbed to *Salmonella minnesota* mutant R595. MAb GGR41 was generated with a GQ1b fraction extracted from bovine brain, which cotained a small amount of GQ1bα as a contaminant (13). The binding specificity of these MAbs was determined by an enzyme-linked immunosorbent assay and an immunostaining on thin-layer chromatogram. Most of these MAbs showed highly restricted binding specificity, reacting only with the immunizing ganglioside. None of other variuos authentic gangliosides or neutral glycolipids were recognized. Although most of the MAbs were of IgM (κ), some MAbs belonged to IgG (κ). The MAbs to gangliosides established in our laboratory are summarized in Table 1.

IMMUNOHISTOCHEMICAL STUDIES OF GANGLIOSIDES IN THE RAT BRAIN

The distribution of major ganglioside antigens in the adult rat brain

Our aim is not to establish and characterize the MAbs to ganglioisdes, but to elucidate the role of gangliosides in the central nervous system. There are a number of applications of their MAbs in life

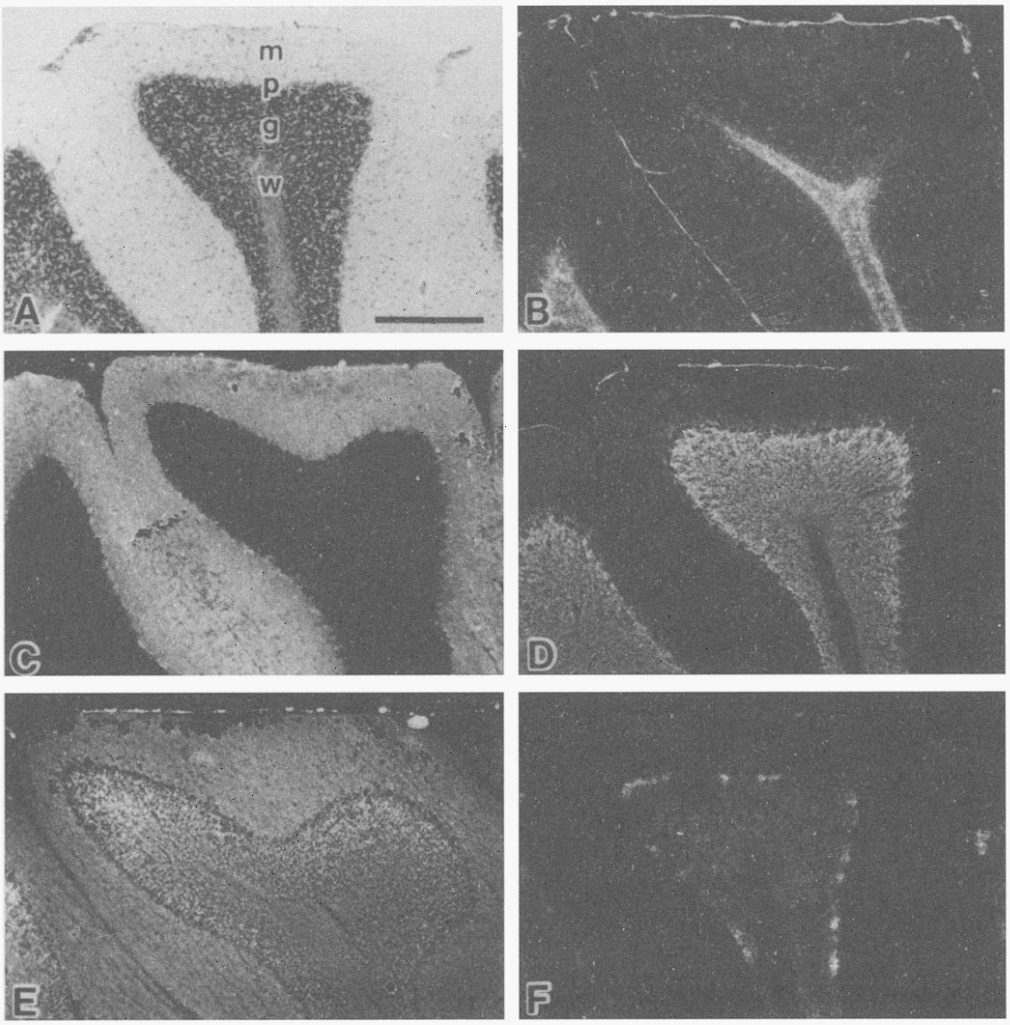

Fig. 2. Indirect immunofluorescence analysis of ganglioside expression in rat cerebellar cortex. Sequential saggital sections were treated with MAbs specific for gangliosides and stained with FITC-labeled goat anti-mouse IgG or IgM antibody. m, molecular layer; p, Purkinje cell layer; g, granular layer; w, white matter. The section was stained with MAbs. A. parallel section stained with K-B technique; B. GMB16 (anti-GM1); C, GMR17 (anti-GD1a); D, GGR12 (anti-GD1b); E, GMR5 (anti-GT1b); F, GMR13 (anti-GQ1b). The bar represents 500 μm. (16)

science as well as glycobiology. At first, we attempted to investigate the localization of major gangliosides in the adult rat brain by an immunofluorescence technique with mouse MAbs. Five MAbs that specifically recognize gangliosides GM1, GD1a, GD1b, GT1b and GQ1b, respectively, were used. We have found that there is a cell type-specific expression of the gangliosides in the rat central nervous system. In cerebellar cortex, GM1 was expressed in myelin and some glial cells. GD1a was detected exclusively in the molecular layer. GD1b and GQ1b were present restrictedly on the granular layer; GD1b was detected on the surface of the granular cell bodies, whereas GQ1b was present in the cerebellar glomerulus. GT1b was distributed intensely in both the molecular layer and the granular layer. In cerebral cortex, GM1 was detected in some glial cells. Dense staining was limited to the white matter. GD1a was distributed in layers I, II/III and Va, and the upper part of layer VI. GD1b was detected beneath layer III. GT1b appeared to be distributed throughout all layers. In other regions, such as hippocampal formation and spinal cord, the expression of the ganglioside was highly localized to a specific cell type and layer (16). The immunohistochemical distribution of major gangliosides in the rat cerebellum is shown in Fig. 2.

The distribution of minor ganglioside antigens in the adult rat cerebellum

As a next step, we studied the distribution of minor gangliosides in the adult rat brain by an immunofluorescence technique with mouse MAbs. Ten MAbs that specifically recognize GM3, GM2, GT1a, GD3, *O*-Ac-disialoganglioside, GD2, GM1b, GM4, IV^{3}NeuAcα-nLc$_4$Cer, and IV^{6}NeuAcα-nLc$_4$Cer, respectively, were used. Our study revealed that there is a cell type-specific expression of minor gangliosides as well as major gangliosides in the rat brain. In the cerebellar cortex, GM3 was expressed intensely in the white matter and slightly in the granular layer. GD3 was present in both the granular layer and the white matter, but not in the Purkinje cell layer or in the molecular layer. A *O*-Ac-disialoganglioside, which was suggested to be *O*-Ac-LD1, was detected exclusively in both the molecular layer and Purkinje cell layer. The presence of GD2 was restricted to the granular layer. GM4 was associated with some astrocytes, but not with myelin or oligodendrocytes. GM2, GT1a, GM1b, IV^{3}NeuAcα-nLc$_4$Cer, and IV^{6}NeuAcα-nLc$_4$Cer gangliosides were not clearly detected in the cerebellar cortex. In other regions, such as cerebral cortex, hippocampal formation, and spinal cord, the expression of the gangliosides was also highly localized to a specific cell type and layer (17).

The distribution of ganglioside antigens during the development of postnatal rat cerebellum

Subsequently, we studied the distribution of gangliosides during the development of postnatal rat cerebellum by an immunofluorescence technique with mouse MAbs. Eleven MAbs that specifically recognize each ganglioside were used. Our study revealed that the expression of each ganglioside changed dramatically during the development. GD3 and *O*-Ac-GD3 were expressed intensely in the external granular layer at 1, 5, and 10 days, whereas GD2 was firstly detected in the internal granular layer at 5 days and GD1b was diffusely detected throughout all layers of the cerebellar cortex at early postnatal days. GD2 and GD1b were more intensely expressed in the granular layer at 20, 30, and 80 days, suggesting that premature granule cells express GD3 and its derivatives, *O*-Ac-GD3, whereas mature granule cells express GD2 and GD1b intensely. On the other hand, GM1 was exclusively detected in the external granular layer and the molecular layer at 1 and 5 days. The staining sites spread gradually from these outer layers into the internal granular layer and the white matter after 10 days. The positive cells in the external granular layer and the molecular layer appeared to be Bergmann glial cells and their radially ascending cytoplasmic processes. The intensity of the staining in these specialized astroglial cells decreased gradually during postnatal days. In contrast, the expression of GQ1b was very faint at birth, but gradually increased during the development and was detected intensely in the internal granular layer, particularly in the cerebellar glomeruli in the adulthood, suggesting that GQ1b expression may be associated with synapse-related structures. The developmental changes of the expression of other gangliosides were also recognized in the postnatal rat cerebellum. These results suggest that specific gangliosides may play an important role in regulating the early events responsible for the orderly formation of the cerebellar cortex (18).

IMMUNOCYTOCHEMICAL STUDY OF GANGLIOSIDES IN PRIMARY CUTLTURED NEURONAL CELLS

Then, we studied the expression of ganglioside antigens in primary cultures of rat cerebellum using an immunocytochemical technique with mouse MAbs specific for various gangliosides. Twelve MAbs that specifically recognize each ganglioside were used. Our study revealed that there is a cell type-specific expression of ganglioside antigens in the primary cultures. A number of b-series gangliosides were detected in the granule cells, whereas a-series gangliosides were not intensely expressed. GD1b was detected in the granule cells. GD2 appeared to be present in a subset of the granule cells or a type of small neurons. GD3 was associated not only with the granule cells, but also with both astrocytes and oligodendrocytes. An *O*-Ac-disialoganglioside, which was suggested to be *O*-Ac-LD1, was restrictedly

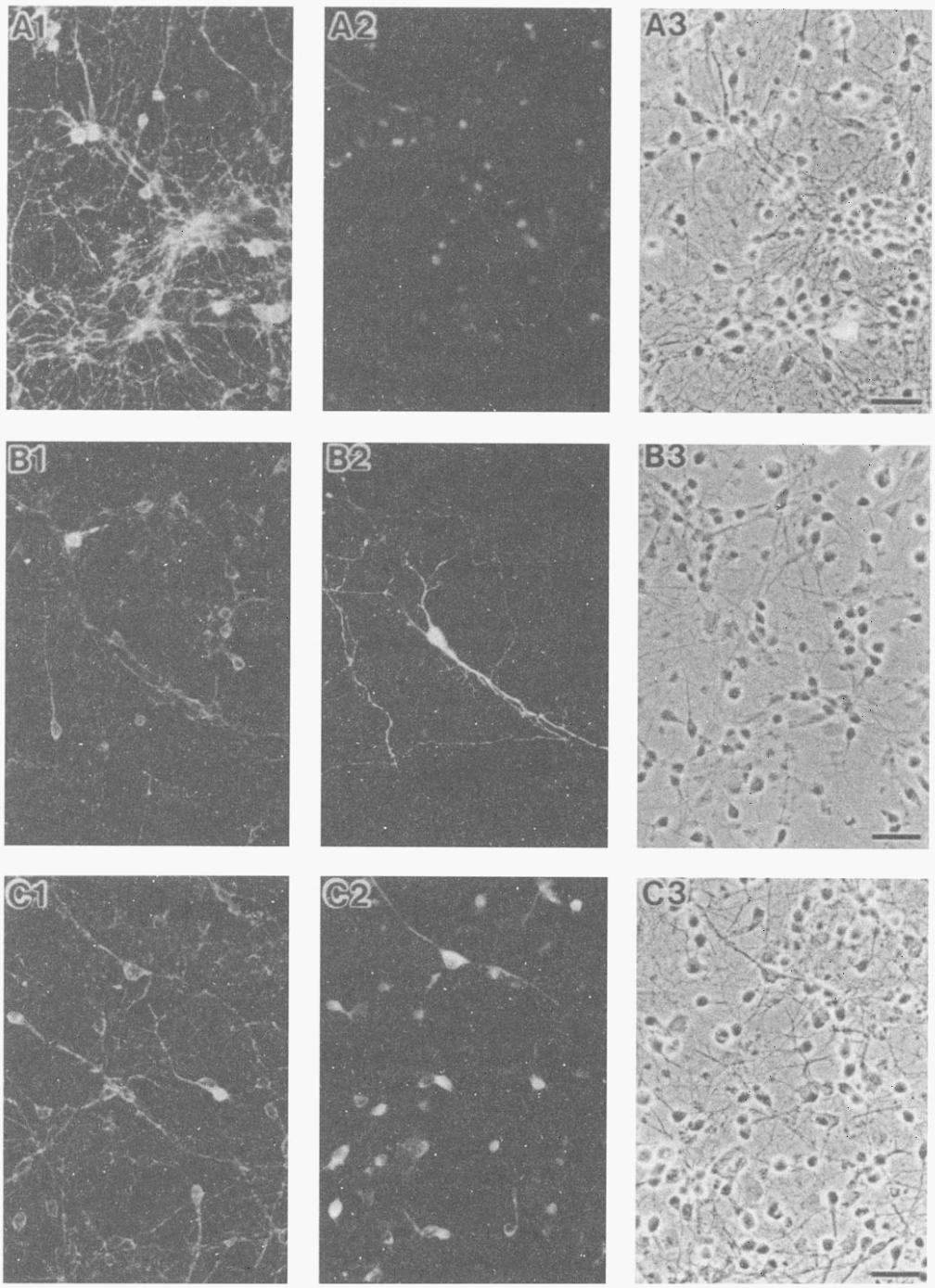

Fig. 3. Indirect immunofluorescence staining of small neurons in granule cell-enriched cultures with MAbs to gangliosides. A: Double-immunostaining with mouse MAb GGR12 (anti-GD1b) (A1) and mouse MAb GMR19 (anti-GD3) (A2), and phase contrast (A3). B: Double-immunostaining with mouse MAb GGR12 (anti-GD1b) (B1) and mouse MAb GMR7 (anti-GD2) (B2), and phase contrast (B3). C: Double-immunostaining with mouse MAb GGR12 (anti-GD1b) (C1) and rabbit anti-NSE polyclonal antibody (C2), and phase contrast (C3). The bar represents 50 μm. (19)

detected in Purkinje cells. The other gangliosides were not detected clearly in these cells. These results suggest that several gangliosides may be useful markers for identifying cells in primary cultures of the rat cerebellum; particularly b-series gangliosides such as GD2 and GD1b for the granule cells and *O*-Ac-LD1 for Purkinje cells (19). The immunocytochemical study of gangliosides in the primary neurons is shown in Fig. 3. Some caution must be used in interpreting the expression of ganglioside antigens based on immunocytochemistry, since a lack of immunorecognition of ganglioside epitope on cells does not necessarily mean that a ganglioside is absent. In fact, several gangliosides such as GM3, GM1, GD1a, GD1b and GT1b were detected in TLC immunostaining of the ganglioside fraction from the granule cell-enriched primary cultures. However, these gangliosides except GD1b were not clearly detected in the granule cells by means of immunofluorescence technique. MAb GMB16 (anti-GM1) reacted intensely with the granule cells after treatment with sialidase, but not with intact cells. Since the affinity of the MAbs to GM1, GD1a, GD1b, and GT1b is similar each other (9, 10), the present results suggest that (i) a major ganglioside accessible to the MAbs in the granule cells may be GD1b; (ii) some gangliosides on the cells in primary cultures may be cryptic. There are indications that a number of factors are involved in influencing the reactivity of MAbs with specific cells: (i) the density of ganglioside on cells is involved in the reactivity of antibodies; (ii) other components of the cell surface may influence antibody reactivity; and (iii) the ceramide portion of gangliosides may be involved in the reactivity (20-22). Further study will be needed for elucidating the precise mechanisms of immunoreactivity, particularly in normal cells, since previous reports were based mainly on the studies of cancer cells. An immunoelectron microscopic study will be necessary to further evaluate the localization of the ganglioside in cells in the rat brain.

MOLECULAR CLONING OF cDNA ENCODING PROTEINS INVOLVED IN GLYCOLIPID EXPRESSION

We isolated a protein cDNA (34A34) that induced GalCer expression and morphological changes in COS cells from a rat brain cDNA library using a eukaryotic cell transient expression system (23). GalCer was detected in the transfected COS cells by means of FACS analysis with anti-GalCer MAb and thin-layer chromatogram. The cDNA insert encoded a polypeptide of 704 amino acid residues with a calculated molecular weight of 78,271 Daltons. The deduced amino acid sequences was shown to have a number of significant characteristics; (i) two proline-rich domains, in which the proline residues amount to 21 % of amino acids in the C terminus, (ii) a zinc-finger domain, composing a number of cystein and histidine residues, (iii) three YXXM motifs. The cDNA hybridized to a single mRNA of 3.1 kb in all rat organs examined. The level of the mRNA was highest in the testis. The COS cells transfected with the cDNA clone showed morphological changes dramatically; the transfected cells appeared to be fibroblast-like cells, whereas parent COS cells was typical epithelial-like cells. The amino acid sequences of the protein cDNA revealed no similarities with UDP-galactose: ceramide; galactosyltransferase, which was recently reported by several groups (24-26). The characterization of the cellular protein is now under investigation.

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