

Binding of laminin to brain gangliosides and inhibition of laminin-neuron interaction by the gangliosides

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Binding of laminin to glycolipids of neuronal membranes was studied with a thin-layer chromatography overlay assay. The major brain ganglioside GD_{1A} was the main binding component, when chromatograms containing the same molar amount of the different brain gangliosides and the brain sulfatide were incubated with laminin at physiological ionic strength. The possible role of laminin binding to brain gangliosides in laminin-neuron interactions was studied with adhesion assays. It was found that binding of rat brain neurons to laminin is blocked by 10–40 μ M brain gangliosides but not by sulfatide. The inhibition by the gangliosides is suggested to be due to competition with the cell surface interaction sites of laminin and not to binding of the gangliosides to the cells. Our findings support the idea that the adhesive and neurite-promoting effect of laminin is dependent on its interaction with gangliosides at the neuronal cell surfaces.

Laminin; Ganglioside; Cell adhesion; Neurite outgrowth; (Brain)

1. INTRODUCTION

Laminin is a high- M_r glycoprotein first characterized as a constituent of basement membranes [2]. It may be involved in the interactions of peripheral neurons with surrounding basal lamina and in those of central neurons with glial cells, which can express laminin [3].

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Abbreviations: BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 0.7 mM CaCl₂, 0.5 mM MgCl₂, pH 7.4); SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TLC, thin-layer chromatography. The shorthand nomenclature of Svennerholm [1] is used for gangliosides

Previous studies have shown that initiation of neurites in neuroblastoma cells on surfaces coated with conditioned media from C6 glioma cells can be blocked by an excess of gangliosides in assay media [4]. It was therefore suggested that cell surface gangliosides are involved in the mechanism of contact-dependent neurite outgrowth [4]. Because the neurite-promoting effect of various conditioned media appears to be mainly due to laminin [5], we decided to study whether the effect of gangliosides could be due to an interaction with laminin. This study shows that laminin binds to neuronal gangliosides, and this binding may be important in cell attachment to and neurite initiation on laminin.

2. EXPERIMENTAL

2.1. Materials

Laminin was purchased from Bethesda Research Laboratories (Neu Isenburg, FRG), or was

purified from mouse sarcoma by gel filtration [2] followed by affinity chromatography on heparin-Sepharose [6]. Anti-laminin was purchased from Bethesda Research Laboratories. The laminin bands of 200 and 400 kDa transferred from SDS-PAGE [7] to nitrocellulose [8] were used for the purification of anti-laminin [9].

Bovine brain sulfatide (galactosylceramide sulfate), heparin and colominic acid were purchased from Sigma (MO, USA). Gangliosides were extracted from pig brain and purified by anion-exchange chromatography [10] followed by chromatography on Iatrobeads 6RS-8060 silica gel

[11]. The concentrations of different gangliosides were measured with a resorcinol method [12].

2.2. Binding of laminin to glycolipids separated on TLC

Glycolipids were chromatographed on aluminum-backed high-performance TLC plates precoated with silica gel 60 (Merck, Darmstadt) in chloroform/methanol/2.5 N ammonia (60:35:8, by vol.). The plates were air-dried, and strips cut from the plates were dipped for 1 min in 0.1% (w/v) polyisobutylmethacrylate (Röhm, Darmstadt) in *n*-hexane [13]. To block non-specific binding sites, the TLC strips were sprayed with 10 mg/ml of BSA in PBS and then incubated in the same buffer for 1 h. The strips were washed twice with PBS, and were then incubated with 10 μ g/ml of laminin in PBS containing 5 mg/ml of BSA (buffer A) for 3 h at room temperature. The

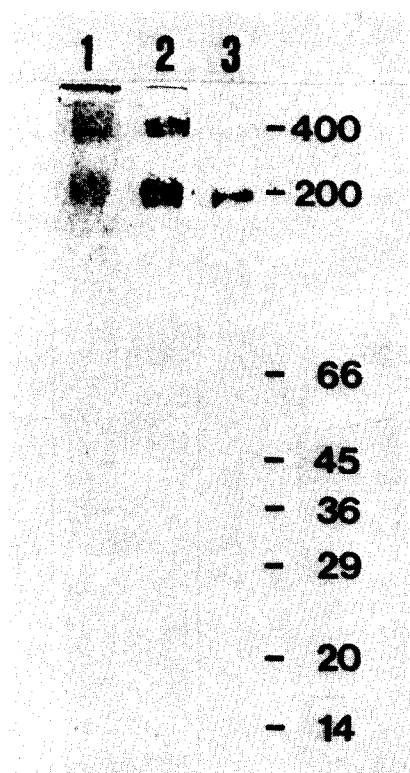


Fig.1. SDS-PAGE and immunoblotting of laminin. Lane 1, 10 μ g laminin on a 5–20% polyacrylamide gel stained with Coomassie blue. Lanes 2 and 3, 3 μ g laminin separated by SDS-PAGE and transferred to nitrocellulose at 100 V for 4 h in a buffer containing 25 mM Tris and 192 mM glycine, pH 8.3. Detection with a commercial antibody (1:1000, Bethesda Research Laboratories) and with an antibody affinity-purified thereof is shown on lanes 2 and 3, respectively. The numbers on the right give the calibration in kDa.

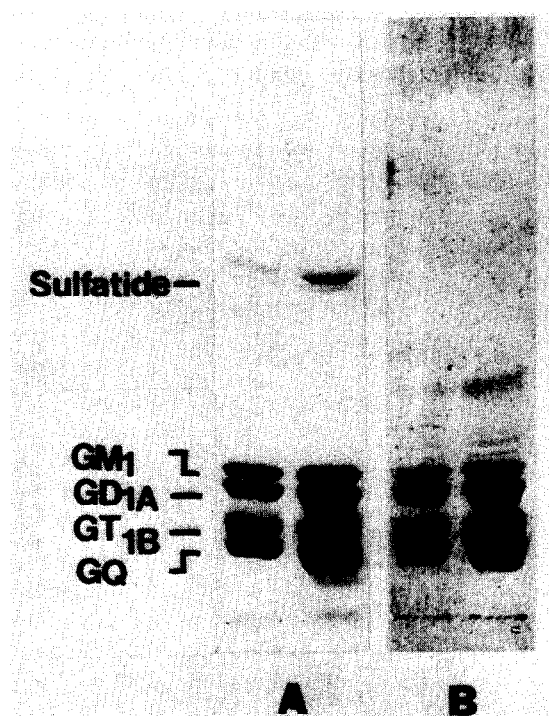


Fig.2. Equimolar mixtures of purified glycolipids analyzed by TLC and laminin overlay. (A) A TLC strip stained for hexose with 0.1% orcinol-3% H_2SO_4 . Left lane, 3 nmol of each glycolipid. Right lane, 6 nmol of each glycolipid. (B) A replica analyzed by laminin overlay (see section 2 for details of the assay).

bound laminin was detected by incubating the strips with the commercial anti-laminin (1:1000 in buffer A for 1 h) followed by peroxidase-conjugated goat anti-rabbit IgG (BioRad, Richmond, USA; 1:3000 in buffer A for 1 h). The strips were washed between the incubations three times with PBS. The peroxidase was detected with 4-chloro-1-naphthol according to the manufacturer's instructions (BioRad).

2.3. Cell attachment and neurite initiation on surfaces coated with laminin

Cells from cerebral hemispheres of 17–19-day-old rat embryos were dispersed and allowed to adhere on poly(L-lysine)-coated tissue culture dishes as in [14]. The cells were used for adhesion assays within 4 days. Fluorescence microscopy of cells stained with monoclonal anti-neurofilament, anti-glial fibrillary acidic protein and anti-vimentin (Labsystems, Helsinki; staining according to the manufacturer's instructions) indicated that 70–80% of the cells used in the experiments were neurofilament-positive neurons. The rest of the

cells were mainly astrocytes staining for glial fibrillary acidic protein. Adhesion assays were carried out with cells metabolically labeled with ^{14}C -amino acids as described for neuroblastoma cells [4]. Laminin (25 $\mu\text{g}/\text{ml}$) was coated for ~20 h at 4°C on polystyrene wells (Greiner; diameter 3.5 cm) that were washed with PBS and coated with 1 mg/ml of BSA for 1 h at room temperature before addition of cells. Cells were incubated in the wells in serum-free DMEM containing 6 mg/ml glucose (0.75×10^6 cells in 1 ml total volume; 15000–30000 cpm) for 1 h in an atmosphere of 5% $\text{CO}_2/95\%$ air at 37°C. To remove non-adherent cells the wells were then rotated on a Bellco shaker at 140 rpm for 5 min followed by three washes with 1 ml assay medium. About 55–60% of cells adhered to surfaces coated with laminin followed by BSA, and 15–20% to surfaces treated with BSA. Specific binding to laminin in different experiments was calculated as the difference of these values. Neurite outgrowth assays with N 18 neuroblastoma cells were carried out according to [4].

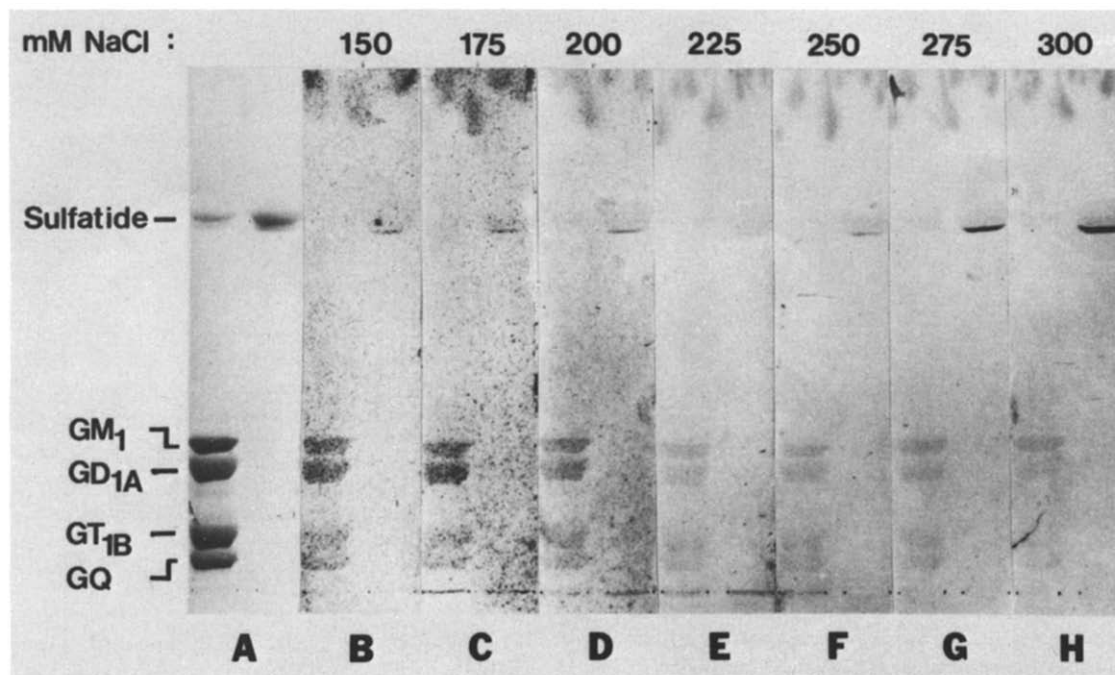


Fig.3. Effect of salt concentration on the binding of laminin to glycolipids. (A) Glycolipids stained for hexose with 0.1% orcinol-3% H_2SO_4 . Left lane, 3 nmol of each glycolipid; right lane, 15 nmol sulfatide. Strips B–H contain the glycolipids as in A; they were analyzed with laminin overlay at increasing concentrations of NaCl.

3. RESULTS

3.1. Laminin binds to brain gangliosides

The purified laminin was composed of the characteristic 200- and 400-kDa polypeptides, as shown on SDS-PAGE stained with Coomassie blue (fig.1, lane 1). Western blotting of the isolated laminin showed that the commercial anti-laminin used detects the 200- and 400-kDa polypeptides (fig.1, lane 2), whereas the affinity-purified antibody only detects the 200-kDa polypeptide (fig.1, lane 3).

An equimolar mixture of the major brain gangliosides and the brain sulfatide (3 and 6 nmol each) was used in a TLC overlay assay to study the binding of laminin to glycolipids. Fig.2A shows a TLC strip stained with orcinol for hexose, and

fig.2B a replica incubated with laminin, which was then detected with anti-laminin followed by a peroxidase-conjugated anti-rabbit IgG as was also used in the Western blotting (fig.1, lane 2). The major binding component in the equimolar mixture was the GD_{1A} ganglioside. Lower amounts of laminin were bound to GM₁, GT_{1B} and GQ_{1B} gangliosides, and little if any binding was observed to the sulfatide under the conditions used (fig.2). Overlay with laminin purified by us (fig.1) or with commercial laminin gave similar results. The results were similar, when either the commercial antibody or the affinity-purified antibody was used to detect laminin bound to the TLC bands.

The binding of laminin to gangliosides was strongly dependent on ionic strength during the overlay. Fig.3 shows that laminin binds to brain

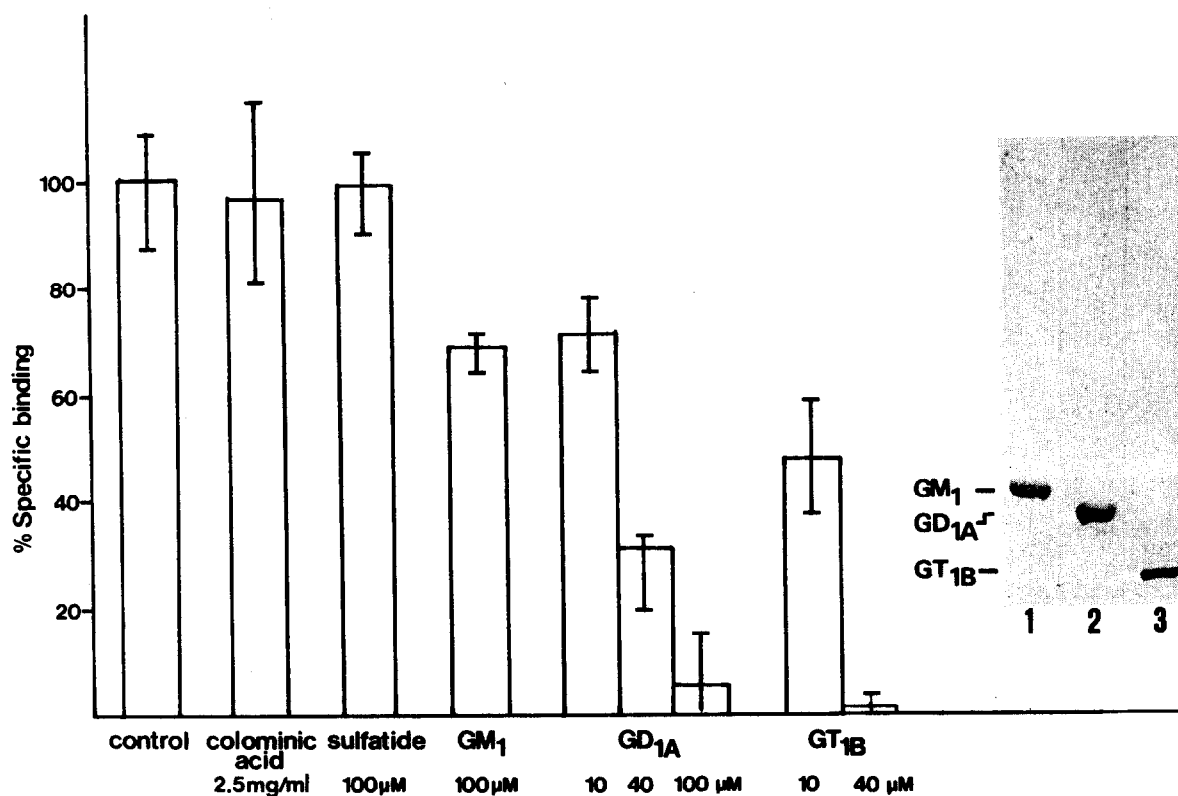


Fig.4. Ganglioside inhibition of binding of rat brain neurons to laminin. The gangliosides and other substances to be tested were dialyzed against the serum-free DMEM used in the adhesion assay. The test substances were added to the wells at 2-times the final concentrations (indicated in the figure), and were allowed to bind to the surfaces for 0.5 h at 37°C. The cells were then added, and the adhesion assays were carried out as described in section 2. The bars indicate the mean \pm the range from triplicate wells. TLC of purified gangliosides used in the assays is shown in the inset; 3 nmol of each ganglioside stained with 0.1% orcinol-3% H₂SO₄.

gangliosides at physiological ionic strength, but little if any binding is observed at 225–300 mM NaCl. In contrast, laminin binds to sulfatide even better at 250–300 mM salt as compared to physiological ionic strength (fig.3).

3.2. Gangliosides block cell attachment and neurite initiation on surfaces coated with laminin

Binding of rat brain cells to laminin-coated surfaces was inhibited when 10–40 μ M GD_{1A} or GT_{1B} ganglioside was added to the assay media (fig.4). The GM₁ ganglioside was a clearly less potent inhibitor, and colominic acid (polysialic acid) or sulfatide did not block cell attachment to laminin (fig.4). When the laminin-coated surfaces were preincubated for 1 h with 100 μ M GD_{1A} ganglioside, which was then washed away before the cells were added, there was only a slight inhibi-

tion in cell attachment (85% of control in specific binding, cf. fig.4). Respective experiments were carried out, in which the cells were incubated with gangliosides, the cells were centrifuged, resuspended and then immediately subjected to binding assays. No inhibition was observed in such experiments, when the cells were preincubated for 1 h with 100 μ M GD_{1A} ganglioside (107% of control, cf. fig.4) suggesting that the inhibition observed is not due to binding of the gangliosides to cells during the assay.

A similar inhibitory effect was observed when initiation of neuritic processes in the rat brain cells or neuroblastoma cells on laminin-coated surfaces was estimated. This is shown in fig.5 for N 18 neuroblastoma cells. Most cells have initiated neuritic processes within the 5 h incubation on laminin (fig.5A). The neurite-promoting effect of laminin is not blocked by a high concentration of

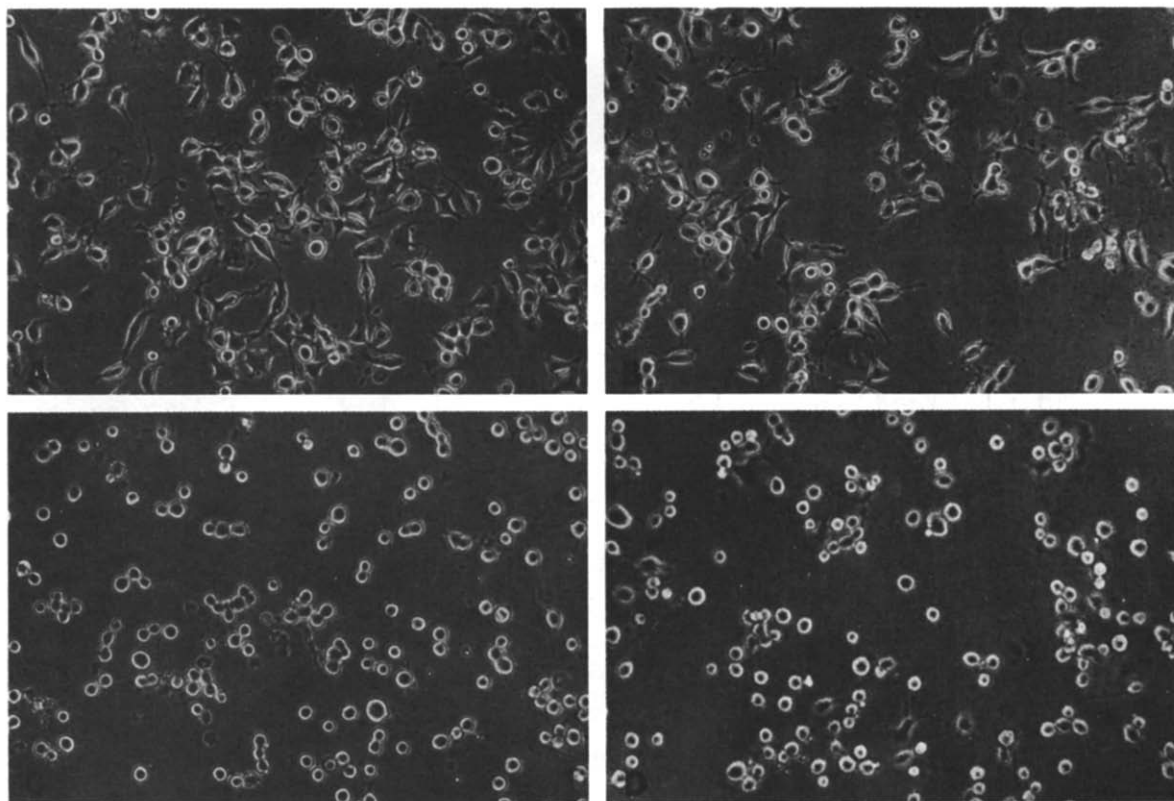


Fig.5. Ganglioside inhibition of neurite initiation in N 18 neuroblastoma cells on laminin in a 5 h assay at 37°C. Surfaces: A, B, and D were coated with laminin (25 μ g/ml); C is a plastic surface. Test substances added to the assay media: A and C, none; B, 5 mg/ml heparin; D, 100 μ M GT_{1B}. Bar, 100 μ m.

heparin (fig.5B) whereas the ganglioside (fig.5D) reduces the extent of neurite initiation to the level seen on plastic (fig.5C).

4. DISCUSSION

Here, we have used a TLC overlay assay to identify neuronal surface structures that might be of importance in laminin-neuron interaction. Laminin bound to the TLC bands has been detected by specific staining with anti-laminin (fig.1), because chemical modification during iodination of laminin tends to reduce its binding to glycolipids (Laitinen, J. and Rauvala, H., unpublished). We have used a physiological salt solution during laminin overlay, and blocked nonspecific binding with about 5000-fold molar excess of BSA. The major binding component under these conditions is the GD_{1A} ganglioside, when different glycolipids are studied at equimolar amounts (fig.2). Binding of laminin to gangliosides is blocked by high salt, whereas laminin appears to bind to sulfatide even better at 250–300 mM NaCl as compared to physiological ionic strength (fig.3).

The possibility that the binding of laminin to brain gangliosides is important in laminin-neuron interaction is suggested by the finding that the adhesive and neurite-promoting effect can be blocked by the GD_{1A} and GT_{1B} gangliosides (figs 4,5), which are major gangliosides of brain and have the same terminal trisaccharide sequences [15]. The concentrations required for the inhibitory effects (10–40 μ M) are reasonably low as compared to the inhibitory concentrations observed in some other adhesion assays [16]. The terminal sialic acid residue is apparently required for the inhibitory effect, because the GM₁ ganglioside has only a slight effect (fig.4). Further experiments are required to determine whether the inhibitory effect on the laminin-neuron interaction is retained by the isolated oligosaccharides or requires clusters of sialic acid-terminating oligosaccharides found in polymeric forms of the GD_{1A} and GT_{1B} gangliosides. Sulfatide, heparin or polysialic acid do not appreciably inhibit the laminin-neuron interaction (figs 4,5). The pattern of and the concentrations required for the inhibitory effects are quite similar as compared to the inhibition of the neurite-promoting effect of the conditioned

medium from C6 glioma cells [4]. The inhibition by gangliosides in both assays can thus be explained by interaction of the gangliosides with laminin, which appears to cause neurite-promoting effects in various conditioned media [5].

Gangliosides have been shown to block the agglutination of glutaraldehyde-fixed erythrocytes by laminin [17]. More recent studies by Roberts et al. [18,19] have, however, indicated that the binding sites of laminin in erythrocytes are provided by sulfatide rather than the gangliosides. Furthermore, pretreatment of erythrocytes by gangliosides followed by washing inhibited laminin-mediated hemagglutination [19]. An indirect effect of gangliosides on the sulfatide-laminin interaction was suggested [19]. In the case of brain neurons we did not observe an indirect effect by pretreatment of the cells with gangliosides. Furthermore, sulfatide did not block brain cell binding to laminin (fig.4). It seems possible that laminin, as a multifunctional protein, has different binding sites in different cells. The differences in TLC overlay assays with laminin are probably due to a higher salt concentration (250 mM ionic strength) used in a previous study [18].

The role of the GD_{1A} and GT_{1B} gangliosides in laminin-neuron interactions appears especially interesting. Studies with a different type of model as compared to the present study have suggested that the GD_{1A} ganglioside is required for neuritic growth in the neuroblastoma cell line GOTO [20]. Interestingly, the content of the GD_{1A} ganglioside in developing brain is strongly increased at the time of extensive neuritic growth [21].

Gangliosides have been previously shown to inhibit adhesive phenomena [16,22–24]. Recent studies by Cheresh et al. [24] have indicated that gangliosides have a general role in cell adhesion in providing the electrostatic environment required in cell binding to various proteins, like collagen, fibronectin and laminin. Whether the ganglioside inhibition in the case of laminin-neuron interaction reflects the general effect of gangliosides on adhesive phenomena or a more specific competition with the cell surface ganglioside receptors is not yet known. At any event, because laminin binds to the gangliosides that are found in high content at the neuronal cell surfaces, it seems reasonable to suggest that the gangliosides or similar carbohydrate chains found in glycoproteins

[25] are involved in the laminin-neuron interactions that initiate neuritic growth.

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