

The L2/HNK-1 Carbohydrate of Neural Cell Adhesion Molecules Is Involved in Cell Interactions

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Abstract. We investigated whether the L2/HNK-1 carbohydrate epitope, expressed by two unusual glycolipids and several neural adhesion molecules, including L1, neural cell adhesion molecule, J1, and the myelin-associated glycoprotein, is involved in adhesion. Monoclonal L2 antibodies, the L2/HNK-1-reactive, sulfate-3-glucuronyl residue carrying glycolipids (L2 glycolipid) and a tetrasaccharide derived from the L2 glycolipid (L2 tetrasaccharide) were added to microexplant cultures of early postnatal mouse cerebellum, and cell migration and process extension were monitored. On the substrate poly-D-lysine, Fab fragments of L2 antibodies, L2 glycolipid, and L2 tetrasaccharide inhibited outgrowth of astrocytic processes and migration of cell bodies, but only L2 glycolipid and L2 tetrasaccharide reduced neurite outgrowth. On laminin, L2 antibodies, L2 glycolipid, and L2 tetrasaccharide inhibited outgrowth of astrocytic processes.

Additionally, L2 glycolipid and L2 tetrasaccharide inhibited cell migration and neurite outgrowth. Several negatively charged glycolipids, lipids, and saccharides were tested for control and found to have no effect on outgrowth patterns, except for sulfatide and heparin, which modified outgrowth patterns in a similar fashion as L2 glycolipid and L2 tetrasaccharide. On astrocytes none of the tested compounds interfered with explant outgrowth. In short-term adhesion assays L2 glycolipid, sulfatide, and heparin inhibited adhesion of neural cells to laminin. L2 glycolipid and sulfatide interfered with neuron to astrocyte and astrocyte to astrocyte adhesion, but not with neuron-neuron adhesion. The most straightforward interpretation of these observations is that the L2/HNK-1 carbohydrate and the sulfated carbohydrates, sulfatide and heparin, act as ligands in cell adhesion.

WE have recently shown that the monoclonal antibody L2 reacts with a common epitope in the carbohydrate moiety of the neural cell adhesion molecules L1 (Schachner et al., 1983, 1985) and N-CAM¹ (Edelman, 1985; Rutishauser and Goridis, 1986), the myelin-associated glycoprotein (MAG) (McGarry et al., 1985; Poltorak et al., 1987), the J1 glycoprotein (Kruse et al., 1985), and other yet unidentified glycoproteins from mouse nervous tissue (Kruse et al., 1984). This group of molecules is also recognized by the monoclonal antibody HNK-1 (Kruse et al., 1984), which reacts with a cell surface antigen of yet unknown functional properties on natural killer cells (Abo and Balch, 1981). We could also show that the population of

N-CAM, L1, and MAG molecules is heterogeneous with respect to the expression of the L2/HNK-1 epitope (Kruse et al., 1984; Poltorak et al., 1987; Faissner, A., manuscript submitted for publication). Sera from patients with gammopathy and peripheral polyneuritis also react with this carbohydrate structure (Braun et al., 1982; Ilyas et al., 1984*b*; Poltorak et al., 1986; Steck et al., 1983). Furthermore, unusual glycolipids from human peripheral nerves and embryonic fetal brain are recognized by the L2/HNK-1 antibodies (Ilyas et al., 1984*a*; Chou et al., 1985, 1986; Noronha et al., 1986; Schwarting et al., 1987). These glycolipids were characterized as sulfate-3-glucuronyl paragloboside and sulfate-3-glucuronyl neolactohexaosyl ceramide (Ariga et al., 1987; Chou et al., 1986). The presence of the sulfate-3-glucuronyl moiety in the lipid was essential for antibody binding (Chou et al., 1986; Ilyas et al., 1986). Indications that the L2/HNK-1 domain was involved in cell interactions came from a study that investigated the effect of Fab fragments of L2 antibodies on neuron-astrocyte and astrocyte-astrocyte adhesion (Keilhauer et al., 1985) or HNK-1 antibodies on neurite outgrowth (Riopelle et al., 1986). However, because antibodies do not only cover the epitope that they are

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1. *Abbreviations used in this paper:* core tetrasaccharide, Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc; L2 glycolipid, sulfate-3-GlcA β 1-3Gal β 1-4Glc-NAc β 1-3Gal β 1-4Glc β 1-1ceramide and sulfate-3-GlcA β 1-3Gal β 1-4GlcNAc β 1-3Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc β 1-1ceramide; L2 tetrasaccharide; sulfate-3-GlcA β 1-3Gal β 1-4GlcNAc β 1-3Gal; LM1 ganglioside, NANA2-3Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc β 1-1ceramide; N-CAM, neural cell adhesion molecule.

directed against, but can also sterically block neighboring domains from function, direct demonstration of the importance of the L2/HNK-1 moiety for cell interactions seemed warranted. We have therefore taken advantage of the possibility to use the L2 glycolipid or the isolated L2 tetrasaccharide of this glycolipid in sensitive culture systems to monitor cell-cell and cell-substrate interactions. Here we show that the L2/HNK-1-reactive carbohydrate moiety, without the attached protein backbone, is able to interfere not only with cell-cell, but also cell-substrate interactions. These observations indicate that the L2/HNK-1 carbohydrate moiety is itself involved as a ligand in cell interactions.

Materials and Methods

Animals

Mice NMRI strain were bred at the animal facilities of the Department of Neurobiology.

Antibodies

Monoclonal L2 antibodies were obtained as described previously (Kruse et al., 1984). L2(336) or L2(412) antibodies were used (Noronha et al., 1986). These antibodies (both IgGs from rat) are designated L2 antibodies by virtue of their reactivity with the L2 glycolipid but may have different affinities and avidities. Fab fragments were prepared by proteolytic digest with papain (Porter, 1959; Rathjen and Schachner, 1984). Rabbit antibodies to glial fibrillary acidic protein from multiple sclerosis plaques (a gift of L. Eng [Stanford University, Stanford, CA]) were used to identify mature astrocytes (Eng et al., 1971; Bignami et al., 1972; Schachner et al., 1977). Guinea pig antibodies to vimentin (a gift of W. W. Franke, German Cancer Research Center, Heidelberg) were used to identify immature and mature astrocytes (Schnitzer et al., 1981). Polyclonal antibodies to the cell adhesion molecule L1 were used to identify neuronal processes (Rathjen and Schachner, 1984). Polyclonal rabbit anti-mouse Thy-1.1 antibodies (a gift of A. Williams [University of Oxford, United Kingdom]) were purified by chromatography on Protein A-Sepharose (a gift of J. Trotter [University of Heidelberg, FRG]). Fluorescein-conjugated, species-specific antibodies were obtained from Cappel Laboratories, Cochranville, PA (via Dynatech, Denkendorf, FRG).

Glycolipids, lipids, saccharides, and liposomes

L2 glycolipid (for structure, see *Abbreviations used in this paper*) was isolated and purified from human sciatic nerve as described previously (Chou et al., 1985, 1986). Desulfated L2 glycolipid was prepared by mild acid hydrolysis (Chou et al., 1985). L2 tetrasaccharide was prepared by reaction of endo- β -galactosidase (*E. freundii*) with L2 glycolipid as described (Chou et al., 1986). Core tetrasaccharide (lacto-neotetraose) was purchased from Biocarb Chemicals (Göteborg, Sweden). LM1 ganglioside was isolated and purified from human sciatic nerve (Chou et al., 1982). Sulfatide (code no. 35692), galactocerebroside (code no. 16474), ganglioside mixture from bovine brain (code no. 22140), ceramide (code no. 16471), phosphatidic acid (code no. 32510), heparin, hyaluronic acid and chondroitin sulfate were purchased from Serva (Heidelberg). Cholesterol was from Fluka (Neu-Ulm, FRG). Phosphatidylcholine (code no. P2772), glucuronic acid, and glucuronic acid-3-phosphate were from Sigma Chemical Co. (Munich). Mannose-2-sulfate, mannose-3-sulfate, and mannose-6-sulfate were gifts of M. Sumper (University of Regensburg, FRG).

For assaying the effects of these reagents in the microexplant culture system, the saccharides were dissolved directly in culture medium (see below). Glycolipids and lipids were suspended by sonication in Earle's basal medium (BME) by first dissolving them in chloroform/methanol (1:1; vol/vol) at a concentration of 1 mg/ml. Aliquots (40 μ g) of this stock solution were dried under a nitrogen stream in sterile glass tubes together with the carrier lipids cholesterol and phosphatidylcholine (200 μ g) at a ratio of 1 to 5 (wt/wt). BME (1 ml) was then added. The mixture was then sonified for 5 min at 4°C in a cup horn device (Branson Sonifier B15, Heinemann, Schwäbisch Gmünd, FRG). In some experiments, glycolipids and lipids were also tested without carrier lipids by suspending and sonifying them in BME as described for the preparation of liposomes with carrier lipids.

Carboxyfluorescein-labeled liposomes were prepared by sonication of

glycolipids in the presence of carrier lipids as described above, but in the presence of 20 mM carboxyfluorescein (Sigma Chemical Co.).

Microexplant Cultures

Cerebella were taken from 6-d-old mice and freed from meninges, choroid plexus, and deep cerebellar nuclei. The remaining tissue was then forced through a Nitrex nylon mesh, pore size 300 μ m, and washed three times in serum-free hormone-supplemented medium (Fischer, 1982) as described previously (Fischer et al., 1986). Explants were plated on glass coverslips (6 or 16 mm diam) coated with poly-D-lysine (10 μ g/ml in water) or laminin from Englebreth-Holm swarm sarcoma (20 μ g/ml in BME; Bethesda Research Laboratories, Karlsruhe, FRG). Coverslips were then placed in 24- or 96-well plates (Nunc, Wiesbaden, FRG) or bacteriologic Petri dishes (3.5 cm diam, three coverslips per Petri dish in 1 ml of culture medium). Coverslips placed in microtiter plates were maintained in 0.5 ml (for 24-well plates) and 100 μ l (for 96-well plates) of culture medium. When explants were maintained on poly-D-lysine-coated coverslips, reagents (antibodies, glycolipids, lipids, or saccharides) were added 16 h after plating. When laminin-coated coverslips or monolayers of astrocytes were used, reagents were added 4 h after plating, because outgrowth of processes and cell bodies was much more rapid than on poly-D-lysine. Explants were maintained in a CO₂ incubator at 35.5°C for 3 d (on poly-D-lysine) and 1-2 d (on laminin and astrocytes) without change of culture medium.

Astrocyte monolayers were prepared as described previously (Fischer et al., 1982). In short, single-cell suspensions of 2-d-old mouse cerebellum were cultured in a chemically defined medium to select for epidermal growth factor-sensitive astrocyte precursors. After 2 wk the cells were subcultured to obtain homogeneous cultures of these astrocyte precursors. 2 d later the chemically defined medium was replaced by medium containing 10% horse serum to induce differentiation of the cells with respect to expression of glial-fibrillary acidic protein. 7 d later the medium with 10% horse serum was replaced by the chemically defined medium used for culture of neurons (Fischer, 1982). 1 d later the microexplants were added. The cultures were stained for L1 antigen 1 d later to visualize outgrowth of neuronal processes.

Immunocytologic Procedures

Immunofluorescence staining of microexplant cultures for glial fibrillary acidic protein, vimentin, L2/HNK-1 carbohydrate epitope, or L1 antigen was carried out by indirect labeling procedures as described previously (Fischer et al., 1986; Kruse et al., 1985; Rathjen and Schachner, 1984; Schnitzer and Schachner, 1981; Schnitzer et al., 1981).

Cell Adhesion Assay

Adhesion of single-cell suspensions of enriched populations of neurons and astrocytes from 6-d-old mouse cerebellum to monolayer cultures of these enriched populations was carried out as described previously (Keilhauer et al., 1985). In adhesion assays with laminin as substrate, single-cell suspensions without previous enrichment for different cell types were used. Single-cell suspensions were labeled by uptake of fluoresceindiacetate (Keilhauer et al., 1985) and used as probe cells. Their adhesion to the monolayer target cells in 30 min at room temperature was quantified by fluorescence microscope examination. Glycolipids were added to the probe and target cells for 20 min on ice before the adhesion assay. Laminin-coated glass coverslips (prepared as described for microexplant cultures) were preincubated with lipids (40 μ g/ml) or heparin (100 μ g/ml) for 2 h at 35°C in the CO₂ incubator before addition of the cell suspension. Final concentrations of lipids and heparin were 20 and 50 μ g/ml, respectively. Adhesion of cells was quantified after 1 h at room temperature using the same washing protocols as for monolayer target cells.

Results

Influence of L2 Monoclonal Antibodies, L2 Glycolipid, and L2 Tetrasaccharide on Outgrowth Patterns in Microexplant Cultures on Poly-D-Lysine

When microexplants of early postnatal mouse cerebellum were plated onto poly-D-lysine-coated glass coverslips, a characteristic outgrowth pattern could be observed during 3 d in culture (Fischer et al., 1986). Microexplants attached to

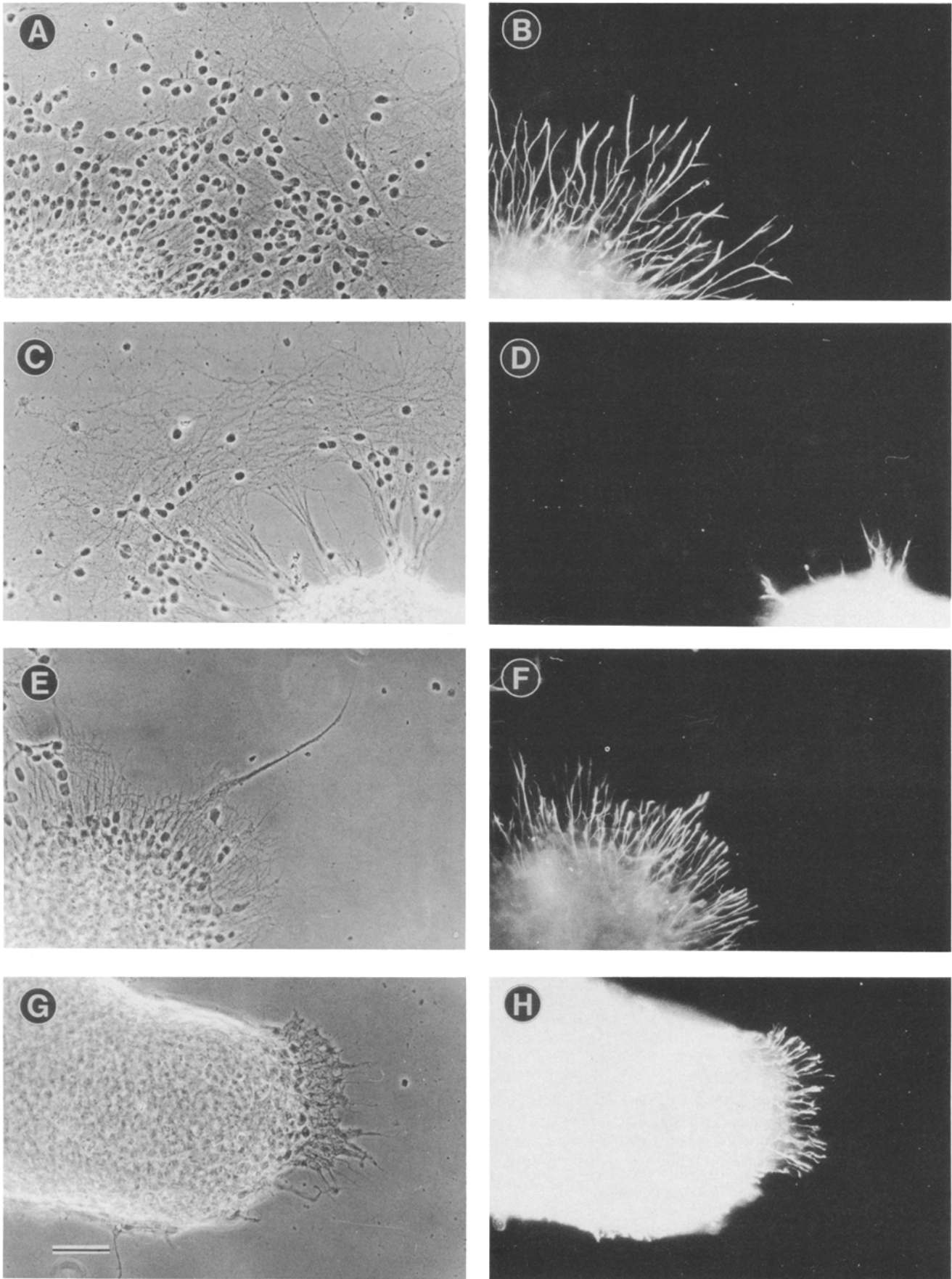


Figure 1. Cerebellar microexplants were cultured on poly-D-lysine for 3 d (*A, B*) without or (*C, D*) with Fab fragments of monoclonal L2 (412) antibodies (0.5 mg/ml) or with L2 glycolipid at (*E, F*) 2 µg/ml or (*G, H*) 5 µg/ml. Antibodies or lipids were added to the medium 16 h after plating. (*B, D, F, H*) Indirect immunofluorescence staining for glial-fibrillary acidic protein and (*A, C, E, G*) corresponding phase-contrast micrographs. (*E, F*) In the left upper corner the outgrowth zone of an adjacent explant is visible. Note the increasing inhibition of outgrowth of cellular processes with increasing concentrations of L2 glycolipid. Bar, 50 µm.

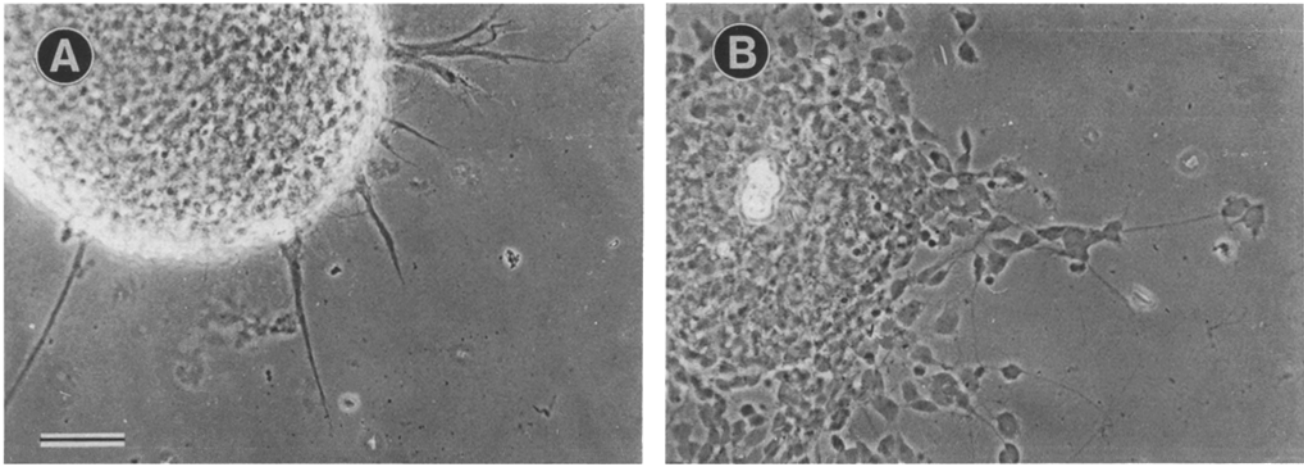


Figure 2. Phase-contrast micrographs of cerebellar microexplants cultured on (A) poly-D-lysine or (B) laminin in the presence of L2 tetrasaccharide (50 $\mu\text{g/ml}$). The tetrasaccharide was added (A) 16 or (B) 4 h after plating. Explants were fixed (A) 3 or (B) 2 d after plating. Note the strong inhibition of migration of cell bodies which is more pronounced on poly-D-lysine and outgrowth of cell processes which appears more pronounced on laminin. Control for B is shown in Fig. 3. Bar, 50 μm .

the substrate within a few hours. Tetanus toxin and L1 antigen-positive neurites were the first to leave the explant. They could be readily discerned already after 1 d of culture and reached a length of several explant core diameters after 3–4 d. In this time period astrocytic processes extended from the explant core in a somewhat radial fashion to a maximal distance of approximately one core diameter (Fig. 1). Cell bodies of astrocytes were sometimes also observed to leave the explant, but did not reach the outer limits of neurite outgrowth. Fasciculation of neurites was detectable in the astrocyte-free periphery. Movement of small, tetanus toxin receptor-positive neuronal cell bodies from the explant also occurred. Neuronal cell bodies started their movement at the time of astrocyte outgrowth, but could be shown to migrate on an astrocyte-free substrate in $\sim 50\%$ of all explants (Fischer et al., 1986). The maximal distance reached by neuronal cell bodies was attained within 3–4 d after plating, by which time the extent of the outgrowth zone of neuronal cell bodies corresponded roughly to the area covered by astrocytic processes. Neurites and astrocytes were stained with L2 antibodies by indirect immunofluorescence (not shown).

When Fab fragments of monoclonal L2 antibody (336 or 412) were added to the explants 16 h after plating, cultures were modified in their outgrowth patterns at concentrations of 500 and 700 $\mu\text{g/ml}$. 3 d after explant plating (~ 2.3 d after addition of antibody) the following pattern was observed by phase-contrast microscopy and immunofluorescence staining with antibodies to L1 (not shown) and glial fibrillary acidic protein (Fig. 1): only few explants (~ 10 – 20%) remained attached to the substrate. Explants that remained attached extended neurites which were strongly fasciculated in the vicinity of the explant core. Astrocytic processes or neuronal cell bodies rarely left the explant core. Cells appeared healthy in the presence of antibodies throughout culture times. When detached explants were removed from the cultures 2 d after antibody addition, washed, and replated, the outgrowth pattern was essentially normal, indicating that antibodies did not reduce cell viability.

When Fab fragments of monoclonal L2 antibodies were added at concentrations of 300 $\mu\text{g/ml}$, more explants re-

mained attached to the substrate (~ 60 – 70%) and fasciculation of neurites and movement of astrocytic processes and neuronal cell bodies from the explant core was retarded with respect to the control (Fig. 1, A and B), but not as much as at concentrations of 500 $\mu\text{g/ml}$ (Fig. 1, C and D) and higher (not shown). For control, the effect of Fab fragments of different antibodies on the outgrowth patterns was monitored: L1 and N-CAM antibodies reduced the extent of neurite fasciculation (Fischer et al., 1986), whereas polyclonal rabbit antibodies to mouse Thy-1 antigen did not interfere with the outgrowth pattern (not shown).

The effects of the L2 glycolipid and the L2 tetrasaccharide on the outgrowth pattern of explants were generally comparable to each other and different from the effect observed with monoclonal L2 antibodies. When either L2 glycolipid or L2 tetrasaccharide were added to the cultures 16 h after plating at concentrations of 2–5 $\mu\text{g/ml}$ (1.3–6.5 nmol/ml; Fig. 1, E and F and G and H) and 50–200 $\mu\text{g/ml}$ (62–248 nmol/ml; Fig. 2 A), respectively, ~ 40 – 50% of the explants detached. Neurites generally extended only as short, stubby fascicles away from the explant core especially at higher concentrations of the additives (Fig. 1, E–H, and 2 A). Migration of neuronal cell bodies and outgrowth of astrocytic processes from the explant core was also strongly inhibited (Figs. 1 and 2). The same results were obtained, when the L2 glycolipid was used without the carrier lipids cholesterol and phosphatidylcholine (not shown).

To test the specificity of the effect of L2 glycolipid and L2 tetrasaccharide other glycolipids and saccharides were tested (Table I). No effect on outgrowth pattern was observed, when desulfated or desulfated and carboxymethylated L2 glycolipid, LM1 ganglioside (differing from the L2 glycolipid by the replacement of sulfated glucuronic acid by *N*-acetylneuraminic acid), GM1 or GD1a ganglioside, a ganglioside mixture, globoside, paragloboside, ceramide, ceramide trihexoside, phosphatidic acid, or the mixture of the carrier lipids cholesterol and phosphatidylcholine were added to the cultures. Interestingly, sulfatide, but not its desulfated analogue galactocerebroside, reduced attachment of explants and modified the outgrowth pattern in a similar manner as the L2

Table I. Concentrations of Monoclonal L2 Antibody, Glycolipids, Lipids, Saccharides, and Glycosaminoglycans That Modify or Do Not Modify the Outgrowth Pattern of Cerebellar Explant Cultures on Poly-D-lysine-coated Glass Coverslips

Reagent	Modification		No Modification	
	$\mu\text{g/ml}$	μm	$\mu\text{g/ml}$	μm
L2 antibody (Fab fragments)	≥ 500			
L2 glycolipid	≥ 2	1.3		
Desulfated L2 glycolipid			≤ 20	13
Desulfated and carboxymethylated L2 glycolipid			≤ 10	6.7
LM1 ganglioside			≤ 20	12
GM1 ganglioside			≤ 20	10
GD1a ganglioside			≤ 10	5
Ganglioside mixture			≤ 20	10
Paragloboside			≤ 10	7
Globoside			≤ 10	7
Sulfatide	≥ 5	5.8		
Galactocerebroside			≤ 20	25
Ceramide			≤ 20	33
Ceramide trihexoside			≤ 10	9
Phosphatidic acid			≤ 20	26
Cholesterol/phosphatidylcholine mixture			≤ 100	65/100
L2 tetrasaccharide	≥ 50	62		
Core tetrasaccharide			≤ 200	260
Glucuronic acid			≤ 500	2,580
Glucuronic acid-3-phosphate			≤ 500	1,840
Mannose-2-sulfate			≤ 500	1,900
Mannose-3-sulfate			≤ 500	1,900
Mannose-6-sulfate			≤ 500	1,900
Heparin	≥ 0.1			
Hyaluronic acid			≤ 50	
Chondroitin sulfate			≤ 50	

Reagents were added to the culture medium 16 h after plating the explants. 1–3 d later patterns of neurite outgrowth and migration of neuronal and astrocytic cell bodies were monitored using morphologic criteria (Fischer et al., 1986). The observed morphologic modifications are described in Results. The category "no modification" was used when outgrowth patterns were not significantly different from control cultures without added reagents. See *Abbreviations used in this paper* for structure of L2 glycolipid, LM1 gangliosides, L2 tetrasaccharide, and core tetrasaccharide. 2 μg of L2 glycolipid and 50 μg of L2 tetrasaccharide correspond to 1.3 and 62 nmol, respectively. Molarities for glycosaminoglycans are not indicated, because exact molecular weights and degree of sulfation are not known.

carbohydrate-containing compounds. Also, heparin, but not hyaluronic acid or chondroitin sulfate, detached microexplants when added at concentrations of 1 $\mu\text{g/ml}$ 16 h after explant plating. At a concentration of 0.1 $\mu\text{g/ml}$ heparin reduced outgrowth of cellular processes and migration of cell bodies and promoted fasciculation of neurites (not shown). The saccharides glucuronic acid, glucuronic acid-3-phosphate, mannose-2-sulfate, mannose-3-sulfate, mannose-6-sulfate, or core tetrasaccharide (the L2 carbohydrate chain without sulfated glucuronic acid) did not modify the outgrowth pattern even at high concentrations (Table I).

To investigate whether sulfated glycolipids bind specifically to the cells whose behavior was modified in their presence, carboxyfluorescein-labeled liposomes containing sulfatide were incubated with cultures for 2 h at room temperature. No specific binding to neurons or astrocytes was seen when compared with the negatively charged gangliosides LM1 or GM1 or galactocerebroside.

Influence of Monoclonal L2 Antibodies, L2 Glycolipid and L2 Tetrasaccharide on Outgrowth Patterns in Microexplant Cultures on Laminin

When laminin was used as a substrate for cerebellar microexplants, an outgrowth pattern different from the one on

poly-D-lysine was observed. Outgrowth of neurites was detectable already 2–3 h after plating. Outgrowth of astrocytic processes and migration of neuronal and astrocytic cell bodies was also enhanced in time and space, i.e., neurons and astrocytes were often observed even at the periphery of the outgrowth zone of neurites. Neurite fasciculation was reduced, when compared with poly-D-lysine as substrate. Because of the speedier explant outgrowth on laminin, cultures were monitored after 2 d *in vitro* (Fig. 3).

When Fab fragments of monoclonal L2 antibody (336 or 412) were added to the cultures 4 h after plating, neuronal migration and extension of neurites were only slightly affected, whereas outgrowth of astrocytic processes and cell bodies was inhibited at concentrations of 500 $\mu\text{g/ml}$ (Fig. 3, A and B and C and D). Fasciculation of neurites was slightly enhanced over control cultures.

Addition of L2 glycolipid (5–10 $\mu\text{g/ml}$; 6.5–13 nmol/ml) 4 h after plating the explants reduced migration of neuronal and astrocytic cell bodies and outgrowth of neurites and, to a smaller extent, astrocytic processes (Fig. 3, E and F). As on poly-D-lysine only short, stubby fascicles of neurites were observed. In contrast to the effects of L2 glycolipid on poly-D-lysine explants hardly detached from laminin.

Addition of L2 tetrasaccharide (200 $\mu\text{g/ml}$; 248 nmol/ml)

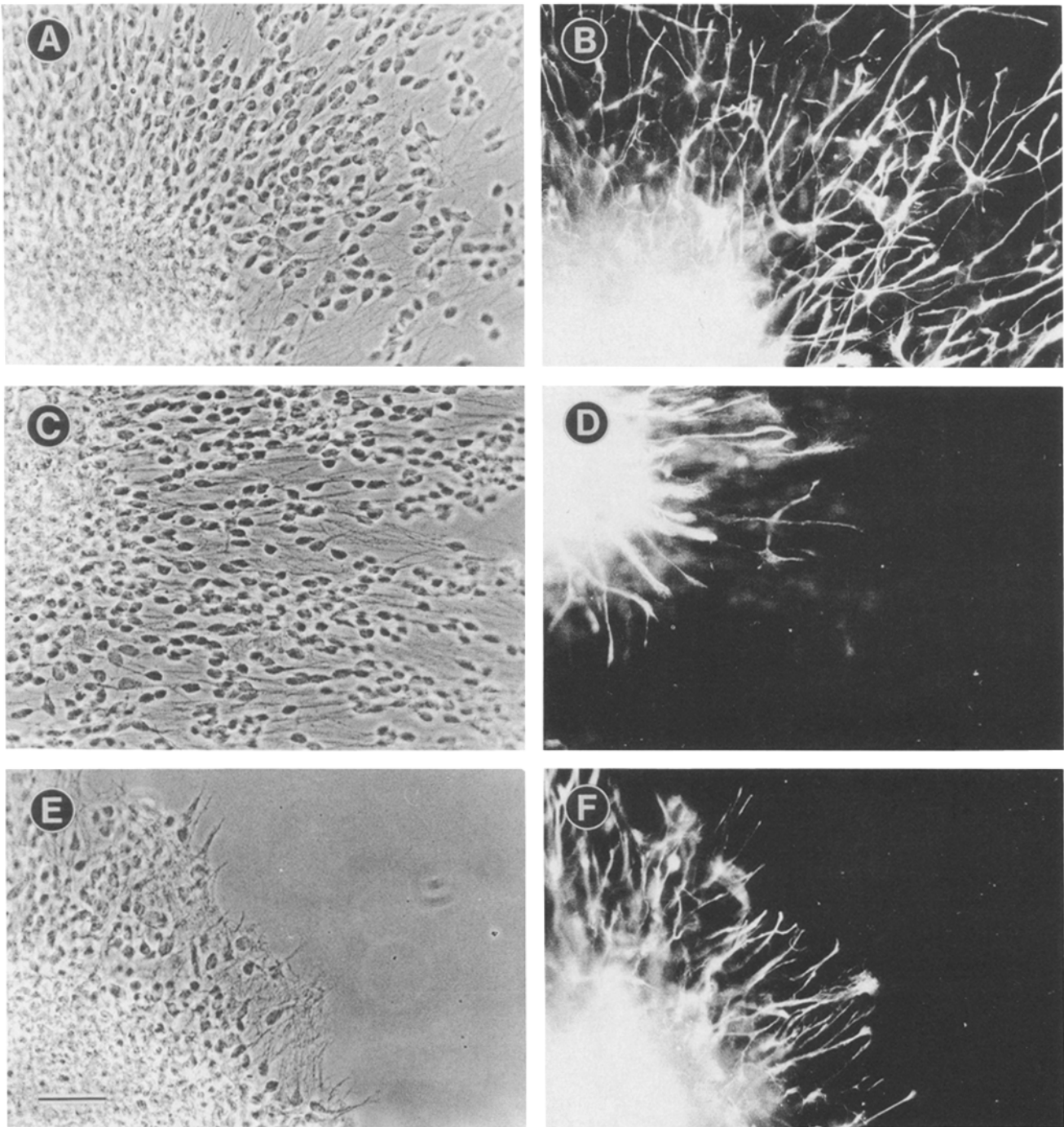


Figure 3. Cerebellar microexplants were cultured on laminin for 2 d (*A, B*) without or (*C, D*) with Fab fragments of monoclonal L2 (412) antibodies (0.5 mg/ml) or (*E, F*) with L2 glycolipid (10 $\mu\text{g/ml}$). Antibodies or lipids were added to the medium 4 h after plating. (*B, D, F*) Indirect immunofluorescence labeling for glial-fibrillary acidic protein and (*A, C, E*) corresponding phase-contrast micrographs. Note the inhibition of outgrowth of astrocytic processes by L2 antibodies and inhibition of outgrowth of cellular processes and migration of neurons by L2 glycolipid. Bar, 50 μm .

4 h after plating reduced outgrowth of neurites, astrocytic processes and migration of neural cell bodies from the explants (Fig. 2 *B*). Most explants remained on the substrate. At 50 $\mu\text{g/ml}$ inhibition of outgrowth was less than that at 200 $\mu\text{g/ml}$, but migration of neural cell bodies and outgrowth of astrocytic processes and neurites was still considerably reduced over control cultures.

Sulfatide (10 $\mu\text{g/ml}$) inhibited neurite outgrowth and cell

migration drastically (Fig. 4 *A*). The ganglioside mixture (20 $\mu\text{g/ml}$) and galactocerebroside (20 $\mu\text{g/ml}$) did not influence the outgrowth pattern. When heparin (1 $\mu\text{g/ml}$) was added to the cultures 4 h after plating, explants remained attached to the substrate, but showed slightly decreased outgrowth of neurites and astrocytic processes and reduced migration of neuronal cell bodies. These effects became more prominent with increasing concentrations of heparin (see Fig. 4 *B* with

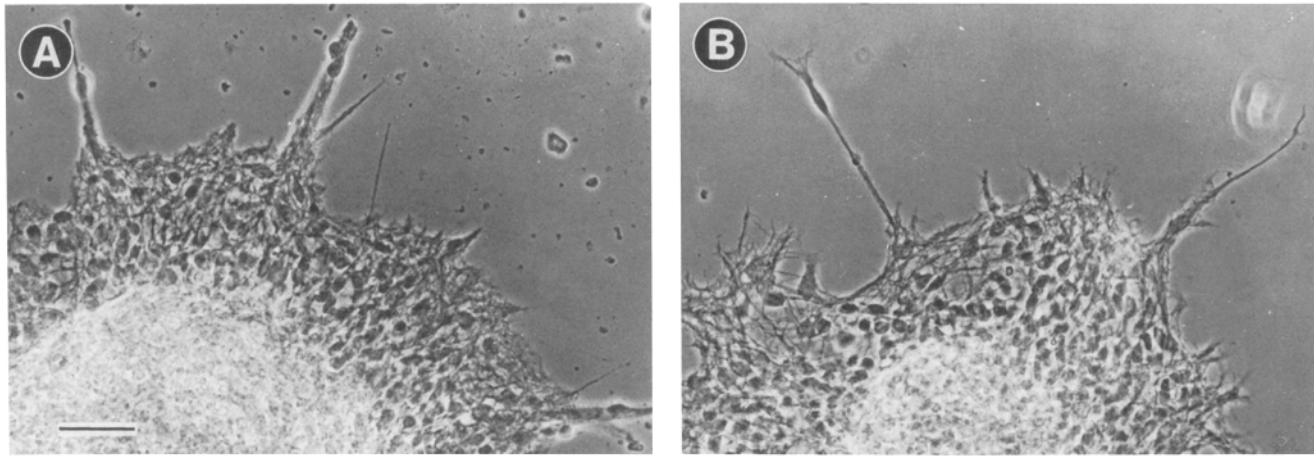


Figure 4. Cerebellar microexplants were cultured on laminin for 2 d in the presence of (A) sulfatide (10 µg/ml) or (B) heparin (30 µg/ml) added 4 h after plating. Inhibition of neurite outgrowth is similar to the one observed with L2 glycolipid (see Fig. 3, E and F). Bar, 50 µm.

30 µg/ml). Hyaluronic acid or chondroitin sulfate (50 µg/ml) did not modify the outgrowth pattern (not shown).

Influence of L2 Glycolipid on Outgrowth Patterns in Microexplant Cultures on Astrocyte Monolayers

When cerebellar microexplants were cultured on astrocyte monolayers neurites extended for several hundred micrometers within 1 d. As on laminin, neurites fasciculated only slightly (Fig. 5, A and B). In the presence of L2 glycolipid (10 µg/ml; Fig. 5, C and D), sulfatide (10 µg/ml; Fig. 5, E and F) or heparin (30 µg/ml; Fig. 5, G and H) the outgrowth pattern was not significantly altered. It is unlikely that astrocytes metabolize the added compounds below their active concentrations.

Influence of L2 Glycolipid and Sulfatide on Neural Cell Adhesion

To investigate more directly whether the L2/HNK-1 carbohydrate epitope is involved in cell-cell interactions, enriched

populations of neurons and astrocytes from early postnatal mouse cerebellum were monitored in a short-term adhesion assay (Table II). Adhesion between enriched populations of small neurons and astrocytes from early postnatal mouse cerebellum was assayed in the presence and absence of L2 glycolipid and sulfatide using the ganglioside mixture and galactocerebroside as controls. The proportion of L2-positive cells was ~60% in single-cell suspensions and monolayer cultures of both neurons and astrocytes when stained with L2 (336) antibodies (Keilhauer et al., 1985), but >90% when stained with L2 (412) antibodies. Immunofluorescence staining with L2 (412) antibodies was also more intense. No inhibition of neuron-neuron adhesion was detectable in the presence of L2 glycolipid or sulfatide. However, L2 glycolipid and sulfatide decreased adhesion of neurons to astrocytes, astrocytes to neurons, and astrocytes to astrocytes. Inhibition of only 10% was observed when concentrations of glycolipids were 10 µg/ml. At 100 µg/ml sulfatide inhibited adhesion of neurons to astrocytes and also, reciprocally, astrocytes to neurons by ~30%. Adhesion of astrocytes to as-

Table II. Inhibition of Adhesion between Neurons and Astrocytes in the Presence of L2 Glycolipid and Sulfatide

Lipid	Concentration µg/ml	Inhibition of adhesion			
		Neuron* to neuron‡	Neuron* to astrocyte‡	Astrocyte* to neuron‡	Astrocyte* to astrocyte‡
		%	%	%	%
None		0 ± 4	0 ± 3	0 ± 4	0 ± 4
L2 glycolipid	10	-2 ± 4	10 ± 1	8 ± 1	9 ± 1
	100§	0 ± 2	16 ± 3	17 ± 3	17 ± 3
Sulfatide	10	2 ± 3	12 ± 3	14 ± 1	10 ± 3
	100	5 ± 3	27 ± 5	27 ± 3	18 ± 7
Galactocerebroside	10	0 ± 3	-5 ± 4	0 ± 2	-2 ± 2
	100	0 ± 2	-4 ± 3	-1 ± 2	-3 ± 4
Ganglioside mixture	10	1 ± 3	0 ± 1	1 ± 2	-1 ± 3
	100	1 ± 2	1 ± 4	2 ± 3	0 ± 5

Fluorescein diacetate-labeled single-cell suspensions of enriched populations of small neurons or astrocytes from early postnatal mouse cerebellum were used as probe cells (*) to adhere to monolayer cultures (‡) of these cell populations. Percent inhibition in the presence of glycolipids was calculated by: % inhibition = ([adhesion (control)] - adhesion (+glycolipid)/adhesion [control]) × 100. Numbers are mean values of three (or two§) experiments ± standard deviation. Each experimental value was run in quadruplicate. The inhibition by L2 glycolipid and sulfatide in neuron-astrocyte, astrocyte-astrocyte, and astrocyte-neuron adhesion, is significantly different from the other values ($P < 0.001$, Student's *t* test).

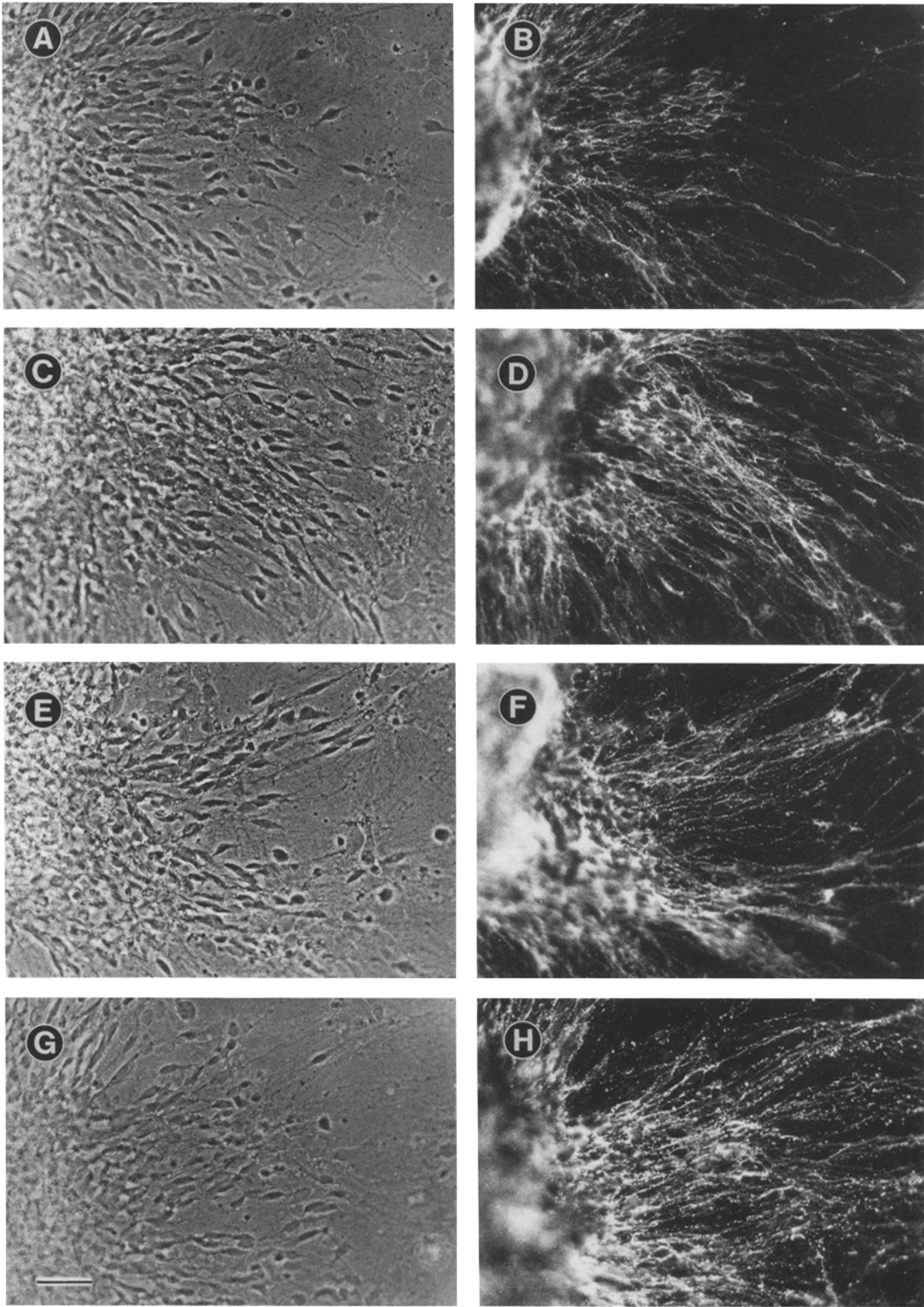


Figure 5. Cerebellar microexplants were cultured on astrocyte monolayers for 1 d (*A, B*) without or with addition of (*C, D*) L2 glycolipid (10 $\mu\text{g/ml}$), (*E, F*) sulfatide (10 $\mu\text{g/ml}$), or (*G, H*) heparin (30 $\mu\text{g/ml}$). (*B, D, F, H*) Indirect immunofluorescence labeling for L1 antigen and (*A, C, E, G*) corresponding phase-contrast micrographs are shown. The additives did not inhibit outgrowth of neurites. Bar, 50 μm .

trocytes was reduced by ~20%. Inhibition of adhesion by L2 glycolipid at 100 µg/ml was almost 20% for neuron-astrocyte, astrocyte-neuron, and astrocyte-astrocyte adhesion. These inhibition values are remarkably similar to those observed with L2 antibodies (Keilhauer et al., 1985). L2 tetrasaccharide or higher concentrations of L2 glycolipid could not be used in this assay because of the limited amounts of material available.

Influence of L2 Glycolipid, Sulfatide, and Heparin on Neural Cell Adhesion on Laminin

Single-cell suspensions from 6-d-old mouse cerebellum were used as probe cells to investigate neural cell adhesion on laminin (Table III). >90% of the cells were stained by indirect immunofluorescence with L2 (412) antibodies. Laminin-coated glass coverslips were preincubated with L2 glycolipid (40 µg/ml), sulfatide (40 µg/ml), or heparin (100 µg/ml) before the addition of suspended cells which reduced the final concentration of additives by a factor of two. A significant decrease in cell adhesion could be observed in the presence of these additives ranging from 40% to 70%, whereas galactocerebroside influenced cell adhesion only slightly. In the cell suspensions used, vimentin-positive immature and mature astrocytes amounted to ~10% within the total cell population. To investigate whether the additives blocked adhesion of astrocytes cells attached to coverslips were stained for vimentin. The percentage of vimentin-positive cells was similar in control and glycolipid- or heparin-treated cultures.

Discussion

In this study we could show that the L2/HNK-1 epitope is involved in cell-cell and cell-substrate interactions. The evidence comes from observations in two culture systems designed to probe sensitively for cell contacts, monitoring outgrowth of neurons and astrocytes from tissue explants on different substrates and adhesion either between neural cells or between cells and laminin. The fact that not only antibodies reactive with this epitope, but also the isolated L2/HNK-1 epitope carrying glycolipid and tetrasaccharide interfere with cell-cell and cell-substrate interactions in these culture systems, indicates that the carbohydrate itself without adjacent protein backbone domains subserves a functional role. The most straightforward way to interpret the present data is to envisage a competitive binding of the membrane- or substrate-associated L2/HNK-1 carbohydrate and the exogenously added carbohydrates to the L2/HNK-1 binding site or "receptor." The present findings thus support and focus a previous postulate that all L2/HNK-1 reactive epitope carrying glycoproteins are indeed involved in adhesion (Kruse et al., 1984, 1985).

Several carbohydrate structures were tested to gain information about the structural requirements underlying the L2/HNK-1 dependent adhesion. Of all compounds tested, only sulfatide and heparin interfere with cell interactions in a similar manner as the L2 carbohydrate-containing compounds. Both compounds are structurally related to the L2/HNK-1 carbohydrate in that they contain sulfate groups at the 2' or 3' hydroxyl groups of hexose sugars. The sulfate group appears to be the decisive factor in ligand activity, in that removal of sulfate from the L2 glycolipid or sulfatide

Table III. Inhibition of Adhesion of Cerebellar Cells to Laminin in the Presence of L2 Glycolipid, Sulfatide, Galactocerebroside, and Heparin

Reagent	Concentration µg/ml	Molar ratio µM	Inhibition of Adhesion	
			a	b
			%	%
None	—	—	0 ± 21	0 ± 13
L2 glycolipid	20	13	52 ± 10	64 ± 6
Sulfatide	20	23	40 ± 13	61 ± 11
Galactocerebroside	20	25	3 ± 16	29 ± 19
Heparin	10		64 ± 12	68 ± 8

Fluorescein diacetate-labeled single-cell suspensions were incubated with laminin-coated glass coverslips. Percent inhibition was calculated as described in legend to Table II. Columns *a* and *b* contain mean values from two independent experiments ± standard deviation. For each experiment eight microscopic fields (selected randomly) on each of two duplicate coverslips were scored. A minimum of 500 cells was counted per experiment.

leads to complete abolishment of the inhibitory activity. It should be emphasized that negative charges per se are not effective ligands, and not all sulfated compounds tested show inhibitory activity. It remains to be seen whether the L2/HNK-1 carbohydrate-containing compounds, sulfatide and heparin, exert their effects via different or similar molecular mechanisms.

It is noteworthy that in the explant culture system the isolated L2 glycolipid and tetrasaccharide were more potent in modifying the pattern of neurite outgrowth on poly-D-lysine or laminin than the monoclonal L2 antibodies. The fact that the L2 tetrasaccharide is at least 50 times less efficient on a molar basis than the L2 glycolipid in interfering with cell interactions is not unexpected, because monomers have been found to show a lower affinity to their binding sites than multimers (see, e.g., Yamada et al., 1981) which, in our case, would be represented by the lipid micelles containing the L2 glycolipid. Furthermore, the extent of inhibition of migration of neurons and astrocytes and outgrowth of cellular processes depended on the substrate: Inhibition tended to be less pronounced on laminin than on polylysine, but was not detected on astrocytes. This implies, that added L2 glycolipid and tetrasaccharide (as well as sulfatide and heparin) do not prevent neurons to extend neurites by interfering with cellular metabolism, but rather interfere with adhesion itself. The fact that adhesion of neural cells to laminin is inhibited to a greater extent by L2 glycolipid at a given concentration than adhesion between neurons and astrocytes or among astrocytes themselves suggests that the L2/HNK-1 carbohydrate epitope is more prominent in cell to substrate than in cell-cell interactions that may be governed by multiple adhesion mechanisms.

Some of our observations indicate that the L2/HNK-1 carbohydrate epitope may be involved in binding to laminin. Laminin is a natural constituent of extracellular matrix and superior to polylysine in promoting neurite outgrowth and migration of cell bodies (Baron van Evercooren et al., 1982; Manthorpe et al., 1983; Rogers et al., 1983). Laminin can bind specifically to sulfated glycolipids (Roberts et al., 1985, 1986), and this binding can be inhibited by heparin but not chondroitin sulfate or hyaluronic acid (Roberts et al., 1986). Besides the sulfatide-binding site laminin has an additional, high-affinity heparin-binding site (Roberts et al., 1985). This

heparin-binding domain is involved in promoting neurite outgrowth (Edgar et al., 1984). It is possible that the L2/HNK-1 carbohydrate epitope is responsible for a putative binding of adhesion molecules to laminin. If this were so, the pronounced inhibitory effect of the soluble L2/HNK-1 carbohydrate on laminin in comparison to the inhibition by L2 antibodies could be explained by the following possibility: in the presence of L2 antibodies other, similar, but immunologically not cross-reactive cell surface structures, such as heparan sulfate could still interact with laminin, whereas by addition of L2 epitope-bearing molecules more binding sites on laminin could be blocked. With polylysine as substrate one should keep in mind that neurite outgrowth starts with a lag period of ~ 1 d and is slower than on laminin. It is therefore conceivable that not polylysine itself, but a cell-conditioned substrate, possibly an astrocyte-derived laminin (Liesi et al., 1983) or an L2/HNK-1 epitope bearing molecule, possibly the extracellular matrix constituent J1 (Kruse et al., 1985; Sanes et al., 1986), may interact with outgrowing neurites.

Our present experiments have shown that besides its involvement in cell to substrate adhesion the L2/HNK-1 carbohydrate epitope also mediates cell-cell interactions, as it was suggested by previous experiments (Keilhauer et al., 1985). Interestingly, neuron-neuron interaction does not appear to be dominated by the L2/HNK-1 carbohydrate in the short-term adhesion assay or in fasciculation of neurites. These findings are noteworthy, since neurites and neurons express this carbohydrate epitope in our assay systems as do astrocytes (see Keilhauer et al., 1985). However, in conjunction with N-CAM the L2/HNK-1 carbohydrate epitope could be shown to be functional in adhesion among neurons (Keilhauer et al., 1985), suggesting that it is also involved in adhesion, but not as the only and possibly minor ligand. These observations beg the question as to the molecular nature of the cellular receptor(s) for the L2/HNK-1 carbohydrate. Whether the receptors for this carbohydrate are the adhesion molecules themselves or yet unknown cell surface constituents remains to be resolved. An interesting hypothesis put forward by Cole, Glaser, and colleagues (1986a, b; Cole and Glaser, 1986) is worth mentioning in this context. They could show that N-CAM has binding sites for heparin and the cell surface. They suggested that the heparin- and cell-binding domains may be identical and speculated that the L2/HNK-1 carbohydrate may well bind to the heparin-binding site. It is, therefore, interesting that the L2/HNK-1-carrying epitope maps to the cell-binding domain of N-CAM (Cole and Schachner, 1987). It is noteworthy that only subpopulations of the adhesion molecules L1, myelin-associated glycoprotein, and N-CAM (both in its embryonic and adult forms), express the L2/HNK-1 carbohydrate moiety (Kruse et al., 1985; Poltorak et al., 1986a; Faissner, A., manuscript submitted for publication). Therefore, additional binding mechanisms besides the one suggested by Cole and Glaser and our experiments may exist between adhesion molecules.

It is tempting to speculate that the L2/HNK-1-carrying carbohydrate moiety subserves a particular function in conjunction with others on a multifunctional glycoprotein as it has been suggested for the hormone chorionic gonadotropin (Calvo and Ryan, 1985). This hormone binds with its protein backbone to a receptor that is distinct from the binding site for the hormone's carbohydrate moiety which is necessary for activation of adenylyl cyclase. Enzyme activation, how-

ever, occurs only when protein backbone and carbohydrate are simultaneously bound to the cell surface, possibly inducing a crosslinking of the two receptors. Whether a similar cooperativity exists between protein backbone and carbohydrate moiety of adhesion molecules and whether the L2/HNK-1 carbohydrate plays a role in activation of second messenger systems will have to be investigated.

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