Neurite Outgrowth of Neuroblastoma Cells: Dependence on Adhesion Surface–Cell Surface Interactions

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ABSTRACT Neurite outgrowth of C 1300 neuroblastoma cells, which were dispersed from adherent cultures or grown in suspension, was studied on different protein-coated surfaces. Of 29 different surface structures studied, including surfaces treated with various fibronectins, lectins, glycosidases, or glycosyltransferases capable of stimulating fibroblast spreading, only the surfaces coated with plasma fibronectin or with a protein mixture secreted by C6 glioma cells displayed an extensive activity in the sprouting assay. Neurite outgrowth was inhibited by brain gangliosides and by colominic acid (a sialic acid polymer). A 50% inhibition of neurite outgrowth of N18 neuroblasts induced by the glioma cell proteins was observed at the following approximate concentrations: 100 μM (0.2 mg/ml) GD_{1A} ganglioside, 20 μM (0.04 mg/ml) GT_{1B} ganglioside, and 5 mg/ml colominic acid. Specificity of inhibition was suggested by the finding that a few polyanionic substances tested were not inhibitory in the sprouting assay, and that the type of gangliosides inhibiting sprouting were found to be major sialoglycolipids of the neuroblasts. A hypothesis is discussed, according to which neurite outgrowth of neuroblasts is stimulated by adhesion involving interactions of the adhesion-mediating protein with cell surface carbohydrates characteristic of brain gangliosides.

Neuronal cells are dependent on adhesive contacts with their surrounding surface in order to form neurites (1–3). Little is known however, of the molecular mechanisms of adhesive interactions capable of promoting neurite outgrowth. A study was therefore undertaken, in which the capability of neuroblasts to extend neurites on different surfaces was examined. The C 1300 neuroblastoma cells were used as a convenient model system, since clones of these cells are known to sprout long neurite-like processes (1, 2).

In an attempt to study the relationship of the adhesiveness of a surface to its capability of enhancing axonal sprouting, various adhesive surface structures were prepared by adsorbing proteins on polystyrene or tissue culture surfaces as in studies of fibroblast attachment and spreading (4–6). The data of these studies suggest that various adhesive surface structures containing lectins or enzymes capable of enhancing fibroblast spreading (4, 5, 7), fail to enhance neuroblast sprouting. In contrast, plasma fibronectin and a protein mixture secreted by C6 glioma cells stimulate neurite outgrowth by acting as contact sites on adhesion surfaces.

Inhibition studies of sprouting were carried out with substances that could compete with the cell surface structure interacting with the adhesion surface to promote neurite outgrowth. Brain gangliosides were found to inhibit strongly neuroblast sprouting. It is suggested that gangliosides or glycoproteins having ganglioside-like sugar sequences could serve as cell surface structures interacting with the adhesion surface in the stimulation of neurite outgrowth from the neuroblasts.

MATERIALS AND METHODS

Materials: Human plasma fibronectin was purchased from Sigma Chemical Co. (St. Louis, MO) and bovine plasma fibronectin from Calbiochem-Behring Corp. (La Jolla, CA). A sample of human plasma fibronectin purified with affinity chromatography according to Vuento and Vaheri (8) was kindly donated by Dr. Jukka Finne (University of Helsinki, Finland). Human plasma fibronectin purified with affinity chromatography on gelatin-Sepharose (9) was used in some experiments.

Bandeiraea simplicifolia lectin was a gift from Dr. Jukka Finne (University of Helsinki, Finland) and soybean agglutinin from Dr. William G. Carter (Fred Hutchinson Cancer Research Center, Seattle, WA). All other lectins were from Sigma Chemical Co. *Aspergillus niger* β-galactosidase was a gift from Dr. Jonathan Knowles (University of Helsinki, Finland) and the fucosyltransferases from Dr. Jean-Paul Prieels (Free University of Brussels, Belgium). *Vibrio cholerae* neuraminidase was from Calbiochem-Behring Corp. Other glycosidase were purchased from Sigma Chemical Co.

The gangliosides containing one, two, or three sialic acid residues per molecules (the gangliosides GM_1, GD_{1A}, and GT_{1B}) were purified from pig brain according to previously described procedures (10). Other anionic sub-

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stances tested as inhibitors of axonal outgrowth were purchased from Sigma Chemical Co.

Cell Cultures: The neuroblastoma clones N18, NB 41A, and NIE 115 and the C6 glioma cells were kindly supplied by Dr. Ewen MacDonald, (University of Kuopio, Finland). The cells were cultured in DME supplemented with 10% fetal calf serum, 100 U penicillin G ml^{-1} and 0.1 mg streptomycin ml^{-1} in an atmosphere of 5% CO_2.

Preparation of Conditioned Media: The conditioned media from the C6 cells and the N18 cells were prepared using confluent cultures on 100 × 20 mm Costar tissue culture plates. The cultures were washed from the serum-containing media, and 5 ml DME (containing penicillin and streptomycin) was applied on the plates, which were then incubated for 20 h at 37°C in an atmosphere of 5% CO_2. The media were removed and centrifuged at 20,000 *g* for 0.5 h. The dialyzed supernatants from the C6 cells and the N18 cells contained 22 and 27 μg/ml protein, respectively. The conditioned media from the C6 and N18 cells were prepared in the same way in the presence of 0.11 mM cycloheximide, and they contained 53 and 40 μg/ml protein, respectively.

Adhesion Assays: Adherent cultures of N18 neuroblasts were labeled with 0.25 μCi ml^{-1} [^{3}H]thymidine (Amersham Corp., Arlington Heights, IL) in the complete medium for 20 h. The cells were dispersed, washed, and applied on different adhesion surfaces as in assays of neurite outgrowth (see below). The cells were incubated for 1 h at 37°C, the nonadherent cells were removed by washing, and the radioactivity of the adherent cells was solubilized for counting with 1% SDS in 0.5 N NaOH (4).

Assays of Neurite Outgrowth: Different protein-coated surfaces were prepared by adsorbing fibronectins, lectins, glycosidases, glycosyltransferases, or conditioned media on polystyrene plates (Linbro 96-well microtiter wells), as described previously (4). Proteins having lectin or enzyme activity were used since these proteins are expected to interact with cell surfaces, and could therefore induce adhesion (4, 7) and sprouting. The plates were washed three times with phosphate-buffered saline (PBS), and 75 μl of HEPES-buffered DME (10 mM HEPES, 100 U penicillin G ml^{-1}, and 0.1 mg streptomycin ml^{-1} in DME, pH 7.4; DME-HEPES) was applied on the wells before starting the assays.

Adherent cultures of neuroblasts were dispersed with 10 μg/ml of crystalline trypsin (Sigma Chemical Co.) for 20 min at 37°C, and washed twice in the presence of 40 μg/ml of soybean trypsin inhibitor (Sigma Chemical Co.) as described previously (4). The neuroblasts were centrifuged from DME-HEPES, and suspended in the same buffer at the density 0.25 × 10^6 cells ml^{-1}. The assays were started by adding 75 μl cell suspension to the wells containing 75 μl DME-HEPES. An assay time of 3 h was routinely used since the analysis was aimed at initial interactions taking place in neurite outgrowth, and at 3 h the sprouting vs. nonsprouting cells could be differentiated without difficulties in interpretation (Fig. 1). The assays were stopped by adding 150 μl 4% glutaraldehyde in DME-HEPES to the wells.

To avoid the trypsin treatment, we cultured the N18 neuroblasts in suspension on bacteriological petri dishes in the same medium as the adherent cells. The cells were dispersed by pipetting, washed from PBS and DME-HEPES, and tested for the sprouting activity as the cells from adherent cultures.

The percentage of sprouting cells was determined by scoring 250 cells from randomly selected areas on each well. The cells containing one or more processes, which were more than half the diameter of the undifferentiated round cell (>20 μm), were defined as sprouting neuroblasts (Fig. 1). The maximum amount of sprouting cells using plasma fibronectin required 50 μg/ml protein for surface treatment in 100 μl PBS. On the basis of five duplicate experiments 68.5 ± 3.65 (SD) % of cells were defined as sprouting ones under these conditions. The sprouting index of a surface compares the effect to the sprouting value 68.5% (sprouting index 100). Mean sprouting values of duplicate or triplicate wells, as shown in each experiment, were used for calculation of the sprouting indexes.

Thin-layer Chromatography of Gangliosides from N18 Neuroblasts: Adherent cultures of confluent neuroblasts on 150 × 15 mm tissue culture plates were washed with PBS, and the gangliosides were extracted and partitioned with mixtures of chloroform-methanol-water (11). About 10

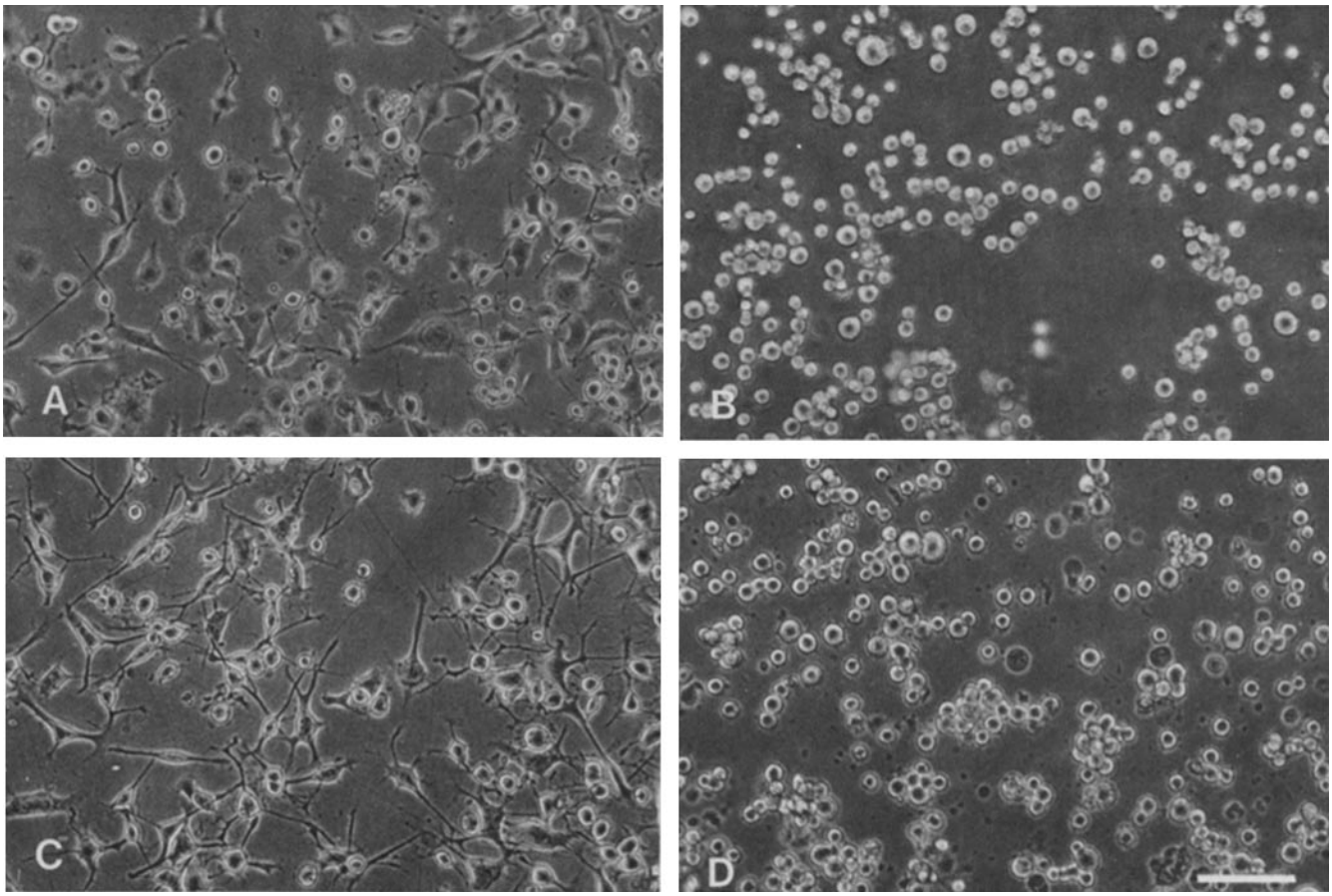

FIGURE 1 Effect of different protein-coated surfaces on neurite outgrowth of N18 neuroblasts. (*A*) human plasma fibronectin (coating concentration 50 μg/ml); (*B*) bovine serum albumin (50 μg/ml); (*C*) proteins secreted by the C6 glioma cells; (*D*) proteins from the C6 glioma cells in the presence of cycloheximide (see Materials and Methods for the preparation of the conditioned media and for details of the assay). Bar, 100 μm. × 100.

nmol of ganglioside neuraminic acid were recovered per plate. Aliquots of the upper phase glycolipids (~5 nmol as neuraminic acid) were analyzed on high-performance thin-layer chromatography. Precoated silica gel 60 plates (E. Merck, Darmstadt, W. Germany) were used with chloroform/methanol/water 60:35:8 (vol/vol/vol) or chloroform/methanol/2.5 M NH_4OH 60:35:8 (vol/vol/vol) as the solvent.

RESULTS

Attachment and Neurite Outgrowth of Neuroblastoma Cells on Different Protein-coated Surfaces

Cells from adherent cultures of N18 neuroblastoma were dispersed and analyzed for their ability to attach to wells, to which different proteins had been adsorbed. Almost no specificity could be observed in neuroblast attachment on different types of surfaces. For example, approximately the same percentage of cells (~70%) were attached on plain tissue culture plastic, concanavalin A or fibronectin in 1-h experiments (Table I).

In contrast to the analysis of cell binding, a clearcut specificity with respect to the contact surface was revealed from an analysis of neurite extension of the N18 cells. The lectins, glycosidases, and glycosyltransferases tested were without effect in the sprouting assay (Table II). Besides the proteins given in Table II, the following proteins were studied and found to have little or no effect on neurite extension (the sprouting index for cells from adherent cultures is given within parentheses): fetuin (0.6), asialofetuin (0.6), ovalbumin (0.9), soybean agglutinin (0.0), wheat germ agglutinin (0.0), β-galactosidase from Jack beans (1.2), β-galactosidase from *Aspergillus niger* (0.3), and neuraminidase from *Vibrio cholerae* (0.0). On the other hand, extensive sprouting was observed on surfaces treated with 10–50 μg/ml plasma fibronectin (Table II) purified in different ways (see Materials and Methods). A soluble protein mixture secreted by C6 glioma cells stimulated strongly neuroblast sprouting, whereas the proteins from the N18 cells itself had little effect (Table III). The cells dispersed from adherent culture were more reactive than the cells grown in suspension (Table II).

Sprouts could be clearly observed at 0.5–1 h after starting the assays, and at 3 h neurites extending up to ~100 μm were observed (Fig. 1). The cells from the neuroblastoma clones NB 41 A and NIE 115 behaved in a similar way (not shown). The NB 41 A cells were able to form only short sprouts rendering the quantitative analysis somewhat difficult.

The stimulating activity derived from the glioma cells is due to a soluble protein since it remains in a 100,000 *g*

TABLE I

Attachment of N 18 Cells on Different Protein-coated Surfaces

Surface	Cells attached
	%
Polystyrene plastic	23
Tissue culture plastic	65
Bovine serum albumin	53
Concanavalin A	72
Fibronectin	65
Conditioned medium from C6 cells	59

Proteins (50 μg/ml in 650 μl PBS) and the conditioned medium from C6 cells (see Materials and Methods) were adsorbed to polystyrene plates (diameter 3.5 cm; Greiner) for 2 h at room temperature. The media were removed, the plates were washed three times with 1.0 ml PBS, and 750 μl DME-HEPES were applied on the plates. Cells that had been labeled with [^{3}H]thymidine were dispersed from adherent cultures and suspended in DME-HEPES (0.25×10^6 cells ml^{-1}; see Materials and Methods for the preparation of cells). The assays were started by adding 750 μl of cell suspension (46,000 cpm) to the wells, which were incubated at 37°C for 1 h. To remove the nonadherent cells, we rotated the wells on a Bellco shaker at 115 rpm for 15 min. The media were removed, and the wells were washed three times with 1.0 ml PBS. Adherent cells were dissolved in 1% SDS in 0.5 N NaOH (4) for counting of the radioactivity. The values given are averages from two determinations.

TABLE II

Neurite Outgrowth of N 18 Cells on Different Protein-coated Surfaces

Surface	Cells from adherent cultures		Cells grown in suspension	
	% Sprouting cells	Sprouting index	% Sprouting cells	Sprouting index
Polystyrene plastic	0.0; 2.0	1.5	0.8; 1.6	1.8
Bovine serum albumin	0.8; 1.2	1.5		
Human plasma fibronectin	57; 74	96	17; 21	28
Bovine plasma fibronectin	64; 68	96	21; 32	39
Bandeiraea simplicifolia lectin	0.0; 1.6	1.2	1.6; 2.4	2.9
Concanavalin A	0.8; 2.0	2.0	0.4; 0.8	0.9
α-Galactosidase	2.4; 2.8	3.8	0.8; 1.2	1.5
Neuraminidase (*Clostridium perfringens*)	0.0; 0.0	0.0		
α-(1-3,4)-Fucosyltransferase	0.8; 2.0	2.0		
α-(1-2)-Fucosyltransferase	2.0; 2.4	3.2		
α-Mannosidase			2.0; 2.8	3.5
Tissue culture plastic			2.0; 5.2	5.3
Human plasma fibronectin*			22; 26	35
Poly-L-lysine*			0.0; 1.6	1.2

Lectins were adsorbed on polystyrene plates at 10 μg/ml in 100 μl PBS for 2 h at room temperature. The medium was removed, and the wells were further treated with 100 μg/ml crystalline bovine serum albumin (Sigma Chemical Co.) in 100 μl PBS for 1 h at room temperature (4). The fucosyltransferases were adsorbed from a glycerol-containing medium, and the wells were further treated with albumin (7). Surface treatments with other proteins (50 μg/ml) were carried out for 2 h at room temperature without further coating with albumin. The media were removed after the coating, the wells were washed three times with 150 μl PBS, and 75 μl DME-HEPES was applied on the wells. The cells were added in 75 μl DME-HEPES, and analyzed for sprouting after 3 h at 37°C from duplicate wells (see Materials and Methods for details of the assay). The average value of the two determinations was used for calculation of the sprouting index.

* Adsorbed on tissue culture plastic (Costar).

TABLE III

Neurite Outgrowth of N 18 Cells on Surfaces Treated with Conditioned Media from C 6 Glioma Cells and from N 18 Neuroblastoma Cells

Surface	% Sprouting cells	Sprouting index
Conditioned medium from C6 cells	61; 61	89
Conditioned medium from C6 cells (heated for 0.5 h at 50°C)	72; 76	108
Conditioned medium from C6 cells (heated for 0.5 h at 80°C)	16; 19	26
Conditioned medium from C6 cells in the presence of cycloheximide	0.4; 0.4	0.6
Conditioned medium from N 18 cells	0.8; 2.0	2.0

Conditioned media from C 6 glioma cells or N 18 neuroblastoma cells were adsorbed on microtiter wells for 2 h at room temperature. The duplicate wells were washed and assayed for their effect on neurite outgrowth of N 18 cells dispersed from adherent cultures (see Materials and Methods for details of the assay).

supernatant during a 1-h centrifugation, is nondialyzable and labile to heating (Table III). The activity was not released to the medium in the presence of cycloheximide (Table III), suggesting that it derives from protein synthesis of the glioma cell. A maximal effect on axonal sprouting was achieved at a protein concentration 3–5 μg/ml. Both the cells from adherent cultures and the cells grown in suspension were able to extend neurites on polystyrene or tissue culture surfaces treated with the glioma cell–derived proteins.

Effect of Proteins in Solution as Compared to the Effect of Proteins Adsorbed on a Solid Surface

Although it appears that polystyrene-immobilized proteins are not released to the medium during cell experiments (4, 5), the possibility was considered that some of the plasma fibronectin or the C6 cell–derived protein would be detached to the medium during the assay, get into the cell, and effect an intracellular stimulation of neurite formation. This possibility seems to be excluded since the proteins displayed little effect when added to the medium after allowing the cells to attach. The effect on sprouting was slight in these experiments even during a 20-h observation time (Table IV). Also, if the plates were pretreated with albumin to inhibit adsorption (4), the effects on sprouting were lost. Therefore, both plasma fibronectin and the C6 cell–derived protein act as surface-bound adhesion sites necessary for neurite outgrowth. Some cell types, including the C6 glioma cells, have been previously shown to release factors that stimulate sprouting when added to neuroblast culture media (12). However, the effects from the culture media require long assay times (reference 12; Table IV), and are slight as compared to experiments, in which the surfaces are pretreated with active proteins (Tables III and IV).

Inhibition of Neurite Outgrowth by Gangliosides and by Colominic Acid

Since both plasma fibronectin and the glioma cell proteins exert their effects on neurite formation by acting as contact

TABLE IV

Effect of Fibronectin and of C 6 Glioma Cell Proteins on Neurite Outgrowth of N 18 Cells from Solution as Compared to the Effects of the Proteins Adsorbed on Plastic Surfaces

Contact surface	Medium	% Sprouting cells	Sprouting index
Tissue culture plastic	DME-HEPES	3.2; 6.4; 8.8	9.0
Tissue culture plastic treated with the glioma cell proteins	DME-HEPES	75; 76; 80	112
Tissue culture plastic	Glioma cell proteins in DME-HEPES	26; 29; 29	41
Tissue culture plastic treated with fibronectin	DME-HEPES	36; 42; 44	59
Tissue culture plastic	Fibronectin in DME-HEPES	2.8; 5.6; 5.6	6.8

Tissue culture wells were incubated in PBS, the conditioned medium from the C6 cells (see Materials and Methods) or 10 μg/ml plasma fibronectin in PBS. The plates were washed, and N 18 cells dispersed from adherent cultures were allowed to attach in DME-HEPES on the surfaces for 1 h at 37°C. The medium was removed and replaced by either DME-HEPES, the glioma cell proteins or plasma fibronectin in DME-HEPES at the same protein concentrations as used to pretreat the surfaces. The wells were incubated for 20 h at 37°C in the atmosphere of 5% CO_2. The sprouting indexes represent the mean values from the triplicate wells.

TABLE V

Effect of Gangliosides and of Various Polyanionic Substances on Neurite Outgrowth of N 18 Cells
A: Effect of Different Anionic Substances as Compared to Ganglioside Effect
B: Effect of Different Gangliosides Containing Increasing Amounts of Neuraminic Acid

Addition	Concentration tested	% Sprouting cells	% of control
A. None		66; 68	100
Dextran sulfate (mol wt 5,000)	5 mg/ml	62; 67	96
Dextran sulfate (mol wt 8,000)	5 mg/ml	62; 66	96
Dextran sulfate (mol wt 500,000)	5 mg/ml	54; 55	81
Heparin	5 mg/ml	62; 68	97
Chondroitin sulfate (from whale cartilage)	5 mg/ml	69; 70	104
Chondroitin sulfate (from shark cartilage)	5 mg/ml	58; 63	90
Colominic acid	5 mg/ml	40; 42	61
Fetuin	5 mg/ml	68; 70	103
GT_{1B} ganglioside	0.21 mg/ml (100 μM)	10; 10	15
N-acetylneuraminic acid	5 mg/ml (16 mM)	69; 71	104
B. None		62; 65	100
GM_1 ganglioside	200 μM	46	72
	100 μM	39	61
GD_{1A} ganglioside	200 μM	17	27
	100 μM	24	38
	50 μM	50	79
GT_{1B} ganglioside	100 μM	9	14
	50 μM	10	16
	25 μM	33	52
	12.5 μM	36	57
	6.25 μM	50	79

The polyanions (Sigma Chemical Co.) and the gangliosides purified from pig brain (10) were dialyzed against PBS and finally against DME-HEPES before the assays. Powdered *N*-acetyl-neuraminic acid (Sigma Chemical Co.) was dissolved in DME-HEPES. Microtiter wells were treated with 100-μl conditioned media from C 6 cells (see Materials and Methods) for 2 h at room temperature. The surfaces were washed, and the test substances at two times the final concentration in 75 μl DME-HEPES were applied to the wells. The N 18 cells from adherent cultures were added to the wells in 75 μl DME-HEPES and analyzed for neurite outgrowth as explained in Materials and Methods.

sites on adhesion surface, they probably interact with some cell surface structure to stimulate sprouting. Experiments were therefore carried out to inhibit the effects of the adhesion surfaces using neuronal cell surface-related substances in the test media. Brain gangliosides were found to have a strong inhibitory effect on neurite outgrowth. A 50% inhibition of neurite outgrowth from N18 neuroblasts induced by the glioma cell proteins required only ~20 μM trisialoganglioside GT_{1B} (Table V). A 50% inhibition of sprouting was achieved at ~100 μM concentration of the disialoganglioside GD_{1A}, whereas the monosialoganglioside GM_1 had little effect (Table V). Fibronectin-treated surfaces were inhibited in a similar way (not shown). Sprouting of the cells from the neuroblastoma clone NIE 115 was also very sensitive to gangliosides. Thus, a complete inhibition of sprouting induced by the C6 glioma cell proteins was observed at 45 μM concentration of GD_{1A} or GT_{1B} ganglioside.

It is unlikely that the inhibitory effect of brain gangliosides on neurite outgrowth would be due to unspecific cell-toxic

reasons, since the gangliosides occur at high concentrations in neuronal cell membranes (13, 14). The possible cell toxic effect of the gangliosides was tested by preincubating the cells in suspension for 3 h at 37°C in the presence of 100 μM GT_{1B} ganglioside in the assay medium. The ganglioside was thereafter removed from the medium by centrifugation of the cells, which were then resuspended and tested for neurite outgrowth. The ganglioside-treated cells gave a sprouting value 65% as a mean of a triplicate determination (62, 66 and 66%, compare to Table V), and the cells incubated in the buffer without the ganglioside gave the mean sprouting value 62% (58, 62, and 67%). Thus, no inhibition can be observed when the cells are preincubated with five times the GT_{1B} concentration than the concentration that gives a 50% inhibition in the sprouting assay (Table V). In contrast, some tendency to an increased sprouting could be often observed, which may be due to incorporation of the ganglioside into the cell surface during the preincubation.

Any of the sulfated polyanions tested did not inhibit neurite outgrowth even at high concentrations (Table V). *N*-acetylneuraminic acid and the sialoglycoprotein fetuin were also without effect (Table V). The sialic acid polymer from *Escherichia coli* (colominic acid) had repeatedly some effect, though the inhibitory concentrations were rather high (Table V).

Gangliosides of the N18 Neuroblasts

Thin-layer chromatography suggested that the type of gangliosides, which inhibit axonal outgrowth, are found in the neuroblasts used in the assay. Thus, a GD_{1A}-type ganglioside was found to be a major fraction in the neuraminic acid–containing glycolipids of the N18 cells (Fig. 2). Therefore, the inhibitory effects of the GD_{1A} and GT_{1B} gangliosides may be due to competition with a cell surface component having a ganglioside-type carbohydrate structure.

DISCUSSION

The results of this study are compatible with the following hypotheses of neuroblast sprouting: (*a*) Neuronal cell adhesion plays an important role in neurite outgrowth. (*b*) Fibronectin and fibronectin-related proteins stimulate neurite outgrowth. (*c*) Gangliosides or glycoproteins having ganglioside-like sugar sequences are cell surface interaction sites in the stimulation of neurite outgrowth. These three postulates are discussed separately below.

Adhesiveness of a Surface and Neurite Outgrowth

The interpretation that adhesion of a neuronal cell has an important role in sprouting of neuroblasts is in agreement with studies of Letourneau (3), who showed by surface-shadowing with heavy metals that axonal outgrowth is facilitated in areas of increased adhesiveness. However, adhesiveness of a surface *per se* may not be a sufficient condition for initiation of neurite outgrowth, although it is a necessary one. This inference is supported by the finding that most adhesive surface structures fail to enhance sprouting in 3-h experiments (Fig. 1, and Tables I and II), and clearcut differences in the extent of neurite outgrowth on different surfaces can be observed at least up to 20 h (Table IV). For example, the cells attach and flatten rapidly on lectin surfaces, but no remarkable neurite outgrowth occurs (Tables I and II). On the other hand, the proteins stimulating axonal sprouting have a far better effect when immobilized on adhesion surfaces as compared to the effects of the same proteins in solution (Table IV). It thus appears that only neuroblast adhesion involving specific interactions of the adhesive surface with the cell surface is capable of stimulating outgrowth of neurites.

Fibronectin and Neurite Outgrowth

Fibronectin has a remarkable effect on axonal sprouting (Fig. 1, Table II). The question of whether fibronectin could stimulate neurite outgrowth in vivo requires further study. It has been suggested that cells from glioma (15) and neuroblastoma (16) synthesize fibronectin, and a fibronectin-like protein occurs in the adhesion sites of neuroblastoma cells (17). However, fibronectin may not be expressed by normal glial or neural cells (18–20). Although aggregates of neural retinal cells (21) and neuroblasts are reactive to fibronectin, it is possible that these effects only reflect a similarity in the cell surface specificity between fibronectin and some other protein occurring in the glial or neural cells. A systematic search of such proteins should be possible on the basis of this study. Preliminary results of studies on glioma cells, on cerebrospinal fluid and on cell membranes of brain suggest that the activities enhancing neurite outgrowth of neuroblastoma cells

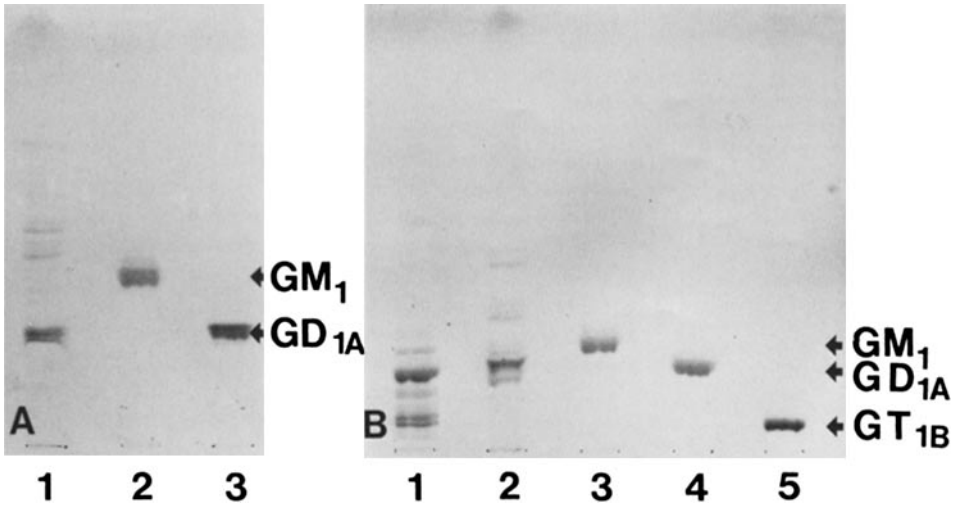

FIGURE 2 Thin-layer chromatography of gangliosides from N18 cells. The plates were developed with a resorcinol-hydrochloric acid spray. (*A*) chromatography using chloroform-methanol-water 60/35/8 (vol/vol/vol) as the solvent. Lane *1*, gangliosides from N18 cells; lane *2*, GM_1 ganglioside from pig brain; lane *3*, GD_{1A} ganglioside from pig brain. (*B*) chromatography using chloroform-methanol-2.5 M NH_4OH 60/35/8 (vol/vol/vol) as the solvent. Lane *1*, gangliosides from rat brain; lane *2*, gangliosides from N18 cells. Gangliosides purified from pig brain are shown on lanes 3–5. Lane *3*, GM_1; lane *4*, GD_{1A}; lane *5*, GT_{1B}.

are due to fibronectin-related proteins.[1] Whether these factors are related to those mediating neurite outgrowth on polyornithine or polylysine surfaces (22–24), remains to be studied.

It is interesting that the effect of fibronectin-like proteins on the morphology of the neuroblastoma cells might be related to the finding that fibronectin partially restores normal morphology to transformed fibroblasts (25). However, the biochemical requirements for adhesion that stimulates neurite outgrowth of neuroblasts are entirely different from those stimulating fibroblast spreading. Thus, whereas many lectins and enzymes are able to stimulate fibroblast spreading in conditions similar to those of the present study (4, 7), these proteins do not stimulate neuroblast sprouting (Table II).

Cell Surface Gangliosides and Neurite Outgrowth

Since only few adhesion surface stimulate sprouting (Tables II and III), a specific interaction with some cell surface structure is necessary for initiation of neurite outgrowth. The inhibition data (Table V) suggest that the interaction sites at the cell surface are neuraminic acid–containing glycoconjugates. Also, treatment of the N18 cells with neuraminidase reduced the extent of sprouting, but the cells were aggregated during the treatment, which made quantitative evaluation of the effect difficult (unpublished results).

The inhibition of axonal sprouting by gangliosides may be related to the ganglioside inhibition of fibronectin-mediated fibroblast attachment (26, 27). Thus, fibronectin-like proteins could interact with anionic cell surface components, like the gangliosides and proteoglycans (28, 29). It is currently difficult to conclude which type of cell surface molecules would be most relevant in the interactions of fibronectin with different cells (30, 31). Since the type of ganglioside, which is inhibitory in the sprouting assay, is a major glycolipid in the N18 cells (Fig. 2), the inhibitory effect may be due to a specific competition with the cell surface. This inference is also supported by reasonably low concentrations of the GD_{1A} and GT_{1B} gangliosides necessary for the inhibition of sprouting (Table V).

The inhibitory effect of purified gangliosides (Fig. 2) is related to increasing amounts of sialic acid in the molecule, but it is not an unspecific polyanionic effect (Table V). It should be recognized that at the rather high concentrations of the monosialoganglioside GM_1 tested (Table V), this molecule is already highly aggregated in aqueous solution (32, 33), and should therefore form a polysialic acid structure. It is however, probable that in contrast to the GD_{1A} and GT_{1B} gangliosides, the sialic residues of this structure are unavailable for interaction due to steric reasons as in neuraminidase–GM_1 ganglioside interactions (32).

Gangliosides have been suggested to serve as cell surface receptors in neurite outgrowth induced by nerve growth factor, and they have been reported to increase the extent of neurite formation when added to neuronal cell cultures (34, 35). The apparently controversial results of the present study may be due to an entirely different method from those used previously. Thus, in the present method the extent of neurite outgrowth is strictly dependent on the structure of the adhesion surface, and no sera or other sources of growth factors are used in the assay of neurite outgrowth. On the other hand, it is important to realize that gangliosides could function as competitive inhibitors in the test media, but they could in contrast enhance sprouting in conditions, which favor incorporation to the cell surface. In fact, pretreatment of cells with high ganglioside concentrations may enhance neurite outgrowth in the present conditions. In any case, both the nerve growth factor and the adhesive protein stimulating neurite outgrowth could act on similar ganglioside-type cell surface structures.

The question of whether the cell surface components, which interact with adhesion surfaces enhancing sprouting, are gangliosides or glycoproteins having ganglioside-like sugar sequences (36) requires further study. Since high concentrations of colominic acid have some inhibitory effect (Table V), the protein enhancing neurite outgrowth may not be strictly specific for gangliosides, but the orientation of clustered sialic acid residues might be the structural feature determining the specificity. It is worth noting that the amount and the structure of the inhibitory type of sugar chains both in glycoproteins (37–40) and in gangliosides (14, 41) change during brain development, suggesting a function in differentiation.

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Note Added in Proof: Recent studies have suggested that laminin adsorbed to polystyrene or linked covalently to glass (Jousimaa, J., J. Merenmies, and H. Rauvala, manuscript in preparation) stimulates neurite outgrowth to a similar extent as fibronectin (see Table II).

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