Isolation and Characterization of a Large, Neurite-associated Glycoconjugate from Neuroblastoma Cells

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ABSTRACT A high molecular weight glycoconjugate has been isolated from neurite-producing neuronal tumor cells in culture and has been designated as I_0 based on its elution characteristics in gel filtration chromatography. This molecule cannot be found in a variety of nonneuronal cells. I_0 is found in the substratum-attached material or cell fraction of neurite-producing neuroblastoma cells, depending upon culture conditions. It is found in the substratum-bound fraction of B104 rat neuroblastoma cells during serum starvation and in the EGTA-detached cell fraction of B104 cells grown in chemically defined N2 medium. It occurs only in the cell fraction of the human neuroblastoma line Platt. Examination of behavioral variants of the B104 rat line further strengthens the association of I_0 with neurite production; the constitutive neurite-producing E_RB9 variant contains I_0 while the non-neurite-producing E_RA11 variant does not. I_0 is large, eluting in the void volume of Sepharose-CL2B columns. Radioiodination of intact cells with lactoperoxidase shows I_0 to be a cell surface component. Metabolic radiolabeling studies show that it contains a high proportion of polysaccharide to protein, does not contain mannose, and is unsulfated. Alkaline borohydride reduction releases two size classes of large polysaccharide chains. The alkaline reduction results, along with the mannose incorporation studies, show the presence of O-glycosidic linkages and few, if any, N-linkages. Resistance to nitrous acid deamination, insensitivity to glycosaminoglycan lyases, and the absence of sulfation, indicate that I_0 does not contain the glycosaminoglycans hyaluronic acid, chondroitin-, dermatan-, or heparan-sulfates. Affinity column chromatography reveals high binding affinity of I_0 to polyornithine and no binding to gelatin (collagen) or the glycosaminoglycans hyaluronate and heparin. These studies describe a unique high molecular weight glycoconjugate on the surface of neurite-producing neuroblastoma cell lines from two species.

Several approaches have been used to examine mechanisms in neuronal cells underlying growth cone adhesion and neurite outgrowth. The effects of physical/chemical properties of the tissue culture substratum on strength of adhesion and rate of neurite outgrowth were initially explored by Letourneau (1). Substrata coated with polycationic polypeptides, especially polyornithine (PORN) and polylysine, were found to optimize growth cone adhesion and neurite outgrowth from embryonic sensory ganglia. A complementary approach has involved a search for factors from cells or their environment that stimulate neuronal differentiation. Studies using embryonic ciliary ganglia have shown the existence of substances in extracts of target tissues (2, 3), conditioned medium from heart cultures (4, 5) and a variety of other tissues (6) that stimulate neurite outgrowth. These substances are active only when bound to PORN-coated substrata (designated PORN-bound neurite-promoting factor:PNPF). Other studies showed that ciliary ganglia can produce their own PNPF microexudate (7). Initial characterization of PNPF from conditioned medium suggests, based on binding affinities, that it is a large, acidic glycoprotein (8). In most of this work, the behavior of neurons in embryonic ganglia has been observed in response to factors allowed to bind to tissue culture substrata.

The approach taken in the present study has been to use an extracellular matrix (ECM) model system and cloned neuronal tumor cell lines for biochemical examination of neuronal cell surface material and cell-substratum adhesion sites. Substratum-attached material (SAM) has been shown to include a subset of cell surface and extracellular matrix material with high concentrations of glycoproteins and glycosaminoglycan (GAG)-containing proteoglycans. SAM is composed of material left behind as cells migrate on tissue culture substrata

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("footprints") and attachment areas ("footpads") pinched or torn off during detachment with EGTA (9). Preparation of SAM can be viewed as a method of concentrating cell surface material involved in cell-substratum adhesion. Glycoprotein and GAG content of cells and SAM can be compared and correlated with cell behavior.

In an earlier study, biochemical analysis of neuroblastoma SAM showed the appearance of a high molecular weight glycoconjugate in SAM from neurite-producing ("differentiated") B104 rat neuroblastoma cells (10). This material was designated I_0 in the analysis scheme and has never been observed in glial cells, muscle cells, or a variety of fibroblastlike cells (11). In this study the association of I_0 with neurite production is further tested using the serum-sensitive B104 rat neuroblastoma cells (12), behavioral variants of that line (13), and Platt human neuroblastoma cells (14) grown under a variety of culture conditions. This paper also describes the isolation and partial biochemical characterization of I_0. Experiments are discussed that establish aspects of the composition of binding affinities of this "neurite-associated" macromolecule.

MATERIALS AND METHODS

Cells and Growth Conditions: B104 rat neuroblastoma cells and their behavioral variants E_RA11 and E_RB9 were grown and subcultured as previously described (10, 13). The human neuroblastoma line, Platt, was grown in Dulbecco's modified Eagles medium (DME) with 10% fetal calf serum (FCS), 10 mM HEPES buffer, 250 U/ml penicillin, 250 μg/ml streptomycin at 37°C in a 10% CO_2-humidified air mixture. B104 cells grown in chemically defined N2 medium (15) were passaged by allowing them to settle in flasks in a small amount of serum-containing DMEM for 1 h. Serum-containing medium was then drained, the flask was rinsed with phosphate buffered saline (PBS), and N2 medium was added. The Platt human neuroblastoma cell line was isolated from a disseminated tumor in a three-year-old, white female patient about four years ago (personal communication, Dr. John Graham-Pole, University of Florida, Gainesville). The B104 and Platt cells were found to be *Mycoplasma*-free by the use of autoradiographic assays and scanning electron microscopic inspection. The E_RB9 and E_RA11 cell lines were found to be contaminated by *Mycoplasma* and were treated with the drug Tylocine (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY), 600 μg/ml, for four to five passages, which suppressed *Mycoplasma* to undetectable levels for several weeks following discontinuation of the drug.

Morphometric Studies: Cells grown in tissue culture dishes for 3 d were photographed using a Nikon Diaphot inverted phase contrast microscope. Cell measurements were made from enlarged photographic prints using a Numonics Electronic Digitizer (Numonics Corp., Lansdale, PA).

Metabolic Radiolabeling: Cells were grown in 100-mm tissue culture dishes containing complete medium plus 5 μCi/ml 6-[^{3}H]-D-glucosamine HCl or 5 μCi/ml 1-[^{3}H]-D-mannose to metabolically radiolabel polysaccharides or 50 μCi/ml [^{35}S]-Na_2SO_4 to radiolabel sulfated GAG's. B104 cells grown in N2-serumless medium were initially seeded into culture dishes, 2×10^6 cells/100-mm dish, in DMEM + 10% FCS and allowed to adhere for 1 h at 37°C. Dishes were then drained and rinsed twice with PBS. N2 medium containing the radiolabeled species was then added. Cells were grown 3–4 d in radiolabeling medium. E_RA11 and E_RB9 variant cells (13) were seeded at 1×10^6 cells/dish. Platt cells were seeded at 7×10^5 cells/100 mm dish or 15×10^6 cells/1750 cm^2 roller bottles. For each preparation, 80 dishes or five to seven roller bottles were grown to 70–80% confluence. Radiolabeled B104, E_RA11 and E_RB9 cells were detached from the substratum with EGTA and SAM extracted with SDS by the method described by Culp et al. (16). An extra step is required to detach the Platt cells: a 20–30 min incubation in Ca^{++}, Mg^{++}-free PBS is added before EGTA to remove these somewhat EGTA resistant cells.

Analysis for Glycosaminoglycans and Other Polysaccharides: Metabolically radiolabeled cells or SAM were processed using the procedures described in detail by Rollins and Culp (17) as originally developed by Cohn et al. (18). Briefly, cell samples were subjected to extensive digestion by DNase and RNase, followed by Pronase digestion in the presence of 0.2% SDS. SAM samples were Pronase-treated in SDS. Nonradioactive carrier GAG's (10 μg each of hyaluronate, chondroitin-4- and -6-sulfate, dermatan sulfate, and heparan sulfate) were added, the extract dialyzed against water, and the GAG's precipitated in ethanol-potassium acetate. The pelleted precipitate was redissolved in water and reprecipitated (3×). After dissolving the precipitate in the appropriate buffer, GAG's were digested with chondroitinase ABC or AC and the chondroitinase digests were chromatographed on paper as in Rollins and Culp (17) to determine chondroitin-4-sulfate, -6-sulfate, dermatan sulfate, and hyaluronate contents. Chondroitinase ABC digests were also posttreated with testicular hyaluronidase (T.H.) to ensure complete digestion of hyaluronate moieties before chromatography on Sepharose CL6B columns (1.4 × 110 cm) in 0.2% SDS, 150 mM Tris hydrochloride buffer at pH 7.4 to separate four classes of polysaccharide (I_0, heparan sulfate sequences, glycopeptide derived from glycoproteins and disaccharides liberated by chondroitinase digestion). An aliquot of [chondroitinase ABC plus T.H.]-treated sample was subjected to nitrous acid deamination or control treatment (19) before column chromatography to determine the percentage of highly N-sulfated heparan sulfate. Radioactivity of fractions was measured by scintillation counting. In this study, column chromatography of samples digested with chondroitinase ABC plus testicular hyaluronidase was used for analytical and preparative purposes. Paper chromatography was used primarily as an internal control in determining the amount of chondroitinase digestible material.

I_0 Analysis: The void volume peak in chromatographic profiles of [chondroitinase ABC plus T.H.]-treated samples (from Sepharose CL6B columns eluted with SDS/Tris buffer) was designated I_0 in these studies (above). Various analytical procedures were performed on this isolated material. I_0 (in 0.2% SDS) was treated with 5 mg Pronase at 55°C overnight or alkaline borohydride reduction to determine the linkage of polysaccharides to protein (20); 7.5% Na Borohydride in 0.1 N NaOH was added 1:1 (vol/vol) to the sample, incubated at 37°C for 24 h, and neutralized with acetic acid. These digests were then chromatographed on Sepharose CL6B columns in SDS-containing buffers as described above. Some isolated I_0 was found to break down after 24 h at 37°C, but this was prevented with the protease inhibitors 2 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine HCl, and 2 mM EDTA.

Affinity Chromatography: Minicolumns were prepared by cross-linking the ligand of interest to cyanogen bromide-activated Sepharose 4B. 2–4 ml of activated Sepharose 4B were reacted with an equal volume of gelatin (10 mg/ml) or polyornithine (PORN, 5 mg/ml) in 0.2 M $NaHCO_3$ (pH 9.5) by the method of March et al. (21). The GAG's heparin (5 mg/ml) and hyaluronate (10 mg/ml) were cross-linked (separately) to the cyanogen bromide-activated Sepharose 4B in 0.2 M $NaHCO_3$ (pH 9.5) by the method of Cuatrecasas (22, 23).

Metabolically radiolabeled I_0 ($\sim 1 \times 10^4$ cpm) was loaded onto each affinity column. Gelatin-Sepharose columns were rinsed extensively in 50 mM Tris-HCl (pH 7.4), 1 mM $MgCl_2$, and 1 mM $CaCl_2$ (TMC buffer), then eluted with 1 M urea followed by 4 M urea in TMC. PORN columns were eluted with TMC buffer followed by 2 M NaCl in TMC. Loaded GAG columns were rinsed with PBS and then eluted with 2 M NaCl in PBS. 250-μl fractions were collected and radioactivity measured by scintillation counting. To prevent nonspecific binding of I_0 to Sepharose, columns were rinsed with 0.1% albumin in the appropriate buffer before loading the sample.

Ion Exchange Chromatography: Irreversible binding of I_0 to DEAE Sephadex-A25 was observed, so columns were rinsed with 0.2% Triton X-100. With Triton, >90% of the radioactive material was recovered. The Triton detergent did not interfere with the exchange properties of these columns. The samples were prepared as follows. Void volume (I_0) peaks isolated on Sepharose CL6B columns were dialyzed extensively against water. A 10×-concentrated Triton/Tris buffer was added to make the samples 0.2% (wt/vol) Triton and 50 mM Tris, pH 7.4. They were chromatographed on a 6-ml column of DEAE-Sephadex A-25 with a gradient ranging from 10 mM NaCl to 2 M NaCl in 0.2% Triton and 50 mM Tris. Refractive indices and radioactivity were determined for each fraction.

Radioiodination: To determine whether I_0 is a cell surface component, Platt cells were radiolabeled with Na[^{125}I] in a method modified from Schenkein et al. (24). Cells were removed from a 70–80% confluent 150 cm^2 flask using EGTA as described above, rinsed four times with PBS containing 100 mg/ml each of $MgCl_2$ and $CaCl_2$ (PBS-II), and incubated in suspension in complete culture medium for ½ h to permit repair of cell surface damage caused by EGTA. The cells were centrifuged, rinsed in PBS-II, and resuspended in radiolabeling medium containing 0.2 mg/ml lactoperoxidase, 10^{-6} M KI, 5 mg/ml glucose, 200 μCi Na[^{125}I], and 0.1 U/ml glucose oxidase in PBS II for 10 min at room temperature. This medium was removed, the cells were rinsed three times with 10^{-3} M KI in PBS-II and then were processed through the GAG analysis (see above).

Materials: Plastic tissue culture roller bottles were purchased from Falcon Labware (Oxnard, CA); tissue culture dishes from Lux Scientific Corp. (Newbury Park, CA); 6-[^{3}H]-D-glucosamine HCl (22 Ci/mmole) and [^{35}S]-$NaSO_4$ were from Amersham International Ltd.; 1-[^{3}H]-D-mannose and Na[^{125}I] from New England Nuclear (Boston, MA); ribonuclease A, bovine pancreas deoxyribonuclease I, α-D-glucose, and Triton X-100 from Sigma Chemical Co. (St. Louis, MO); Pronase

and lactoperoxidase from Calbiochem-Behring Corp. (American Hoechst Corp., San Diego, CA); Chondroitinases ABC and AC from Miles Laboratories, Inc. (Elkhart, IN); testicular hyaluronidase and glucose oxidase from Worthington Biochemical Corp. (Freehold, NJ); Sepharoses CL-6B, CL-2B, 4B, and DEAE-Sephadex A-25 from Pharmacia Fine Chemicals (Piscataway, NJ). Hyaluronate, dermatan sulfate, chondroitin 4-sulfate, -6-sulfate, and heparan sulfate generously supplied by Drs. Matthews and Cifonelli of the University of Chicago.

RESULTS

Cellular Localization of I_0

Several experiments were performed to determine whether I_0 is only associated with neurite production or if it can be made by undifferentiated neuroblastoma cells under special circumstances. Neurite-producing and non-neurite-producing rat B104 neuroblastoma cells and two behavioral variants of the B104 line were examined. The parent B104 cell line is serum-sensitive (12), producing neurites only in the absence of FCS (Fig. 1 *a* and *b*). The variants are clonal lines selected from the parent line under specific sets of cell culture conditions for substratum adhesive differences (13). E_RB9 (Fig. 1 *d*) produces neurites constitutively and has lost the serum-sensitivity (serum inhibition of neurite outgrowth) of the parent line. The E_RA11 (Fig. 1 *c*) line will not produce neurites even in the absence of serum (serum starvation). Morphologically, the E_RB9 cells resemble B104 cells grown without serum (Fig. 1 *b* and *d*) while E_RA11 cells resemble serum-inhibited B104 cells (Fig. 1 *a* and *c*).

Column chromatographic profiles of cell and SAM fractions processed through the GAG/polysaccharide analysis scheme were examined. Neither E_RB9 nor E_RA11 SAM contained I_0 (Table I), but the neurite-containing E_RB9 cell fraction contained significant amounts of this species (Fig. 2*A*). The non-neurite-containing E_RA11 cell fraction had no I_0 (Fig. 2*A* and Table I). The appearance of I_0 in the cell fraction but not the SAM fraction of E_RB9 cells raised the possibility that the appearance of I_0 in the SAM of B104 neurite-positive cells (10) was the result of serum starvation. To test this, serum-sensitive B104 cells were grown in the chemically defined, serum-free

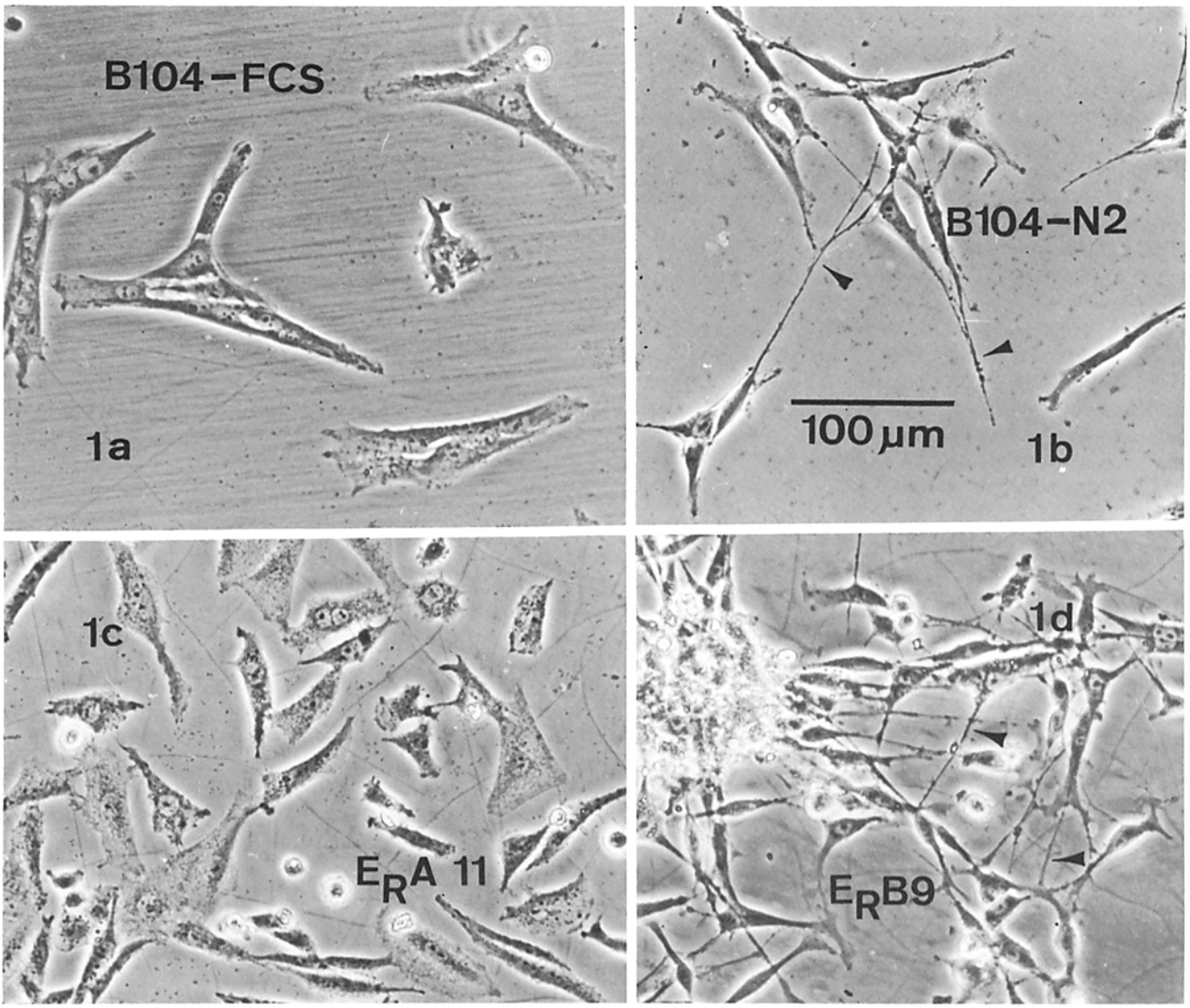

FIGURE 1 Phase contrast micrographs of rat neuroblastoma cells and their variants. (*a*) Small groups of rat B104 neuroblastoma cells shown in culture (*B104-FCS*) 24 h in FCS-containing medium. (*b*) Rat B104 neuroblastoma cells in culture 24 h in serum-free, chemically defined N2 medium (*B104-N2*). Arrowheads indicate some neurites. (*c*) *E_RA 11* neuroblastoma cells, a non-neurite-producing variant of B104 cell in culture 48 h in serum-containing medium. (*d*) *E_RB9* variant cells, constitutive neurite-producing variant of B104 cell 48 h with serum. Arrowheads indicate neurites. Bar, 100 µm. × 235.

TABLE I

Percentage I_0 in Total Polysaccharide Pools of Various Neuronal Tumor Cell Lines

Cell line/fraction	I_0	Cell line/fraction	I_0
	%		%
E_RB9/Cell	16.0	E_RA11 Cell	0
B104 N2/Cell	3.1	B104 N2/SAM	0
Platt/Cell P. 19	2.2	Platt/SAM P. 19	0
Platt/Cell P. 30	13.5	Platt/SAM P. 30	0
Platt/Cell-^{35}S P. 39	0	Platt/SAM-^{35}S P. 39	0

I_0 is tabulated as the percentage it comprises of the total polysaccharide containing material in a given sample. Cell and SAM fractions from B104 cells grown in N2 medium, E_RB9 and E_RA11 behavioral variant lines and Platt cells. The name of the cell line and fraction (cell or SAM) assayed are given. Passage (*P.*) numbers are indicated for the Platt entries. All samples were processed through the glycosaminoglycan analysis scheme and chromatographed on Sepharose-CL6B columns.

N2 medium. In this medium, the cells can both divide and produce neurites (15). Fig. 2*B* shows the cell and SAM chromatographic profiles from B104 cells grown in N2 medium. I_0 is present only in the cell profile. Therefore, the appearance of I_0 in the SAM of serum-starved B104 cells is probably the result of unusual conditions of cell-substratum adhesion. N2 culture medium conditioned by the B104 cells for 3–4 d was also processed through the GAG/polysaccharide analysis protocol. I_0 could not be detected, suggesting that it is either not secreted by the cells or is degraded into smaller species before release into the medium.

To determine whether I_0 is only a component of the rat cell lines, a human neuroblastoma line, Platt (14) was examined. This particular cell line was chosen by surveying three human lines for their ability to adhere to roller bottles in order to facilitate the preparation of sufficient amounts of material to permit chemical characterization. (The B104 rat cells will not adhere sufficiently well to roller bottles.) The Platt cell line produces neurites in the presence of serum. Fig. 3 shows the cell and SAM profiles from Platt (passage 30) cells. A large I_0 peak appears in the cell profile. When Platt cultures were serum-starved, I_0 remained in the cell fraction, unlike the serum-starved B104 cells which contain I_0 in their SAM (10).

There was a significant increase in the relative amount of I_0 with increasing passage of Platt cells (Fig. 4): a 600% increase between passage 19 and 30. The appearance of Platt cells (viewed by phase contrast microscopy) also changed noticeably between early (passage 15–19) and late passage (30–48) numbers (Fig. 5). The cells appeared to be more stellate, with smaller cell bodies, or longer neurites at higher passage numbers. In order to determine the nature of the difference, morphometric studies were done. The length of neurites and the cell body areas of cells from early and late passages were measured (Table II). Neurite length remained unchanged (± 2 SE), while the cell body area decreased significantly (~30%) with increasing time in culture. The measurements confirm the overall impression from the micrographs (see Fig. 5) that the cell body size has decreased. This increase in the relative amount of neurite to cell body could account for the increase in I_0 and strengthens the association of I_0 with neurite production.

To determine whether I_0 is a cell surface component, lactoperoxidase catalyzed iodination was performed and the cells were processed through the GAG/polysaccharide analysis. A Platt radioiodination profile is shown plotted against a Platt

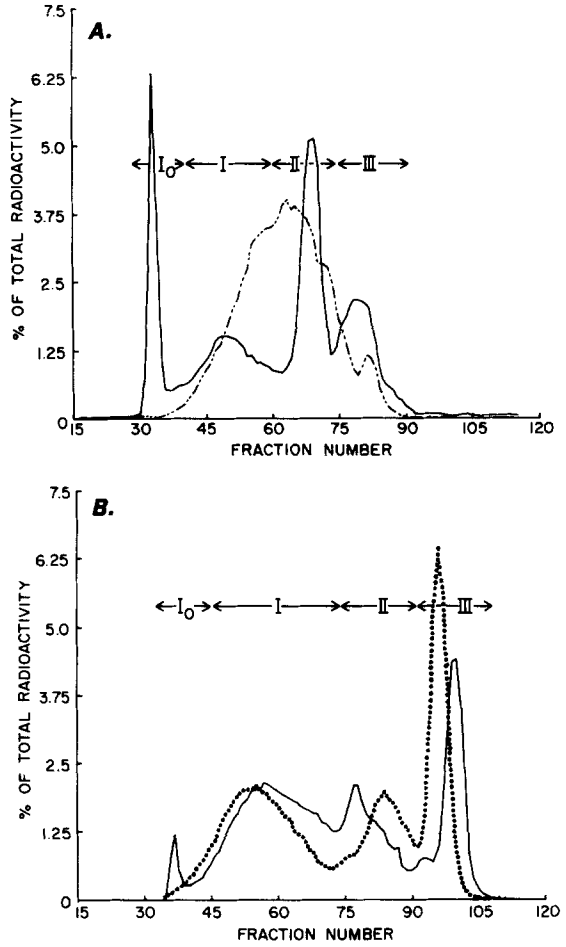

FIGURE 2 Gel filtration chromatography of polysaccharide fractions from three rat neuroblastoma cell lines. SDS-extracted material was processed through the glycosaminoglycan and polysaccharide analysis protocol and chromatographed on Sepharose-CL6B columns in SDS-Tris buffer pH 7.4 (see Materials and Methods). The exclusion (void) volume for Sepharose-CL6B chromatographic columns (see Materials and Methods) shown in Figs. 2–4 and 6–8 is at fraction 30–33; the inclusion (total) volume occurs at fraction 105–108. The elution profile data are plotted by computer: in each profile the radioactivity for that profile and the points were connected directly. (*A*) [^{3}H]-Glucosamine incorporation profile for cell fractions of behavioral variants of rat B104 neuroblastoma line: E_RB9 (——) and E_RA11 (- - - -). (*B*) [^{3}H]-glucosamine incorporation profiles of cell (——) and SAM (·····) fractions from B104 cells grown in chemically defined N2 culture medium (15). Region I_0 of the profile contains a large neurite-associated glycoconjugate; region *I* heparan sulfate; region *II* glycoprotein-derived glycopeptide and region *III* chondroitinase digestion products (17).

profile of [^{3}H]-glucosamine radiolabeled polysaccharide (Fig. 6). There is significant radioiodination of the I_0 peak indicating that I_0 is a cell surface component with a protein moiety accessible to iodination. The relative level of labeling of I_0 is lower in the radioiodination profile (protein) than in the [^{3}H]-glucosamine incorporation profile (polysaccharide). Most of the radioiodinated material is in the glycopeptide material (peak II) derived from iodinated cell surface glycoproteins.

Biochemical Characterization of I_0

An estimate of the size of I_0 was obtained by column chromatography on a Sepharose-CL2B column in SDS-Tris

buffer. I_0 metabolically radiolabeled with [^{3}H]-glucosamine eluted in the void (exclusion) volume of this column (data not shown), giving further indication of the large size of this glycoconjugate.

Several features of I_0 composition were accessible using metabolic radiolabeling with various precursor molecules. Incorporation of [^{3}H]-leucine shows that I_0 contains a protein moiety even after the Pronase digestion included in the GAG/polysaccharide analysis scheme (data not shown). When [^{35}S]-Na_2SO_4 radiolabeled material from Platt cells is processed for GAG/polysaccharide analysis, there are distinct sulfate-containing peaks in the heparan sulfate (*I*), glycopeptide (*II*), and

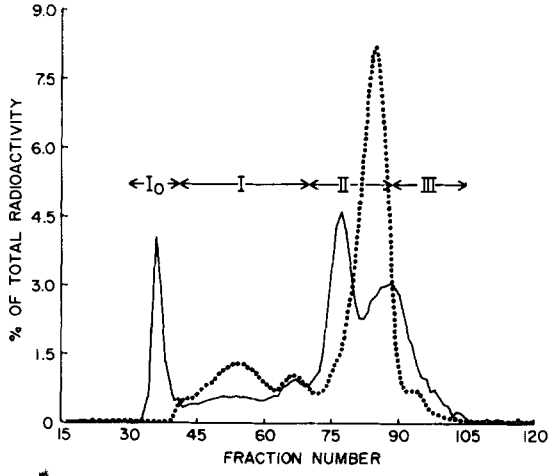

FIGURE 3 Gel filtration chromatography of human neuroblastoma polysaccharide fractions. Comparison of [^{3}H]-glucosamine incorporation for cell (——) and SAM (·····) fractions from passage 30 (*P. 30*) Platt neuroblastoma cells on SDS-eluted Sepharose-CL6B columns as described in Materials and Methods. Regions of profile described in legend of Fig. 2.

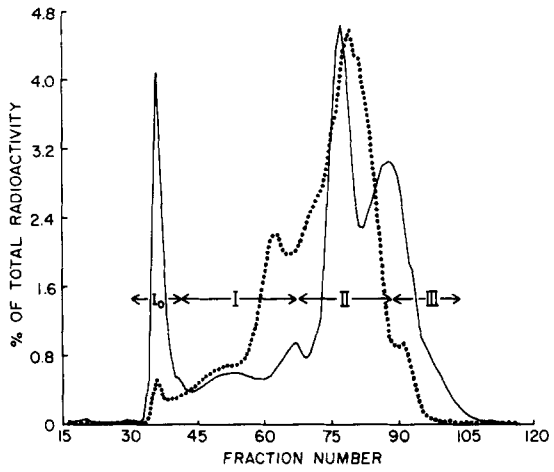

FIGURE 4 Changes in polysaccharide distribution with increasing passage number in human neuroblastoma cells. [^{3}H]-Glucosamine incorporation profile for cell fractions of "early" (passage 19: P.19 ····) and "late" (passage 30: P. 30——) passage Platt cells on SDS-eluted Sepharose-CL6B columns as described in Materials and Methods. Regions defined as in legend of Fig. 2.

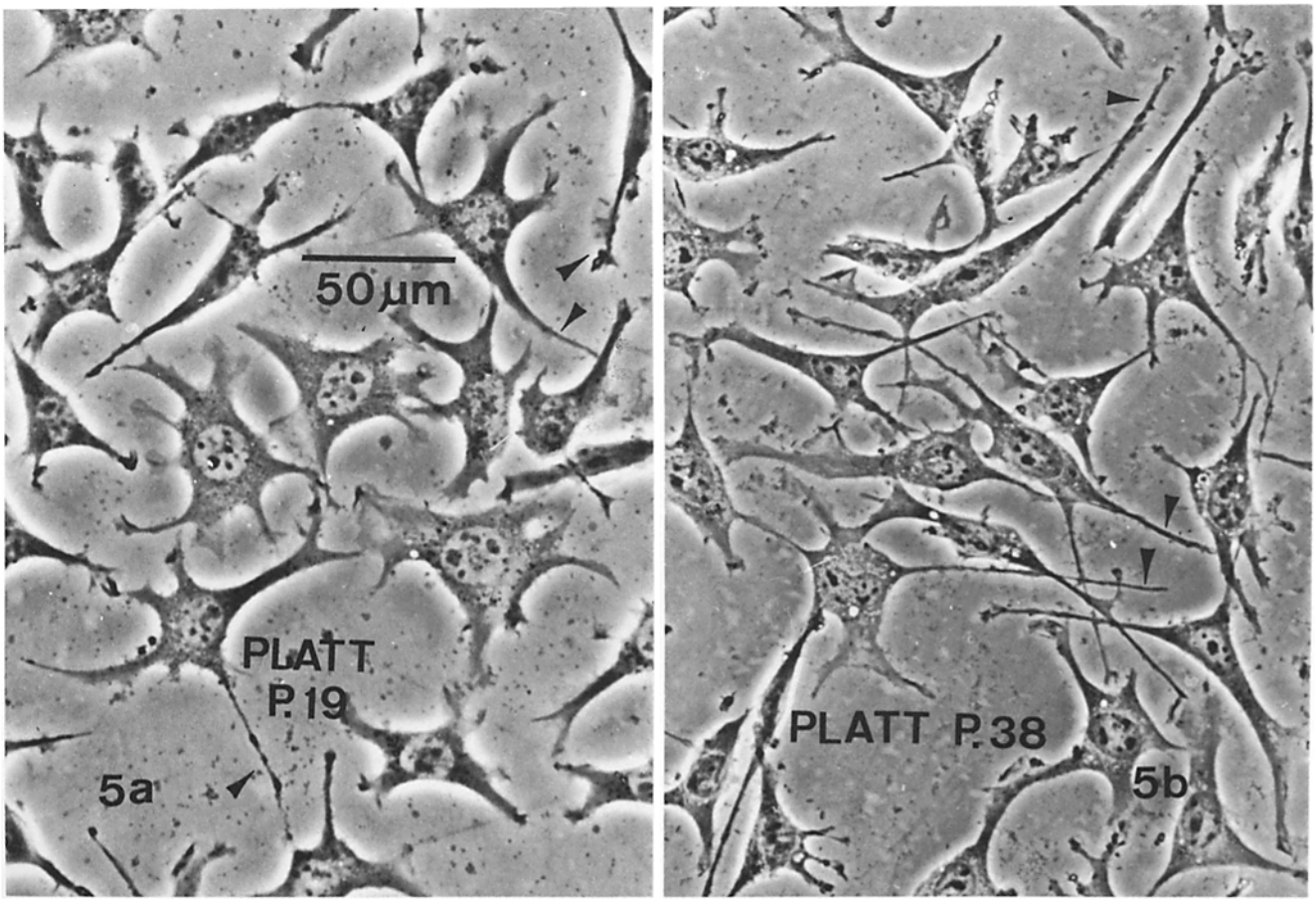

FIGURE 5 Phase contrast micrographs of low and high passage number human neuroblastoma cells. (*a*) "Early" passage (*P. 19*) Platt human neuroblastoma cells 48 h in culture. (*b*) "Late" passage (*P. 38*) Platt human neuroblastoma cells 48 h in culture Arrowheads indicate neurites. Bar, 50 μm. × 400.

TABLE II

Platt Cell Measurements

Passage number	Neurite Length ($\bar{X} \pm 2$ SE)	Cell area ($\bar{X} \pm 2$ SE)
15	43.5 ± 5.2 μm	1,005 ± 83 μm²
38	39.5 ± 4.0 μm	714 ± 100 μm²

Morphometric studies were performed using photomicrographs of cultures of early and late passage Platt cells. Cell areas and neurite length were measured. 100 measurements were made in each category.

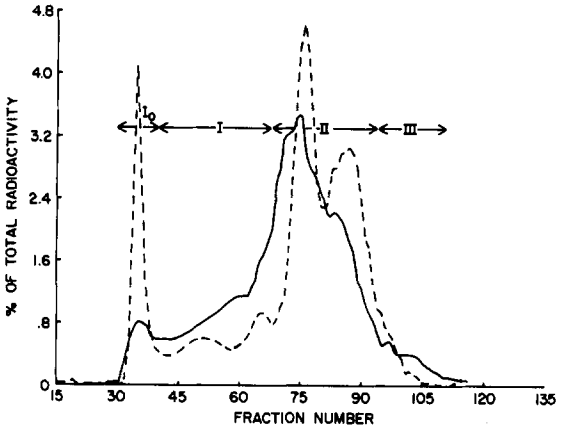

FIGURE 6 Lactoperoxidase-catalyzed radioiodination of I_0. Platt human neuroblastoma cells in culture 3 d were detached from the culture substratum using 0.5 mM EGTA in PBS, pH 7.4. EGTA was removed and cells were suspended in serum-free culture medium and incubated at 37°C for 30 min in an incubator shaker to permit repair to cell-surface membrane. The cells were then subjected to lactoperoxidase iodination as described in Materials and Methods. Iodinated cells were processed for polysaccharide analysis and chromatographed on SDS-eluted Sepharose-CL6B columns as described in Materials and Methods. Chromatographic profile of cell-surface material radiolabeled by ^{125}I-lactoperoxidase iodination (——) is shown with [^{3}H]-glucosamine (^{3}H-GlcN- - - -) profile obtained from metabolically radiolabeled late passage Platt cells in a separate experiment. Regions are defined as in legend of Fig. 2.

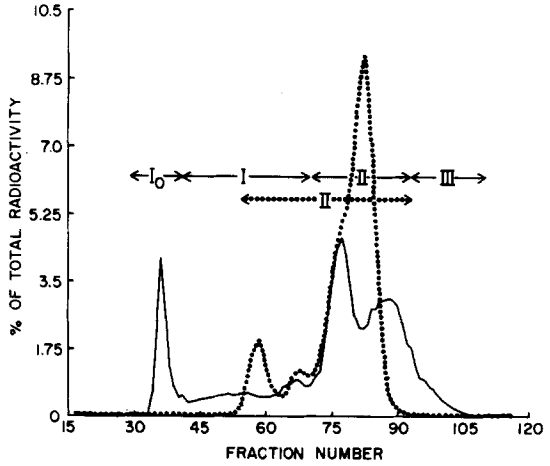

FIGURE 7 Incorporation of various precursors into human neuroblastoma polysaccharides. Comparison of [^{3}H]-mannose (····) and [^{3}H]-glucosamine (^{3}H-GlcN——) incorporation into polysaccharides. Material chromatographed on SDS-eluted Sepharose-CL6B columns as described in Materials and Methods. Regions defined as in legend of Fig. 2.

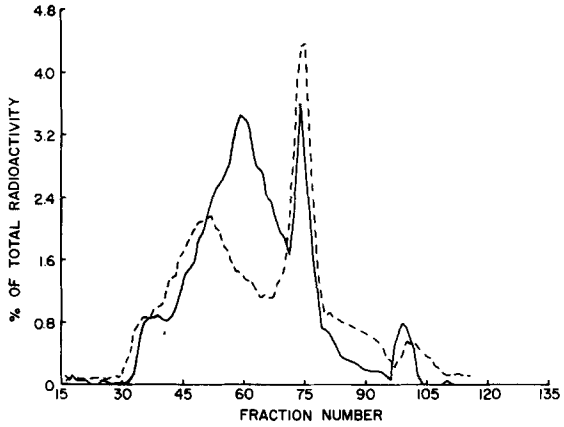

FIGURE 8 I_0 isolated from Platt or B104 cells by column chromatography (see Figs. 2 *B* and 3) was subjected to alkaline borohydride reduction for 24 h at 37°C (see Materials and Methods). The reduced material was re-chromatographed on Sepharose-CL6B columns in SDS-Tris buffer. Chromatographic profiles for alkaline reduced I_0 from Platt cells (——) and from B104 I_0 cells grown in N2 medium (B104/N2 - - - -) are shown.

chondroitin sulfate disaccharide (*III*) regions in the Sepharose-CL6B profile but I_0 is unsulfated (data not shown). Incorporation of [^{3}H]-mannose demonstrates two features (Fig. 7). First, there was no mannose incorporation into I_0, making it unlikely that I_0 contains appreciable numbers of N-glycosidic linkages to asparagine residues. Since there is no mannose in GAG's, all of the mannose incorporation must go into glycoprotein-derived glycopeptides concentrated in region II of the profile. Therefore, mannose incorporation also reveals minor glycopeptide peaks that are "buried" in the heparan-sulfate region in the conventional GAG profile resulting from glucosamine radiolabeling (peak *I*).

Alkaline borohydride reduction was performed on I_0 isolated from Sepharose-CL6B column preparations to determine whether O-glycosidic linkages exist between I_0 polysaccharides and serine or threonine residues in protein portions of the molecule. Fig. 8 shows reduction profiles for isolated [^{3}H]-glucosamine-radiolabeled I_0 from Platt and B104 cells. From both types of cells alkaline reduction yields three size classes of polysaccharide material: there are two major included peaks and a minor peak at the inclusion volume (fraction 105). The first major peak is heterodisperse, while the second is quite sharp (homogeneous). Pronase digestion (5 mg at 55°C for 24 h) yielded a very similar pattern, though the first major peak is not as large or distinct as in the alkaline reduction.

A survey was made of binding affinities of isolated I_0 to extracellular matrix ligands that could be important for neuronal cell adhesion. Material for affinity chromatography was prepared from [^{3}H]-glucosamine-labeled Platt or B104 cells using Sepharose-CL6B columns. SDS was removed from pooled peaks by dialysis to permit affinity chromatography. Gelatin-Sepharose affinity columns were run to test for binding of I_0 to collagen. Binding to the GAG's hyaluronate or the heparan sulfate analogue heparin was also tested. I_0 did not bind to any of these three ligands (data not shown). Binding to PORN-Sepharose was also tested and I_0 was found to have a strong affinity for PORN, eluting with 1 M NaCl (80–90% of I_0 loaded eluted with 1 M NaCl.)

Isolated I_0 was fractionated using DEAE-Sephadex A-25 ion exchange column chromatography to determine the number of components in the Separose-CL6B column void volume material and the relative charge properties of the components. The ion exchange columns were run in the presence of 0.2%

Triton-X 100 nonionic detergent to prevent nonspecific, irreversible binding to the Sephadex. Platt I_0 yielded peaks at 0.1 M, 0.5 M, and 0.8 M salt. The 0.8 M peak (24–26% of total radioactivity) is contaminating heparan sulfate from region I in the GAG analysis (sensitive to nitrous acid). The major fraction of Platt I_0 binds weakly to the ion exchange resin (it is weakly anionic), eluting at a salt concentration of 0.1 M.

DISCUSSION

In this paper we have correlated the presence of I_0, a large glycoconjugate, with neurite production using a variety of approaches. Studies on neurite-plus or -minus behavioral variants of the B104 rat neuroblastoma cell line, on B104 cells grown in serum-free chemically defined culture medium and on a human neuroblastoma cell line show that I_0 is a cell surface component normally present in the cell fraction of neuronal tumor cells from two species. We have also isolated and partially determined the composition of I_0 and characterized some of its binding affinities. I_0 is a very large glycoconjugate, possibly a glycoprotein, with O-glycosidic protein-polysaccharide linkages. It is unsulfated, negatively charged, and has a strong binding affinity to polyornithine.

Neurite-producing neuroblastoma cells were found to produce a type of SAM morphologically distinct from SAM of non-neurite-producing neuroblastoma cells, with the suggestion that this represents growth cone SAM (9). The initial observation (10) of a large glycoconjugate (termed I_0) in the SAM of neurite-producing ($B104^+$) rat neuroblastoma cells suggested the association of this material with neurite production and, possibly, growth cone adhesion. Two sets of experiments here reinforce the association of I_0 with neurite production. The E_RB9 and E_RA11 variants of the B104 rat cells separate neurite production from the serum sensitivity of the parent B104 line. I_0 occurs in the neurite-plus E_RB9 cells and is absent from the neurite-minus E_RA11 cells. In the constitutive neurite-producing human neuroblastoma cell line Platt, a change in the cells with time in culture also correlates I_0 with neurite production. Cell morphology changed so that there was an increase in the relative proportion of neurite to cell body material. This change was accompanied by a several-fold increase in the amount of I_0 in the polysaccharide analysis. Culturing the B104 parent rat neuroblastoma cells in serum-free, chemically defined N2 medium (15) permitted growth of these cells under conditions where they can both divide and produce neurites. This provided another way neurite outgrowth could be separated from the effects of serum-starvation. Under these conditions, I_0 occurred in the cell fraction, not in SAM, unlike the situation with serum-starvation. It seems likely that serum-starvation created unusual conditions for neurite adhesion that caused deposition of I_0 into SAM.

The "normal" occurrence of lactoperoxidase iodinatable I_0 on the surface of these neuronal tumor cells raises several questions. If I_0 occurs on the cell surface and is not normally in SAM, is it involved in growth cone adhesion? It is possible that I_0 is involved in cell-substratum adhesion but is released from the neurite surface instead of being left behind in SAM during EGTA treatment. When conditioned medium was examined, no I_0 was found, however. A remaining possibility is that I_0 is broken down before release from the cell surface.

Molecular sieve column chromatography shows I_0 to be a very large molecule; the nominal void volume for highly glycosylated material on Sepharose-CL2B is 20×10^6 mol wt. This could still be an overestimate of I_0 size due to hydration of polysaccharide chains and conformation of the molecule (25). But even if the nominal size of I_0 from column chromatography is an overestimate, its appearance in the exclusion volume of Sepharose-CL2B columns eluted with SDS-containing buffer indicates that I_0 is much larger than previously described neurite-associated glycoproteins, such as the sodium channel component (about 200,000 mol wt) or the neural cell adhesion molecule isolated from brain (200,000–250,000 mol wt, forming aggregates $0.5–1.2 \times 10^6$ mol wt) (26, 27). Large glycoconjugates are found in extracellular matrix material and associated cell surface components and in exudates of embryonic cells (28–30).

Metabolic radiolabeling shows I_0 to be glycosylated ([^{3}H]-glucosamine) and unsulfated in its polysaccharide portion ([^{35}S]-Na_2SO_4), and it does not contain mannose ([^{3}H]-mannose). [^{3}H]-Leucine incorporation, alkaline borohydride reduction and Pronase digestion of isolated I_0 indicated the presence of amino acid residues in the molecule and it proved to have protein accessible to lactoperoxidase iodination. Alkaline borohydride reduction, plus the absence of mannose residues, shows the existence of O-glycosidic linkages. This type of protein-polysaccharide linkage is present in GAG-protein linkages and in mucin-type glycoproteins. I_0 appears in polysaccharide analysis profiles at the high molecular weight end of the heparan sulfate-containing region. The various steps of the GAG/polysaccharide analysis protocol, and the lack of sulfation, make it unlikely that I_0 is a conventional GAG, however. I_0 is resistant to chondroitinase ABC (digesting CS) and testicular hyaluronidase (digesting HA) and nitrous acid deamination (highly N-sulfated HS).

I_0 did not bind to gelatin- or GAG-Sepharose affinity columns, suggesting that collagen and GAG's would not be involved in I_0/extracellular matrix interactions. Description of a mechanism by which I_0 might participate in neuronal cell adhesion requires further biochemical characterization of this material. I_0 has, on the other hand, a very strong binding affinity to PORN. This has several possible implications. PORN is a polycationic polypeptide (1) for which neuronal cells and growth cones show strong adhesion. The neurite-promoting factors described in the ciliary ganglion studies (2, 4–6) are active only when bound to polyornithine. The PORN affinity of these neurite-promoting factors, neuronal cells, and I_0 might simply involve a net negative charge of cell surface components or the molecules under investigation, or it could have functional significance. PORN may substitute for extracellular matrix or cell surface components that orient neurite-promoting factors or growth cone adhesion components in vivo or in other in vitro situations. In addition, when enough material can be isolated it will be possible to test the neurite-promoting ability of I_0 bound to PORN-coated culture substrata. This may show a functional relationship between the neurite-associated glycoconjugate that we have isolated and characterized and the neurite-promoting factors described in other studies.

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