Adhesion Sites of Neural Tumor Cells: Biochemical Composition†

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ABSTRACT: Since accumulating evidence indicates that the behavior and differentiation of neuronal cells is controlled to some extent by their interactions with extracellular matrices, we have initiated studies into the biochemical mechanism by which various neural cell derivatives adhere to their tissue culture substratum. Purified cold-insoluble globulin from bovine or human serum will substitute for serum by binding to dishes and promoting "normal" spreading and movement of neuroblastoma and glioma cells. Detachment of rat or mouse neuroblastoma cells or rat C6 glioma cells with the Ca^{2+} chelator EGTA leaves cell–substratum adhesion sites firmly bound to the serum-coated tissue culture dish for subsequent compositional analysis. Adhesion sites from neuroblastoma and glioma cells are enriched in some cytoskeletal proteins which differ in distribution between neuroblastoma and glioma cells and under various physiological conditions of adherence. There is also an enrichment in these sites for a cell surface 220K glycoprotein which appears to be fibronectin-like as determined by immunochemical criteria. These sites are also enriched in various glycosaminoglycans—their distribution in adhesion sites is different from that in other cell fractions. Neurite-containing neuroblastoma cells deposit a unique high molecular weight polysaccharide in their adhesion sites. The more motile glioma cells deposit much more hyaluronate and chondroitin in their adhesive material than the less motile neuroblastoma cells. Newly attaching glioma or neuroblastoma cells generate adhesion sites enriched in heparan sulfate and glycoprotein; as cells begin to spread and move on the substratum, hyaluronate and the various chondroitins accumulate in this adhesive material. These results suggest that the biochemical mechanism of adhesion of these neural cell derivatives to an extracellular matrix is similar to that of fibroblasts but with some differences that are related to the differentiated properties of these neural cell derivatives.

Considerable attention has been focused in recent years on the molecular mechanism of fibroblast adhesion to extracellular matrices (Culp, 1978; Grinnell, 1978). These processes appear to be very similar for cells adhering to either collagen or tissue culture dishes and are mediated by cold-insoluble globulin adsorbed to the inert matrix and by cell surface fibronectin and proteoglycans (Culp et al., 1979). Since interaction with extracellular matrices is important for various physiological functions of neuronal and glial cells (Bunge & Bunge, 1978; Letourneau, 1975), such as neurite extension of neuronal cells, we have initiated studies on how neuronal cell derivatives, specifically neuroblastoma and glioma cell lines, adhere to tissue culture dishes. We have isolated and examined the biochemical composition of the cell–substratum adhesion sites (substrate-attached material) of these cells under a variety of physiological conditions. When cells are detached by EGTA[1] treatment, cell bodies round up as a result of cytoskeletal disorganization resulting from Ca^{2+} chelation; they pull away from their substratum adhesion sites and the labile retraction fibers break, liberating cells into suspension and leaving adhesion sites as substrate-attached material (Rosen & Culp, 1977; L. A. Culp and C. Domen, unpublished experiments). Adherence of these neural cells in general appears to be mediated by biochemical processes similar to those observed in fibroblasts, with the exceptions of cell type specific differences in relative amounts of various cytoskeletal proteins and appearance of a unique polysaccharide in adhesion sites of neurite-containing cells.

Materials and Methods

Mouse neuroblastoma C1300 (N115 clone) and rat glioma C6 (both the C62B- and BUdR-resistant C6Bu1 clones were used and generated the same results reported here) cells were obtained from Dr. Gordon Sato, University of California at San Diego (Augusti-Tocco & Sato, 1969; Bottenstein & Sato, 1979; Schubert et al., 1969). Rat neuroblastoma B104 cells were obtained from Dr. David Schubert of the Salk Institute (Schubert et al., 1974). The glioma cells displayed biochemical markers of glial origin, while the neuroblastoma cells extend neurites in serum-free medium and display neurochemical markers indicating their neuronal origin (Rosenberg et al., 1978; Schubert et al., 1974, 1969). All cells were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum, 250 units/mL penicillin, and 250 μg/mL streptomycin at 37 °C in an 10% CO_2–humidified air mixture. For generation of neurite-containing neuroblastoma cultures, the cell layer was rinsed twice with phosphate-buffered saline and fed the same medium as above but without serum; 24 h later, ~70–80% of the cells contained neurites and the cultures were harvested for appropriate analysis. A variety of tests were used to show that these cell lines were not contaminated with *Mycoplasma*.

Metabolic Labeling. Cells were fed complete medium supplemented with 2 μCi/mL [4,5-^{3}H]leucine (New England Nuclear Corp.; sp act. 60 Ci/mmol) to radiolabel proteins or with 5 μCi/mL [6-^{3}H]-D-glucosamine hydrochloride (New England Nuclear Corp.; sp act. 19 Ci/mmol) to radiolabel polysaccharides. Labeling over a 72-h period of exponential growth of cultures brought the cells' specific activity to a

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[1] Abbreviations used: CIg, cold-insoluble globulin (the serum-derived molecule related to, but slightly different from cellular fibronectin); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetate; GAG, glycosaminoglycan; GAP, glycosaminoglycan-associated protein; $NaDodSO_4$, sodium dodecyl sulfate; PBS, phosphate-buffered saline without divalent cations; PBS-II, phosphate-buffered saline plus 100 mg/L $CaCl_2$ and $MgSO_4 \cdot 7H_2O$.

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constant level without adversely affecting cell growth. For isolation of fractions from neurite-containing cells, the cultures were grown in radioactive precursor- and serum-containing medium for 48 h, rinsed twice with PBS, and refed with precursor-containing serum-free medium for 24 h to allow neurites to form. Cell surface iodination with lactoperoxidase was performed as described by Cathcart & Culp (1979) under conditions where only cell surface proteins were radiolabeled and cell viability was not affected. None of the radiolabeling conditions were deleterious to cell viability (by staining and plating efficiency assays).

Preparation of Substrate-Attached Material. Substrate-attached material was isolated as described previously (Culp et al., 1978). In brief, the cell layer was rinsed twice with PBS (divalent cation free) and incubated with 5 mL of 0.5 mM EGTA in PBS [plus 1 mM phenylmethanesulfonyl fluoride (PMSF)] per 100 mm diameter dish with gentle shaking at 37 °C for 30 min. Most cells had detached during this treatment and the remainder were detached by gentle pipetting. Substrate-attached material was rinsed twice with PBS and once with distilled water before quantitatively solubilizing it in 5 mL of 0.2% $NaDodSO_4$ (w/v) (plus 1 mM PMSF) per 100-mm dish by gentle shaking at 37 °C for 30 min. For $NaDodSO_4$–polyacrylamide gel electrophoretic analysis of proteins or glycoproteins, 20–30 100 mm diameter dishes of material were harvested; for carbohydrate analyses, 60–80 dishes were harvested. It was shown previously that fibroblast substrate-attached material is not appreciably contaminated by cellular proteins and polysaccharides secreted into the medium or solubilized by EGTA treatment and then artifactually bound to the substratum (Culp, 1978); similar results were obtained with these neural cells (data not shown). After 72 h of radiolabeling, this substrate-attached material is referred to as *long-term* adhesion sites and contains both adhesion sites that bound the cells to the substratum at the time of the EGTA treatment as well as "footprints" which are adhesion sites pinched off at the cell's trailing edge during natural cell movement (Culp, 1978). To obtain newly-formed adhesion sites free of "footprint" adhesive material, we allowed EGTA-detached and -rinsed cells to reattach to fresh dishes in serum-containing medium for 1 h [this is sufficient time for fibroblasts or these neural cells to generate mature footpad adhesion sites (Rosen & Culp, 1977; L. A. Culp and C. Domen, unpublished experiments)]; the cells were then detached by EGTA treatment, and substrate-attached material was harvested as described above with 0.2% $NaDodSO_4$ (referred to as *reattaching* adhesion sites). The *cell* fraction was obtained by pelleting the EGTA-suspended cells by centrifugation (600*g* for 10 min), resuspending the pellet in PBS-II, and pelleting them again by centrifugation. All of these fractions were treated as described below for various analyses.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. Cell and substrate-attached fractions were analyzed under reducing conditions by $NaDodSO_4$–polyacrylamide gel electrophoresis as described previously (Culp et al., 1978). In brief, the cell pellet was suspended in 0.5 mL of electrophoresis sample buffer (15% glycerol, 0.2% $NaDodSO_4$, 1% mercaptoethanol, 0.075 M Tris–sulfate, pH 8.4, and 0.001% bromphenol blue) containing 1 mM PMSF and dialyzed against this buffer overnight. Substrate-attached fractions were concentrated by vacuum dialysis to a volume of 0.2–0.3 mL and dialyzed against sample buffer overnight. Samples were boiled for 5 min prior to slab gel electrophoresis, which was performed by the Ortec method (Ortec Inc., Oak Ridge, TN) on 10% gels. All samples being compared were run on the same gel. Gels were stained and fluorographed by the quantitative method as described previously (Culp et al., 1978).

Glycosaminoglycan Analysis. These procedures are described in detail by Rollins & Culp (1979a) as originally developed by Cohn et al. (1976). Briefly, the pellet of EGTA-suspended cells (~ 2–4×10^8 cells) was resuspended in 3 mL of PBS-II, frozen–thawed 3 times, and incubated with 100 μg/mL each of DNase I and RNase at 37 °C for 5 h. The extract was then made 0.2% (w/v) in $NaDodSO_4$. Five milligrams of Pronase was added for a 24-h incubation at 55 °C, followed by a second 5-mg addition for another 24 h. Twelve micrograms of carrier hyaluronic acid (HA), heparan sulfate (HS), chondroitin 4-sulfate (C4S), chondroitin 6-sulfate (C6S), and dermatan sulfate (DS) were added, and the extract was exhaustively dialyzed against glass-distilled water. The GAG's were precipitated from solution by addition of 3 volumes of 95% ethanol–1% potassium acetate; the pellets were dried in vacuo overnight before being redissolved in water and two more precipitation processes were performed. The substrate-attached fractions were treated as described above, except that the RNase–DNase treatment is not performed and only 2-mg quantities of Pronase were used.

The polysaccharide was quantitatively digested with chondroitinase ABC to determine the amounts of glucosamine-radiolabeled hyaluronate and chondroitin sulfate derived $\Delta^{4,5}$-unsaturated disaccharides subsequent to paper chromatography as described previously (Rollins & Culp, 1979a). For determination of dermatan sulfate content, an equivalent amount of polysaccharide was digested with chondroitinase AC (which does not hydrolyze DS), and the difference in the amounts of C4S disaccharide was taken as the DS content. For determination of heparan sulfate and glycoprotein-derived glycopeptide content, the chondroitinase ABC digest was passed through a Sepharose CL6B column to separate HS chains, glycopeptide, and the chondroitinase-liberated disaccharides (1.2 × 120 cm); only some of the HS was labile to nitrous acid as shown previously (Rollins & Culp, 1979a) which cleaves HS chains at N-sulfated residues.

Materials. The following materials were used: plastic tissue culture dishes from Lux Scientific Corp., Newbury Park, CA; DMEM from Grand Island Biological Co., Grand Island, NY; fetal calf serum from Kansas City Biologicals; [4,5-^{3}H]leucine and [6-^{3}H]glucosamine hydrochloride from New England Nuclear Corp., Boston, MA; $NaDodSO_4$, acrylamide and bis(acrylamide) from Bio-Rad Laboratories, Richmond, CA; authentic standards of $\Delta^{4,5}$-unsaturated disaccharides from Miles Laboratories Inc., Elkhart, IN; chondroitinase ABC or AC from Seikagaku Fine Chemicals, Inc., Tokyo, Japan. Hyaluronate, dermatan sulfate, choindroitin 4-sulfate, chondroitin 6-sulfate, and heparan sulfate were generously supplied by Drs. Matthews and Cifonelli of the University of Chicago.

Results

Adherence of cells in a physiologically compatible fashion to an inert tissue culture substratum is stringently dependent upon a specific receptor glycoprotein (Culp, 1978; Grinnell, 1978); in the case of fibroblasts, this is cold-insoluble globulin in the substratum-bound serum layer. We tested the ability of neuroblastoma or glioma cells to adhere properly to CIg-coated substrata. As demonstrated in Figure 1, rat B104 neuroblastoma cells adhere and spread effectively on CIg- or serum-coated substrata but not on a bovine albumin coated substratum. This was observed at 2 h (figure 1A,B,C) or 24 h (figure 1D,E,F) after replating EGTA-detached cells. The CIg-attached cells had a propensity to extend neurites (arrow in Figure 1E) in serum-free medium that was not observed

FIGURE 1: B104 neuroblastoma adherence to a CIg-coated substratum. Sixty-millimeter tissue culture dishes were treated at 37 °C for 1 h with 5 mL of 10% fetal calf serum in medium (A and D), 10 μg/mL bovine CIg in serum-free medium (B and E) and purified according to the modified procedure (Engvall et al., 1978) by affinity chromatography on gelatin–Sepharose, or 1 mg/mL bovine serum albumin in medium (C and F); the dishes were then rinsed several times with PBS before receiving 5 mL of protein-free medium. B104 cells were detached by EGTA treatment from other serum-containing cultures as described under Materials and Methods, pelleted by centrifugation, and washed twice with PBS before suspending in protein-free medium. 1×10^6 cells were inoculated into all dishes, except for the dish in (D) which received 2×10^6 cells. For photomicrographs, the cells were rinsed twice with PBS, fixed with trichloroacetic acid, and stained with Coomassie blue. (A) Serum coated, 2 h; (B) CIg coated, 2 h; (C) albumin coated, 2 h; (D) serum coated, 24 h; (E) CIg coated, 24 h; (F) albumin coated, 24 h. The arrow in (E) indicates a neurite extension of the type that become prominent on CIg-adherent cells. (Magnification was 57.2×.)

in the serum-attached cells, particularly by 48 h. The albumin-attached cells never spread and detached within 24 h; the same effects for albumin were noted by using either 10 μg/mL or 1 mg/mL for adsorption. Similar responses to the CIg coating were observed for the mouse C1300 neuroblastoma and rat C6 glioma cells. CIg's from either human plasma or bovine serum were equally effective in mediating these effects at a maximal concentration of 5–10 μg/mL. CIg has also been found effective in long-term culturing of neuroblastoma cells in serum-free, hormone-supplemented medium (J. Bottenstein and G. Sato, personal communication).

EGTA-Mediated Detachment. Both neuroblastoma and glioma cells were easily and quantitatively detached from tissue culture dishes by treatment with 0.5 mM EGTA in PBS for 30 min at 37 °C with gentle shaking and pipetting of the suspension over the surface of the dish. The substrate-attached material was then completely solubilized by $NaDodSO_4$ treatment as shown previously for fibroblasts (Cathcart & Culp, 1979) and subsequently for these neural cells (data not shown) and quantitated by precipitation with trichloroacetic acid. Scanning electron microscopy studies (L. A. Culp and C. Domen, unpublished experiments) indicate that substrate-attached material is composed of footpad adhesion sites of these neural cell derivatives by which they adhere to the substratum. Approximately 1–2% of the cell's total protein content was left as substrate-attached material. However, 4–7% of the glucosamine-radiolabeled polysaccharide was observed in this fraction. These adhesion sites are therefore enriched in a polysaccharide-rich surface region of cells, a conclusion reached previously for fibroblasts (Culp, 1978). No appreciable RNA or DNA was detected in this fraction. The amounts of substrate-attached protein and polysaccharide varied slightly between neurite-containing and nonneurite-containing neuroblastoma cells. Although glioma cells left a comparable amount of protein in their adhesion sites, there was slightly more polysaccharide (6–8%) in glioma adhesion sites when compared to neuroblastoma adhesion sites.

Adhesion Site Composition: Protein. $NaDodSO_4$–polyacrylamide gel electrophoretic analysis was used to define the complexity of the protein components in substrate-attached material (Figure 2). Both C6 and B104 substrate-attached fractions contained many similar components which are enriched in this fraction of the cell. Two leucine-radiolabeled bands in the 5% stacking gel (GAP-1 and -2) coelectrophorese with prominent glycosaminoglycan polysaccharide bands (see below) and are referred to as GAG-associated proteins (although some of the material in the GAP-1 band is denatured protein which becomes trapped at the gel interface). Biochemical evidence indicates that these are true proteoglycan species (Rollins & Culp, 1979b). When compared to enriched surface membrane preparations, substrate-attached material from both cell types (figure 2A) is enriched in a 220K protein (C_0) which appears to be a glycoprotein (see below) and several proteins (C_b; C_c; C_1; C_2) which are not glycosylated and which coelectrophorese with prominent fibroblast cytoskeletal proteins (Culp, 1978; Culp et al., 1979). Immunochemical and biochemical criteria [such as two-dimensional O'Farrel gels (O'Farrel, 1975) and peptide mapping] have identified C_a as myosin, C_1 as a 10 nm filament subunit protein, C_2 as actin, and C_b as some protein other than α-actinin (data not shown). Substrate-attached fractions from C62B or C6Bu1 cells were identical, while mouse neuroblastoma substrate-attached material generated $NaDodSO_4$–polyacrylamide gel electrophoretic profiles very similar to that of B104.

The most prominent difference in the substrate-attached fractions from the two cell types is noted in the C_1 region of Figure 2A and which is further amplified in Figure 2B. Neuroblastoma material contains a very prominent band at 55 K (C_{1b}), whereas the glioma material does not contain a 55K band but a more minor band (relative to the C_2 actin band) at 57K (C_{1a}). The substrate-attached materials from these two cell types also differ in another prominent protein, C_x (50K), which is not observed in nonneurite B104 material but is quite prominent in glioma material. However, the neurite-containing B104 fraction does contain a prominent C_x band (figure 2B, well 4). Also, if B104 cells were grown on a substratum coated with *nonradioactive* C6 substrate-attached material in medium containing radioactive leucine, the radiolabeled adhesion sites deposited by the B104 cells contained a prominent radioactive C_x band (figure 2B, well 3), although these cells did not extend neurites when grown on the coated substratum. A similar result was obtained when B104 cells were grown on substrata coated with B104 substrate-attached material (data not shown). These proteins are probably cytoskeletal since they are not glycosylated, since they are prominent components of Triton cytoskeletons (Brown et al., 1976) of these cells (data not shown), and since they coelectrophorese on one-dimensional or two-dimensional gels with the different and prominent 10 nm filament subunit proteins of glial or neuronal cells (Goldman et al., 1978; Liem et al., 1978). Electrophoresis of proline-radiolabeled adhesive material from glioma or neuroblastoma cells gave similar profiles

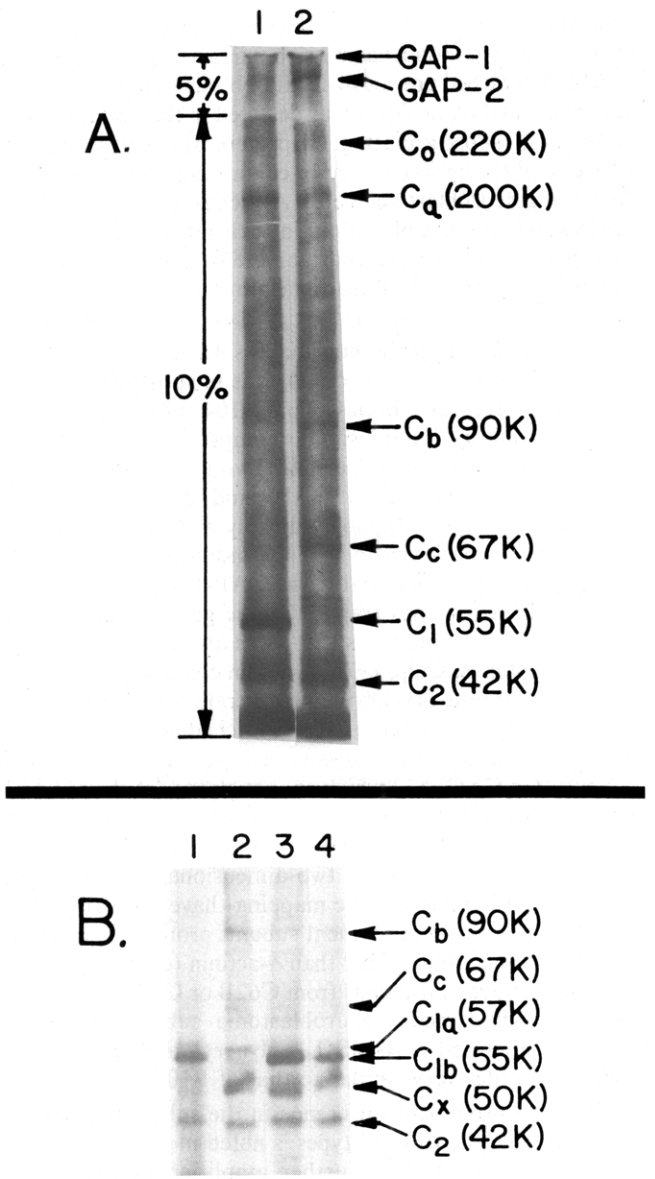

FIGURE 2: $NaDodSO_4$–polyacrylamide gel electrophoretic gel of neuroblastoma and glioma adhesion sites. B104 or C62B cells were grown over a 72-h period in medium containing [^{3}H]leucine and EGTA detached as described under Materials and Methods. Substrate-attached material was isolated by $NaDodSO_4$ solubilization, concentrated, and electrophoresed (10000 cpm of each sample) after thiol reduction on a 10% Ortec slab gel as described under Materials and Methods. Fluorograms of the gels are shown. (A) Well 1, nonneurite B104$^-$ substrate-attached material; well 2, C62B substrate-attached material. (B) A detailed region of the gel fluorogram where cytoskeletal proteins separate in the following samples: well 1, nonneurite B104$^-$ adhesion sites; well 2, C62B adhesion sites; well 3, nonneurite B104$^-$ adhesion sites from cells grown on a substratum coated with C62B substrate attached material (obtained by prior growth of nonradioactive C62B cells to confluence and EGTA-mediated detachment); well 4, neurite-containing B104$^+$ adhesion sites. The locations of various proteins which have been characterized in fibroblasts (Culp, 1978) are indicated to the right in (A): GAP, glycosaminoglycan-associated protein; C_o coelectrophoreses with CIg; C_a, mysosin; C_1, 10 nm filament subunit protein; C_2, actin [see the text and Culp et al. (1979) for evidence that these are specific cytoskeletal proteins]. Molecular weight markers were used to size proteins, including rabbit skeletal muscle actin and myosin, chick ovalbumin, bovine serum albumin, and *Escherichia coli* β-galactosidase.

and failed to reveal any collagen-like polypeptides in these sites.

Substrate-attached material from both cells types is enriched in a 220K cellular glycoprotein (Figure 2A; also see below)

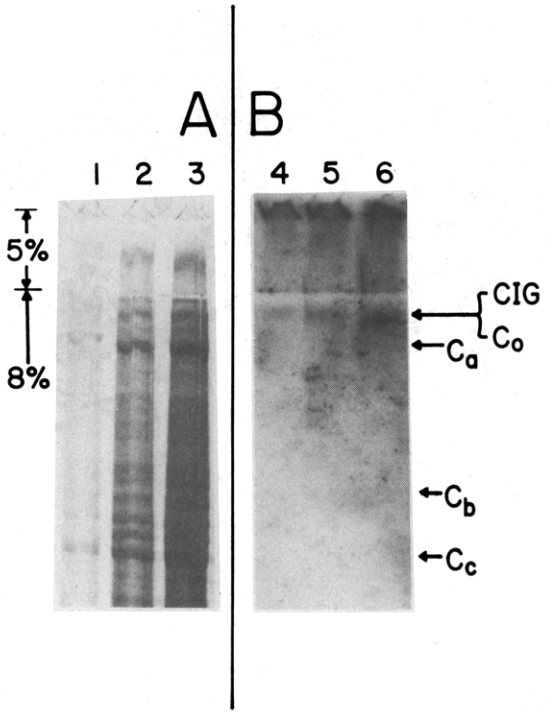

FIGURE 3: Antifibronectin binding to the C_o band from B104 adhesion sites. Nonneurite B104$^-$ cells were grown in complete medium until they were ~70% confluent. Cells were EGTA detached, and the nonradioactive adhesive material was harvested with $NaDodSO_4$ and concentrated as described under Materials and Methods. Several sets of samples of substrate-attached material (wells 1 and 4, 5 μg of total protein; wells 2 and 5, 10 μg; wells 3 and 6, 20 μg) were electrophoresed under reducing conditions on the same 8% Ortec slab gel, along with bovine and human CIg control samples (not shown). One set of samples was Coomassie-stained and the top half of the gel is shown in panel A. Other sets of samples were treated with solvents to extract the $NaDodSO_4$ from the gel as described by Adair et al. (1978) for antibody binding studies. One set of gel samples was treated with 20 μg/mL rabbit preimmune IgG (in PBS containing 100 μg/mL ovalbumin plus sodium azide) by gentle rocking at room temperature for 24 h as described by B. A. Murray and L. A. Culp (unpublished experiments); a second set of samples was treated with 20 μg/mL rabbit antihuman CIg, which had been affinity purified on a human CIg-Sepharose column and subsequently absorbed with bovine CIg (this absorbed adsorbed antibody was reactive with human, rat, or mouse CIg but not with bovine CIg; therefore, bovine CIg absorbed in the serum layer of these cultures and subsequently $NaDodSO_4$ extracted in the substrate-attached samples will not be detected). Both sets were rinsed well over several days with PBS to extract unbound IgG and then treated for 24 h with 5 μCi of ^{125}I-labeled staphylococcal protein A (Amersham Corp.; 30 mCi/nmol) in 20 mL of PBS (plus 100 μg/mL ovalbumin and sodium azide). The gels were rinsed well, dried, and autoradiographed for 4 weeks to detect ^{125}I-labeled protein A–antibody–antigen complexes as described by Adair et al. (1978). Panel B shows the autoradiogram of substrate-attached samples treated with bovine CIg absorbed antihuman CIg. The staining at the top of the gel in panel B was nonspecific and was observed with any preimmune or immune IgG used.

which can be detected in Coomassie-stained preparations (Figure 3A) and which coelectrophoreses with purified CIg from human or bovine serum. To determine if this B104 cell-synthesized glycoprotein is fibronectin-like, we tested its ability to bind antihuman CIg, which had been absorbed with bovine CIg, directly in the $NaDodSO_4$–polyacrylamide gel electrophoretic gel as described by Adair et al. (1978) using radioiodinated staphylococcal protein A; this absorbed antibody was reactive with rat or mouse serum CIg but not with bovine CIg, which is a component of the substratum-bound serum layer (data not shown).

This absorbed antibody does bind effectively to the C_o band of B104 substrate-attached material (Figure 3B) as demon-

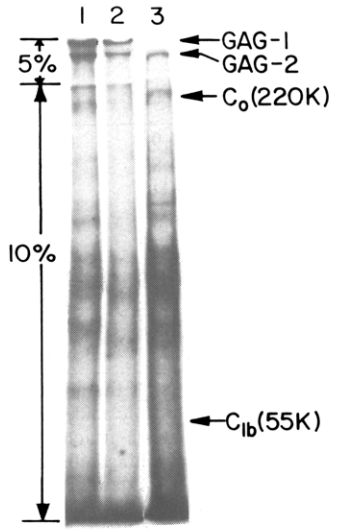

FIGURE 4: $NaDodSO_4$–polyacrylamide gel electrophoresis of glucosamine-radiolabeled B104 cell and adhesion site samples. B104 cultures were grown over a 72-h period in medium containing [^{3}H]glucosamine. Cells were EGTA detached and processed for electrophoresis as described in the legend to Figure 2. Substrate-attached samples were processed as described under Materials and Methods. The following samples (5000 cpm of each) were compared on the same 10% Ortec slab gel run under reducing conditions by fluorography: well 1, nonneurite B104$^-$ adhesion sites; well 2, neurite-containing B104$^+$ adhesion sites; well 3, neurite-containing B104$^+$ cells.

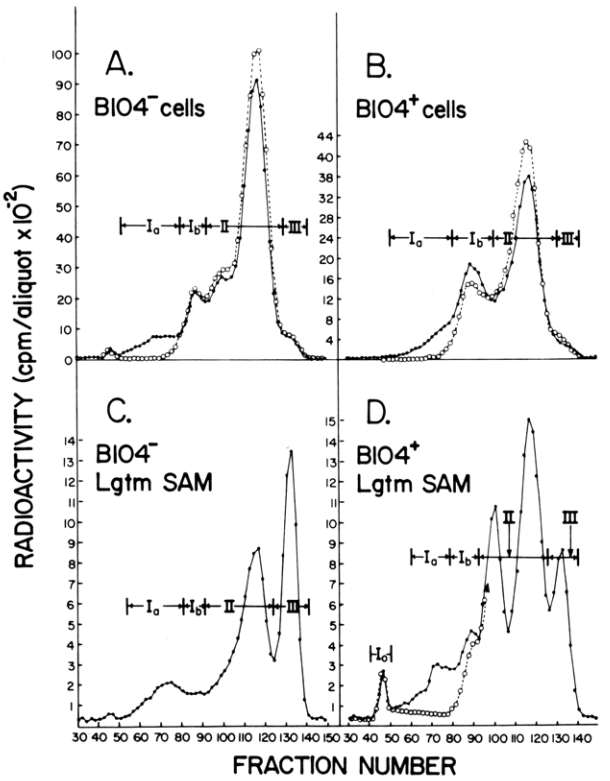

FIGURE 5: Sepharose CL6B chromatography of chondroitinase ABC digests of B104 fractions. B104 cells were grown in [^{3}H]glucosamine-radiolabeling medium under two different sets of conditions—for 72 h in serum-containing medium to generate nonneurite fractions (B104$^-$) or for 48 h in serum-containing medium followed by 24 h in serum-free medium to generate neurite-containing fractions (B104$^+$). The cell and long-term substrate-attached material (SAM) fractions were processed for polysaccharide analysis as described under Materials and Methods section for chondroitinase ABC digestion (Cohn et al., 1976; Rollins & Culp, 1979a) which completely hydrolyzes hyaluronate, the chondroitins, and dermatan sulfate to $\Delta^{4,5}$-unsaturated disaccharides. The digests were applied to a 1.2 × 120 cm column of Sepharose CL6B and eluted into 1.5-mL fractions with 150 mM Tris, pH 7.4 (0.2% $NaDodSO_4$); radioactivity in fractions was determined by scintillation counting. The void volume of the column was at fraction 40, while low molecular weight markers eluted at fractions 130–140. In some cases, before chromatography the digests were split in half for control hydrolysis (●) or for nitrous acid treatment (○) which breaks down N-sulfated heparan sulfate to low molecular weight species; the details of this process have been described previously (Rollins & Culp, 1979a). Rollins & Culp (1979a) have established that region I_a of the elution profile contains nitrous acid labile HS, I_b contains acid-resistant HS, II glycoprotein-derived glycopeptide [this glycoprotein is not covalently bound to the proteoglycan species (Rollins & Culp, 1979b)], and III contains choindroitinase digestion products.

strated by subsequent reactivity of the antigen–antibody complex in the gel with ^{125}I-labeled staphylococcal protein A and autoradiographic detection. Antibody did not bind to any other bands in substrate-attached or cell preparations; preimmune IgG did not bind to any bands in the gel, including the C_o band. Therefore, the metabolically labeled C_o glycoprotein in these adhesive preparations must be a cell-synthesized fibronectin-like molecule. Similar data were obtained for both C6 glioma and C1300 neuroblastoma substrate-attached fractions. This glycoprotein is a cell surface component since it can be radioiodinated with lactoperoxidase (Cathcart & Culp, 1979) (data not shown).

Adhesion Site Composition: Carbohydrate. Enrichment of certain carbohydrate species in B104 substrate-attached material is further explored in Figure 4. Both neurite-containing and nonneurite-containing adhesive fractions are enriched in the GAG-1 and GAG-2 bands in a similar relative distribution. They are also enriched in the 220K C_o glycoprotein. The cell fraction contains only one prominent proteoglycan species in the 5% stacking gel and many prominent glycoproteins distinguishable in the 10% separating gel, including the 220K glycoprotein.

Pronase digestion of substrate-attached extracts, followed by gel filtration of the polysaccharide material, demonstrated that most of the carbohydrate was of high molecular weight and therefore probably glycosaminoglycan (data not shown). A detailed analysis of the GAG components in the substrate-attached and cell fractions under various physiological conditions was then initiated. Figure 5 shows the Sepharose CL6B profiles of glucosamine-radiolabeled cell and long-term substrate-attached preparations which have been exhaustively digested with chondroitinase ABC under the protocols defined by Rollins & Culp (1979a); this enzyme completely hydrolyzes (under the pH conditions used here) hyaluronate, unsulfated and sulfated chondroitins, and dermatan sulfate to $\Delta^{4,5}$-unsaturated disaccharides which can be separated as a group by Sepharose chromatography from undigested components or individually by paper chromatography. The identities of the components in the various peaks of Figure 5 are also biochemically defined by Rollins & Culp (1979a,b). Neurite-containing B104 cells differ in their carbohydrate content from nonneurite-containing cells by having smaller amounts of nitrous acid labile HS (I_a), larger amounts of nitrous acid resistant HS (I_b), and no shoulder of higher molecular weight glycopeptide (fractions 100–110 of peak II). Long-term substrate-attached material from the two cell types differs considerably from their respective cell profiles in containing a large pool of chondroitinase-digested material (peak III) and in containing larger relative amounts of nitrous acid labile HS (peak I_a). The substrate-attached material from neurite-containing cells (Figure 5D) also differs from that of nonneurite-containing cells (Figure 5C) in a very high molecular weight polysaccharide (I_o), which could not be easily detected

Table I: Glycosaminoglycan Distribution[a]

cell fraction[b]	I_o[c]	HS		HA	C6S	C4S	COS	DS
		I_a	I_b					
B104⁻ cells	ND[d]	23.9	32.5	40.7	0.7	1.9	0.2	ND
B104⁻ Lgtm[e] adhesion sites	ND	30.7	13.6	13.8	13.6	14.8	5.4	8.0
B104⁻ Reatt adhesion sites	ND	37.4	45.5	17.1	ND	ND	ND	ND
B104⁺ cells	ND	16.2	52.9	28.4	0.2	0.8	0.3	1.1
B104⁺ Lgtm adhesion sites	6.7	29.6	28.4	15.2	5.8	7.3	2.0	5.0
B104⁺ Reatt adhesion sites	30.1	19.6	47.1	ND	ND	3.3	ND	ND
C6BU1 cells	ND	10.0	26.6	51.4	0.6	11.0	0.4	ND
C6BU1 Lgtm adhesion sites	ND	7.8	12.0	48.3	2.8	28.0	1.1	ND
C6BU1 Reatt adhesion sites	ND	15.8	36.0	37.5	ND	10.7	ND	ND

[a] The percentage of each of the following GAG's to the total GAG content is given: HS = heparan sulfate [I_a is the nitrous acid labile fraction and I_b is the acid-resistant fraction as demonstrated in Figure 5 and Rollins & Culp (1979a)]; HA = hyaluronic acid; C6S = chondroitin 6-sulfate; C4S = chondroitin 4-sulfate; COS = unsulfated chondroitin; DS = dermatan sulfate. [b] Cell and substrate-attached preparations from glucosamine-radiolabeled cultures are described under Materials and Methods. B104⁻ denotes nonneurite-containing cells, while B104⁺ denotes neurite-containing cells. [c] This undefined polysaccharide is also included as a percentage of total. [d] None detected. [e] Lgtm, long term; Reatt, reattaching.

in the cell fraction, and in displaying two distinguishable pools of glycoprotein-derived glycopeptide (peak II). The chemical nature of I_o is uncertain at this point, although it is not labile to nitrous acid degradation, to Pronase, to either of the condroitinases, or to testicular hyaluronidase. In any case, the most dramatic differences occur between homologous substrate-attached and cell fractions in their chondroitinase-resistant and -sensitive polysaccharide components and between neurite-containing and noneurite-containing adhesive fractions.

Long-term substrate-attached material contains both footpad-derived and "footprint" adhesive material which complicates the data described above (Rollins & Culp, 1979a). For examination of the composition of newly formed adhesion sites without complications by "footprint" material, EGTA-detached and glucosamine-radiolabeled cells were washed well and permitted to reattach to fresh serum-coated dishes for 1 h [which is sufficient time for mature footpad adhesions to form (Rosen & Culp, 1977)] before EGTA-mediated detachment and isolation of reattaching substrate-attached material. The chondroitinase digests of these substrate-attached fractions are shown in Figure 6 after Sepharose CL6B chromatography. It should be noted that the reattaching cell fraction contains the same specific radioactivity and distribution of polysaccharide species as shown in Figure 5. The most striking change from long-term adhesive material is the paucity of chondroitinase-digestible polysaccharide in reattaching material (peak III). Therefore, heparan sulfate and glycoprotein may be important factors in the formation of new adhesion sites, whereas chondroitinase-labile polysaccharides are not. Reattaching substrate-attached material from neurite-containing cells also contains considerable I_o.

When polysaccharide analyses are performed on C6 cell and substrate-attached fractions (Figure 7), similar distributional differences are noted. Long-term material contains much more chondroitinase-labile GAG's than the cell fraction or B104 long-term substrate-attached material (see part C or D of Figure 5). Reattaching C6 material is also enriched in heparan sulfate and glycoprotein relative to the condroitinase-labile GAG's. There was no I_o polysaccharide observed in any of the C6 fractions analyzed.

The precise distribution of the various GAG's of cell and adhesion site fractions is given in Table I. There are appreciable amounts of all the chondroitin species, hyaluronate, and dermatan in long-term substrate-attached fractions from both neurite-containing and nonneurite-containing B104 cells. The reattaching material from these cells contains little of these chondroitinase-digestible GAG's, except for some hyaluronate

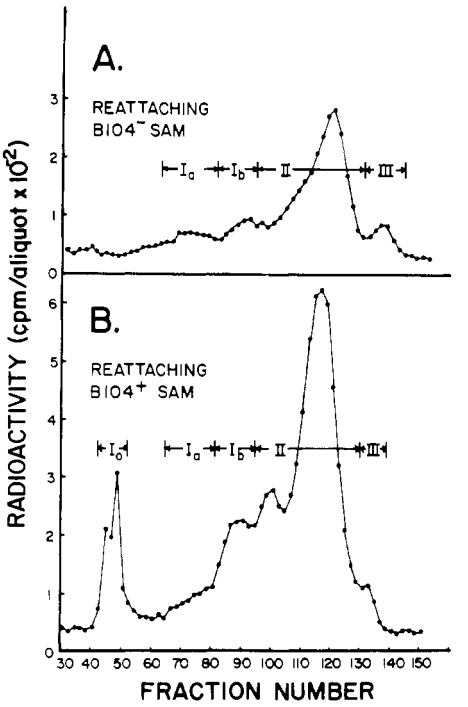

FIGURE 6: Chondroitinase digests of substrate-attached material (SAM) from reattaching B104 cells. Nonneurite (B104⁻) and neurite-containing (B104⁺) neuroblastoma cells which had been grown in [³H]glucosamine-containing medium as described in the legend to Figure 5 were EGTA detached, rinsed twice with PBS, and permitted to reattach to fresh serum-containing dishes for 1 h. The substrate-attached fractions (SAM) from these reattaching cells were then harvested, processed for polysaccharide analysis as described under Materials and Methods, and treated with chondroitinase ABC. These digests were analyzed by Sepharose CL6B chromatography as described in the legend to Figure 5.

in nonneurite cells. It is also clear that the unknown polysaccharide I_o is considerably enriched in the reattaching material of neurite-containing cells.

Discussion

Although surface membranes of fibroblasts have been studied extensively, only limited attention has been devoted to the biochemistry of cell surface glycoproteins of neural cell derivatives. Brown (1971) and Glick et al. (1973) reported differences in the glycopeptide distributions of membrane preparations from differentiated and undifferentiated mouse neuroblastoma cells. Truding et al. (1974) compared the glycoprotein composition of an enriched surface membrane

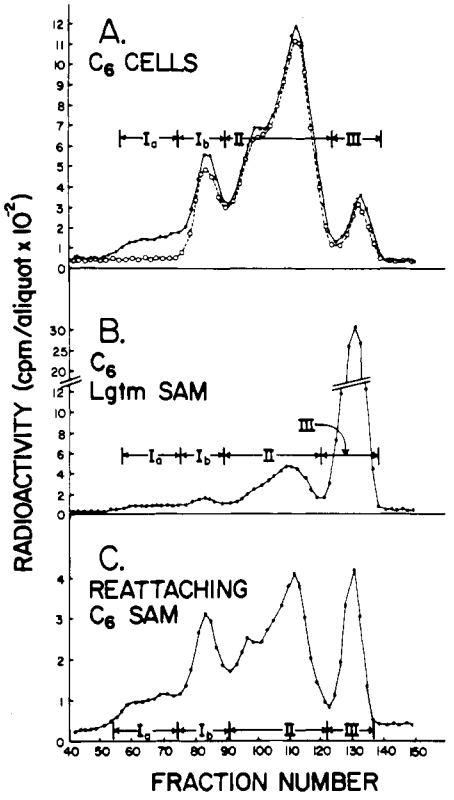

FIGURE 7: Chondroitinase digests of C62B cell and substrate-attached fractions. C62B cells were grown for 72 h in [^{3}H]glucosamine-containing medium, detached by EGTA treatment, and split into two pools. One pool was processed for polysaccharide analysis as C6 cells as described in the legend to Figure 5; the long-term substrate-attached material (SAM) was processed from these cells as described under Materials and Methods. The second pool of cells was rinsed twice with PBS before reattachment to fresh serum-containing dishes for 1 h; this reattaching SAM was processed as described in the legend to Figure 6. All three fractions were digested with chondrotinase ABC. The substrate-attached fractions were chromatographed on Sepharose CL6B as described in the previous figures. The cell digest was split in half with one portion mock-hydrolyzed (●) and the second portion nitrous acid hydrolyzed (○) before Sepharose chromatography as described in the legend to Figure 5.

fraction from differentiated and undifferentiated mouse neuroblastoma cells and found quantitative differences in 105K and 78K species. Akeson & Hsu (1978) have reported a cell surface glycoprotein from mouse neuroblastoma cells of high molecular weight that does not appear to be fibronectin. Estridge & Bunge (1978) have recently described methods for separating axons from cell bodies of primary cultures of adrenergic autonomic ganglia; the polypeptide composition of the two fractions was remarkably similar.

Initial studies have also pointed to the importance of the chemical nature of the substratum in facilitating neurite extension of neuronal cells. Letourneau (1975) observed considerable variation in axonal elongation on tissue culture substrates coated with polymers or heavy metals. Since chemically different inert substrata bind a differing array of serum proteins (Haas & Culp, 1979), axon growth cone formation and extension may require a specific array of serum receptor molecules. This is supported by evidence reported in this study whereby EGTA-detached neuroblastoma cells (either mouse or rat) extend neurites on a CIg-coated substratum more readily than on a serum-coated substratum during short incubation periods (both cultures in serum-free medium). For primary rat sensory ganglia, contact of Schwann cells with a rat tail collagen matrix was required to generate ensheathment of nerve fibers and myelination of axons (Bunge & Bunge, 1978); it is also interesting to note that rat tail collagen in serum-containing medium has CIg bound to it, a situation analogous to the experiment in Figure 1 of this study.

Because of the importance of the substratum in promoting differentiation of neural cell derivatives, we initiated these studies on the biochemical composition of the cell–substratum adhesion sites of neuroblastoma and glioma cells by following protocols which have proven effective in fibroblast adhesion studies (Culp, 1978; Culp et al., 1978, 1979). Schubert (1977) has previously shown that substrate-attached material from neural cells contains a prominent 55K protein. Glimelius et al. (1978) have shown that substrate-attached material of normal glial cells contains more heparan sulfate than the material from many glioma cell lines. Our studies have shown that all three cell types—glioma, undifferentiated neuroblastoma, and differentiated neuroblastoma—generate comparable amounts of protein and polysaccharide in their adhesive material which comprises ~1–2% of the cell's protein content. This material is enriched in glycosaminoglycan-containing proteoglycans, the 220K C_0 glycoprotein, and some cytoskeletal proteins. This biochemical evidence plus scanning electron microscopic evidence to be presented later (L. A. Culp and C. Domen, unpublished experiments) establish that substrate-attached material is principally composed of cell–substratum adhesion sites which have pinched off from the retracting cell surface and not larger pieces of the cell or cell-secreted components artifactually bound to the substratum.

The polypeptide composition of the three cell types was very similar in its content of the C_0 glycoprotein, *total* proteoglycan, myosin, actin, and several unidentified proteins. The major differences were found in the NaDodSO$_4$–polyacrylamide gel electrophoretic gel regions where the 10 nm filament subunit proteins were found (below the migration region for glial or neuronal tubulins) (Gordon et al., 1978; Jorgensen et al., 1976; Yen et al., 1976). Glioma adhesion sites contained much smaller relative amounts of 57K protein than the quite prominent 55K protein found in neuroblastoma cells under all physiological conditions tested. On the other hand, glioma sites contained a prominent 50K protein which was not apparent in undifferentiated neuroblastoma sites. This may reflect biochemical differences in the subunit proteins of 10–nm filaments noted previously for glial (50K) or neuronal cells (55K) (Goldman et al., 1978; Liem et al., 1978). A 50K protein was observed in differentiated neuroblastoma adhesion sites or in sites of undifferentiated cells adhering to substrata coated with substrate-attached material from glial or neuronal cells. More extensive analyses will be required to identify the cell surface signal which induces these presumptive cytoskeletal rearrangements and to conclusively establish that they are 10 nm filament proteins. Since cells apparently facilitate motility by pinching off adhesion sites via cytoskeletal reorganization (Culp, 1978), these differences may reflect important properties in the social behavior and differentiation of these neural cell derivatives; for example, C6 glioma cells are much more motile on the substratum than neuroblastoma cells, and the latter cell retains the ability to extend neurites by growth cone formation.

The 220K glycoprotein synthesized by these cells and enriched in their adhesion sites appears to be a fibronectin-like component on the cell surface. Its cell surface location is confirmed by lactoperoxidase-catalyzed iodinatability (L. A. Culp and C. Domen, unpublished data). Proline-enriched, collagen-like proteins are not detected in the adhesion sites of these cells. This situation is similar to that of some fi-

Table II: Ratios of Various Polysaccharide Classes[a]

cell fraction[b]	ratio HS/gp	HS/HA	HA/Σ(CS + DS)
B104⁻ cells	0.23	1.4	14.2
B104⁻ Lgtm[d] adhesion sites	0.33	3.2	0.33
B104⁻ Reatt adhesion sites	0.37	4.8	*c*
B104⁺ cells	0.62	2.4	11.4
B104⁺ Lgtm adhesion sites	0.28	3.8	0.75
B104⁺ Reatt adhesion sites	0.29	*c*	*c*
C6BU1 cells	0.32	0.71	4.2
C6BU1 Lgtm adhesion sites	0.29	0.41	1.5
C6BU1 Reatt adhesion sites	0.29	1.4	3.5

[a] The ratio of each GAG type or glycoprotein-derived glycopeptide (gp) is determined from the distributions calculated in Table I and Figures 5–7. The HS fraction is I_a plus I_b; the nomenclature is the same as Table I. ΣCS is the sum of all the chondroitin species in the fraction. [b] As described in Table I. [c] These values cannot be calculated because one of the ratio values is zero. [d] Lgtm, long term; Reatt, reattaching.

broblast cell lines (Culp & Bensusan, 1978) where fibronectin and CIg binding to GAG-containing proteoglycans in the absence of collagen appears to be the principle determinant of cell–substratum adhesion and subsequent detachment of cells (Culp et al., 1979).

The analogy with fibroblasts is even further reinforced by compositional analyses of the glycosaminoglycans of these neural cells. (a) Adhesion sites of all three cell types is considerably enriched in GAG when compared to other cell fractions. (b) Reattaching sites contain principally heparan sulfate and glycoprotein; as cells begin to spread and move on the substratum, chondroitinase-digestible GAG, particularly hyaluronate, accumulates with time. (c) The ratio of HS/glycopeptide in all substrate-attached fractions for all three cell types is invariant (Table II). (d) The ratios of the other polysaccharide classes are highly variable (Table II). (e) The highly motile and easily detachable glioma cells deposit much more hyaluronate and chondroitin in their adhesion sites than the *relatively* immobile neuroblastoma cells. All of these data agree with similar data found in fibroblasts (Culp et al., 1978,1979) suggesting that heparan sulfate and glycoprotein (probably fibronectin) are direct mediators of substratum adhesion of these cells while hyaluronate–chondroitin species play some unknown role in facilitating motility and detachability of cells (Atherly et al., 1977). These cells differ from fibroblasts by depositing substrate-attached material with much less chondroitin, particularly unsulfated chondroitin (Culp et al., 1978).

The most striking difference in the polysaccharide analyses was the identification of the high molecular weight, amino-sugar-radiolabeled I_0 polysaccharide found exclusively in the substrate-attached fraction of neurite-containing cells. It is not a hyaluronate or chondroitin-, dermatan, or keratan sulfate type of GAG, and more extensive biochemical analyses will be required to characterize this interesting polysaccharide. There are also no data bearing on whether this polysaccharide is localized to the growth cone class of adhesion sites, which would suggest a unique function in neurite formation.

Our studies indicate that the overall biochemical aspects of adhesion of these neural cells to a serum-coated tissue culture matrix are very similar to those processes of fibroblasts. Notable differences between these cells and fibroblasts include very different distributions of certain presumptive cytoskeletal elements, indentification of a unique polysaccharide in neurite-containing sites, and the reduced levels of the various chondroitin species. Our studies will continue on the functional significance these similarities and differences.

Added in Proof

We have recently isolated three types of behavioral variants of rat neuroblastoma cells which differ in their ability to extend neurites or their tumor-inducing capacity (Culp, 1980). The protein and polysaccharide compositions of the adhesion sites of these variants will be of particular interest to substantiate the conclusions drawn from this study.

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Lateral Diffusion of M-13 Coat Protein in Mixtures of Phosphatidylcholine and Cholesterol†

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ABSTRACT: The translational diffusion of fluorescent-labeled M-13 phage coat protein (FITC-M-13), an integral membrane protein, has been measured in mixtures of phosphatidylcholines and cholesterol, using a pattern photobleaching technique. At temperatures below the chain-melting transition temperature of dimyristoylphosphatidylcholine (DMPC) (23.8 °C), the lateral diffusion coefficient of M-13 shows a marked increase when the cholesterol concentration is increased above 20 mol %. A similar marked increase in the lateral diffusion coefficient of a fluorescent phospholipid is also observed. At temperatures above the chain-melting transition temperature of DMPC, a minimum is observed in the lateral diffusion coefficient of FITC-M-13 for cholesterol concentrations in the vicinity of 25 mol %. This minumum in the diffusion coefficient of FITC-M-13 is also observed at 25 mol % cholesterol in egg phosphatidylcholine at 15 °C. No such minimum is observed for the lateral diffusion coefficient of the fluorescent lipid. The lateral diffusion coefficient of FITC-M-13 is large ($>10^{-9}$ cm^2/s) at all cholesterol concentrations for temperatures above the chain-melting transition temperature of the phosphatidylcholine. Several other proteins contain hydrophobic regions similar to that of the M-13 coat protein. We anticipate that a number of proteins of this type will show similar diffusional behavior, in particular exhibiting rapid diffusion throughout a wide range of lipid composition.

In recent work the lateral diffusion coefficient of the M-13 phage coat protein in dimyristoylphosphatidylcholine (DMPC)[1] was measured by using a technique involving fluorescence redistribution after pattern photobleaching (Smith & McConnell, 1978; Smith et al., 1979a). In other recent work we have observed a surprising effect of cholesterol concentration on the lateral diffusion coefficient of fluorescent phospholipid in binary mixtures of phosphatidylcholines and cholesterol at temperatures below the chain-melting transition temperature of the phosphatidylcholine; increasing cholesterol concentration in the bilayer above ~20 mol % leads to a sharp, order-of-magnitude increase in the diffusion coefficient of a fluorescent phospholipid (Rubenstein et al., 1979). This unusual effect has now been accounted for in terms of a remarkable lipid bilayer structure in which stripes of pure solid phosphatidylcholine alternate in a regular way with stripes of fluid lipid, the fluid lipid containing 20 mol % cholesterol (Copeland & McConnell, 1980; Owicki & McConnell, 1980; Rubenstein et al., 1980). The several studies described above prompted the present work. Thus, it was anticipated that a comparable abrupt change in the lateral diffusion of this intrinsic membrane protein would be observed when cholesterol concentration is increased above 20 mol % in binary mixtures of DMPC and cholesterol at temperatures below the chain-melting transition temperature of the phospholipid (23.8 °C). This expectation is confirmed here.

A second motivation for the present study stems from the paucity of biophysical studies of the effects of cholesterol on the properties of integral membrane proteins. [For two references see Kleeman & McConnell (1976) and Warren et al. (1975).] In contrast, extensive studies have been made of the biophysical properties of phosphatidylcholine–cholesterol mixtures. [For a review, see Demel & De Kruyff (1976).]

A recent report on the lateral diffusion of an amphipathic apolipoprotein bound to phosphatidylcholine and phosphatidylcholine–cholesterol bilayers has been made by Vaz et al. (1979). Their results are consistent with those presented here in that rapid diffusion of the apolipoprotein is reported.

Materials and Methods

Materials. Dimyristoylphosphatidylcholine (DMPC) was obtained from Sigma and used without further purification. A stock solution in methanol was stored at 4 °C. Egg phosphatidylcholine was isolated as described by Ross & McConnell (1977). A stock solution in ethanol was stored at −20 °C. Concentrations of the phospholipid stock solutions were determined by phosphate assay (McClare, 1971). Cholesterol was recrystallized twice from ethanol and stored in ethanol and under argon at −20 °C. The concentration of

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[1] Abbreviations used: DMPC, dimyristoylphosphatidylcholine; FITC, fluorescein isothiocyanate; FITC-M-13, M-13 phage coat protein conjugated with FITC; *D*, lateral diffusion coefficient; NBD egg PE, fluorescent-labeled egg phosphatidylethanolamine; NBD-DMPE, a fluorescent-labeled dimyristoylphosphatidylethanolamine, *N*-(4-nitrobenzo-2-oxa-1,3-diazolyl)-L-α-dimyristoylphosphatidylethanolamine.

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