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Glycopeptides from the Surface of Human Neuroblastoma Cells[1]

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SUMMARY

Glycopeptides suggesting a complex oligosaccharide composition are present on the surface of cells from human neuroblastoma tumors and several cell lines derived from the tumors. The glycopeptides, labeled with radioactive L-fucose, were removed from the cell surface with trypsin, digested with Pronase, and examined by chromatography on Sephadex G-50. Human skin fibroblasts, brain cells, and a fibroblast line derived from neuroblastoma tumor tissue show less complex glycopeptides. Although some differences exist between the cell lines and the primary tumor cells, the similarities between these human tumors and animal tumors examined previously are striking.

INTRODUCTION

Specific groups of glycopeptides are expressed on the surface of virus-transformed (1, 8, 13) and tumor cells (7, 8, 14, 16). These glycopeptides appear to be a general characteristic of many virus-transformed cell surfaces. Some chemically transformed cells do not show these glycopeptides when grown in culture, however, the cells from tumors derived from these cultured cells have membrane glycopeptides similar to other tumor cells (7). The glycopeptides, which are unique to the pathological cell surface, show them to contain oligosaccharides more complex than those found on the surface of the nontransformed cell (3).

Recently, several cell lines from human neuroblastoma tumors have been established in culture (10), making it possible to examine the surface of these human tumor cells. In addition, the availability of biopsy material from human tumors has made it possible to compare the cells in culture with those of the tumor by techniques similar to those used in the examination of the hamster cell systems (8). Thus, as part of our studies searching for unique membrane properties, we have examined the glycoproteins from these cells. Our results show that the surface glycopeptides derived from the human tumors are complex, similar to those found in other animal cell tumors. However, some variation in glycoproteins exists between the neuroblastoma cell lines in culture.

MATERIALS AND METHODS

Cell Culture. Human cell lines CHP-126, CHP-134, and CHP-100 have been described previously (10). CHP-168 was obtained subsequently by similar methods (H. Schlesinger, unpublished data). The first 2 cell lines (CHP-126 and CHP-134) were obtained from tumors of patients diagnosed as neuroblastoma and were derived from a mass surrounding the kidney and the left adrenal, respectively. The 3rd line was derived from an epidural tumor surrounding the spine and diagnosed as consistent with neuroblastoma. The 4th cell line, CHP-168, diagnosed as neuroblastoma, was from a mass surrounding the left kidney. Another cell line, IMR-32, obtained from a human neuroblastoma tumor, has been described (12). A clone of IMR-32 was obtained from Dr. F. Gilbert, Department of Human Genetics (IMR-32C_8), and was subsequently recloned and designated IMR-32$C_{8/4}$. CHP-100 was passed through a nude mouse and a cell line (CHP-100NM) was derived from these tumor cells. All of the cell lines examined contained enzymes characteristic of neurotransmitter synthesis, acetylcholinesterase, and choline acetyltransferase.

Two cell lines also had enzyme activity for tyrosine hydroxylase. The cell lines were examined for these enzyme activities by Dr. C. Palfrey and Dr. Y. Kimhi of the Weizmann Institute, Israel, subsequent to the studies reported here. Their results are given in Table 1. TF-3 is a clone of fibroblasts grown from an abdominal tumor diagnosed as neuroblastoma. A normal brain cell line and homologous skin fibroblasts were obtained from Dr. P. Koldovsky, Children's Hospital of Philadelphia. All of these cells were grown at 37° on Falcon or Corning flasks containing McCoy's 5A modified medium with 15% fetal bovine serum, supplemented with 20 mM glutamine, penicillin (100 μg/ml), and streptomycin (50 μg/ml), in an atmosphere of 5% CO_2. With the exception of IMR-32, all of the cells were below the 40th passage unless otherwise stated. All of the neuroblastoma cell lines were tested for *Mycoplasma* and found to be negative.

Tumors. Human tumors obtained at autopsy or surgery and diagnosed as neuroblastoma were minced with scalpels into approximately 10-mm pieces and dispersed by mechanical aspiration. The cell suspension was layered on a Ficoll-Hypaque gradient (9) and centrifuged at 500 × *g*. The cells were removed from the interface, washed 3 times with McCoy's 5A modified medium, and placed into 25-sq mm Falcon plastic flasks. The cells were incubated for 24 hr. After this time, fresh McCoy's medium containing 7 μCi of L-[5,6-^{3}H]fucose (4.3 Ci/mmole) was added and incubation was continued for 48 hr. The cells were removed from the flask and trypsinized after washing 4 times with TBS,[2] and the surface glycopeptides were prepared as described

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[2] The abbreviation used is: TBS, 0.2 M Tris-HCl, pH 7.5, in 0.16 M NaCl.

Table 1

Enzyme activities of human cell lines

The cell lines were grown to confluency, harvested, frozen, and subsequently assayed in triplicate according to the method of Schrier *et al.* (11) for the activities of tyrosine hydroxylase, choline acetyltransferase, and acetylcholinesterase.

	pmoles product/min/mg protein		
Cell line	Tyrosine hydroxylase	Choline acetyltransferase	Acetylcholinesterase
CHP-126	75	495	12,000
CHP-134	21	654	495
CHP-100	1	166	848
CHP-100NM	0	164	585
TF-3	0	13	100
IMR-32	7	408	1,220
IMR-$32C_8$	77	200	1,500
IMR-$32C_{8/4}$	0	34	635

below for the cell lines. After this procedure, the tumor cells were 70 to 85% viable as determined by the uptake of trypan blue.

Preparation of Surface Glycopeptides. All of the cell lines examined were reseeded at 2×10^6 cells/75-sq mm flask and cultured for 72 hr in the presence of 5 μCi of L-[1-^{14}C]fucose (50.8 mCi/mmole) or 7 μCi of L-[5,6-^{3}H]fucose (4.3 Ci/mmole) obtained from New England Nuclear Corp., Boston, Mass. After this time, the cells, in late logarithmic phase of growth, were washed 4 times with TBS and removed from the monolayer by trypsinization with 1 mg of trypsin (3 times crystallized; Worthington Biochemical Corp., Freehold, N. J.) in 1 ml of TBS. After 5 min at room temperature, an equivalent amount of soybean trypsin inhibitor (purified; Worthington Biochemical Corp.) was added. The cells were centrifuged at $500 \times g$ for 5 min. The supernatant solution, containing the membrane glycopeptides was further centrifuged at $40{,}000 \times g$ for 30 min and lyophilized. This fraction is referred to as "trypsinate" and represented 15 to 25% of the total radioactivity incorporated.

In 1 experiment, the cells were removed from the monolayer by mild trypsinization (trypsinate A), as reported previously (5), and surface membranes were prepared from these cells by the zinc ion procedure (15). Trypsinate A obtained by the mild first step contains loosely associated glycopeptides while the surface membranes contain the more tightly associated glycopeptides (3).

Characterization of the Membrane Glycopeptides. The trypsinates containing the radioactive glycopeptides were dissolved in water and the fractions to be compared were combined and digested exhaustively with Pronase (Calbiochem, La Jolla, Calif.). Separation of the Pronase-digested glycopeptides was by gel filtration on a column (1.5×100 mm) of Sephadex G-50 fine. All details of these procedures have been described (1, 5). Fractionation of these glycopeptides by DEAE-cellulose chromatography revealed that the peak areas separated by Sephadex G-50 chromatography represented a family of glycopeptides (3), so that reference is made to groups of glycopeptides rather than a single glycopeptide.

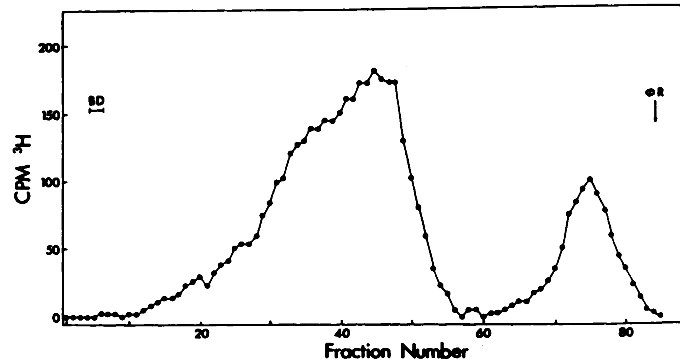

Chart 1. Chromatography on Sephadex G-50 of Pronase-digested glycopeptides removed by trypsin from a human neuroblastoma tumor. The dispersed tumor cells were placed in culture for 48 hr with L-[^{3}H]fucose. All details are in the text. *BD*, the fractions where blue dextran eluted from the column. Phenol red (ϕR) eluted at Fraction 85.

RESULTS

Surface Glycopeptides from Tumor Cells. Glycopeptides removed by trypsin from the surface of primary neuroblastoma tumor cells labeled for 48 hr with L-[^{3}H]fucose were digested with Pronase and examined by chromatography on a Sephadex G-50 column. Chart 1 shows that the fucose-containing glycopeptides eluted over a broad area that can be divided arbitrarily into several major groups, Fractions 20 to 28, 29 to 38, and 39 to 52. The fucose-containing material that migrated just prior to the phenol red (Fractions 70 to 80) consisted of dialyzable material including free fucose; therefore, in discussing the glycopeptides these latter fractions are not included. Six different neuroblastoma tumors were examined by this technique and the results showed patterns similar to that shown in Chart 1. The enrichment of glycopeptides in Fractions 25 to 35 (Chart 1) was typical of the pattern obtained from hamster tumor cells (7, 8).

Glycopeptides of a Neuroblastoma Cell Line Compared with Other Human Cells in Culture. The cell surface glycopeptides containing radioactive fucose were examined from a clonal cell line (IMR-$32C_8$) that was derived from a human neuroblastoma tumor. Chart 2 shows the pattern obtained after chromatography on Sephadex G-50 of the Pronase-digested membrane glycopeptides from this cell line. There appear to be several groups of fucose-containing glycopeptides migrating rapidly, Fractions 11 to 25 and 26 to 38 and a larger group, Fractions 39 to 50, eluting more slowly from the column. Similar to the tumor cells (Chart 1), the material that migrated in Fractions 65 to 79 contained free fucose and other dialyzable fucose-containing compounds which are currently under investigation. This low-molecular-weight material is seen in all trypsinates (G. Mihalik and M. C. Glick, unpublished observations). The Sephadex G-50 patterns differ somewhat from those of the primary cells from the human tumors, because in the latter more material was eluted in Fractions 29 to 38 (Chart 1). This cell line (IMR-$32C_8$) was recloned and the subsequent population of cells (IMR-$32C_{8/4}$) retained a similar glycopeptide pattern (Chart 2C), even though the activities of the enzymes for neurotransmitter synthesis were reduced (Table 1). Regardless of the details of the chromatography patterns, the cells

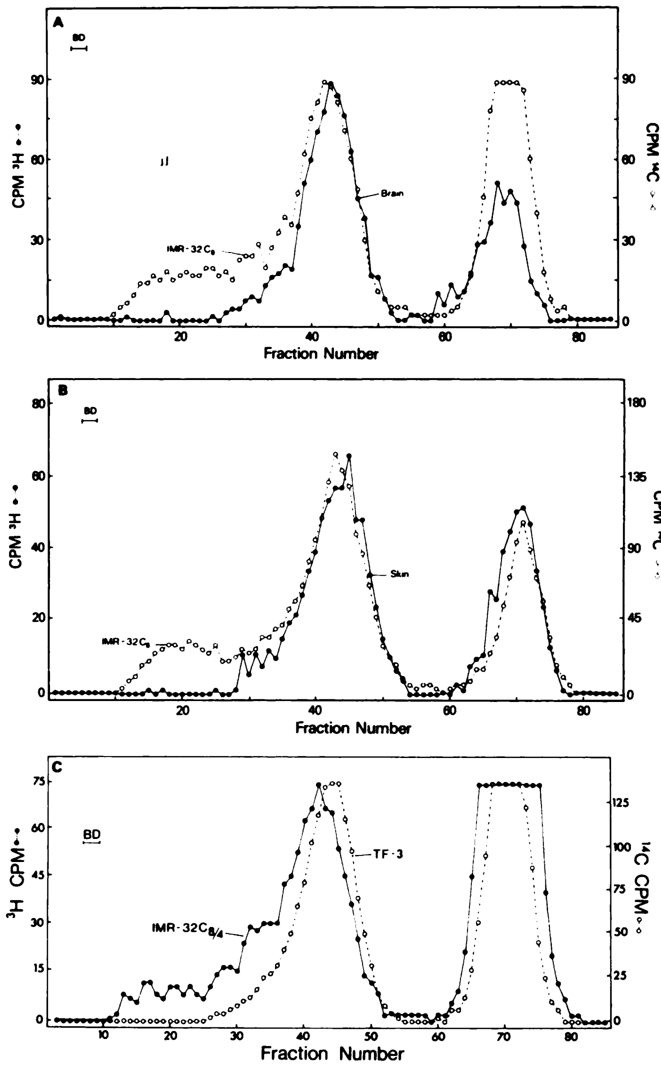

Chart 2. Chromatography on Sephadex G-50 of Pronase-digested trypsinates of a human neuroblastoma cell line, IMR-32C$_8$ (○), (*A*) brain cells (●), (*B*) skin fibroblasts (●) (*C*) a recloned clonal line, IMR-32C$_{8/4}$ (●), and fibroblasts from tumor tissue, TF-3 (○). The trypsinates, made radioactive by growth of the cells in L-[^{3}H]- or [^{14}C]fucose for 72 hr, were combined before Pronase digestion. See Chart 1 and text for further details. ◐, coinciding points.

derived from a human neuroblastoma have surface glycopeptides more characteristic of tumor cells than of human brain cells or skin fibroblasts (Chart 2, *A* and *B*). Both the brain cells and skin fibroblasts have only 1 group of fucose-containing glycopeptides when examined by these techniques (Fractions 38 to 50). Fibroblasts (TF-3) that grew from neuroblastoma tissue had insignificant levels of the enzymes for neurotransmitter synthesis (Table 1). The surface glycopeptides of these cloned fibroblasts were more similar to the brain cells than to the neuroblastoma cells (Chart 2, *A* and *C*).

Surface Glycopeptides from Other Neuroblastoma Cell Lines. Using the same technique, chromatography on Sephadex G-50 of the Pronase-digested fucose-containing glycopeptides was removed from the cell surface with trypsin, a remarkable similarity was obtained with 3 other human neuroblastoma cell lines, CHP-134, CHP-168, and CHP-126. Chart 3 shows the patterns of the surface glycopeptides obtained from CHP-168 and CHP-134 and that the similarity with IMR-32 (Chart 2) is striking. Again, more rapidly migrating material is present in all of these cell lines, Fractions 17 to 38. The membrane glycopeptides of CHP-126 showed an identical pattern.

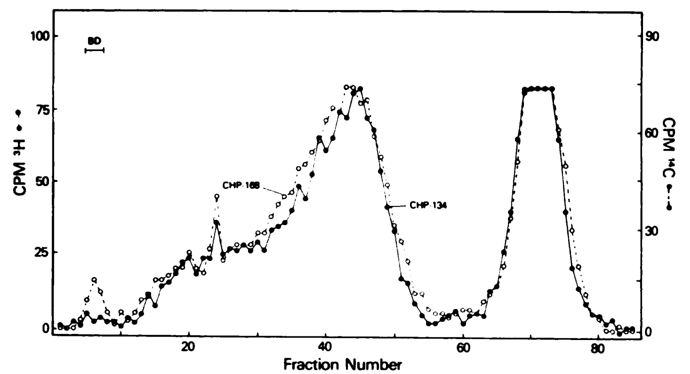

Chart 3. Chromatography on Sephadex G-50 of Pronase-digested trypsinates of human neuroblastoma cell lines, CHP-134 (●) and CHP-168 (○). The glycopeptides were made radioactive by growth of the cells for 72 hr in L-[^{3}H]- or [^{14}C]fucose, respectively, and the trypsinates were combined before Pronase digestion.

The chromatographic pattern of the Pronase-digested trypsinate of CHP-100 is shown in Chart 4*A*. CHP-100 is compared with a cell line (CHP-100NM) obtained after passage through a nude mouse (10). Both of these cell lines exhibit a similar group of fucose-containing glycopeptides and have the unique feature of appearing more like human brain cells (Chart 2*B*) than the other tumor cells. Both of these cell lines show the enzyme activities of choline acetyl-

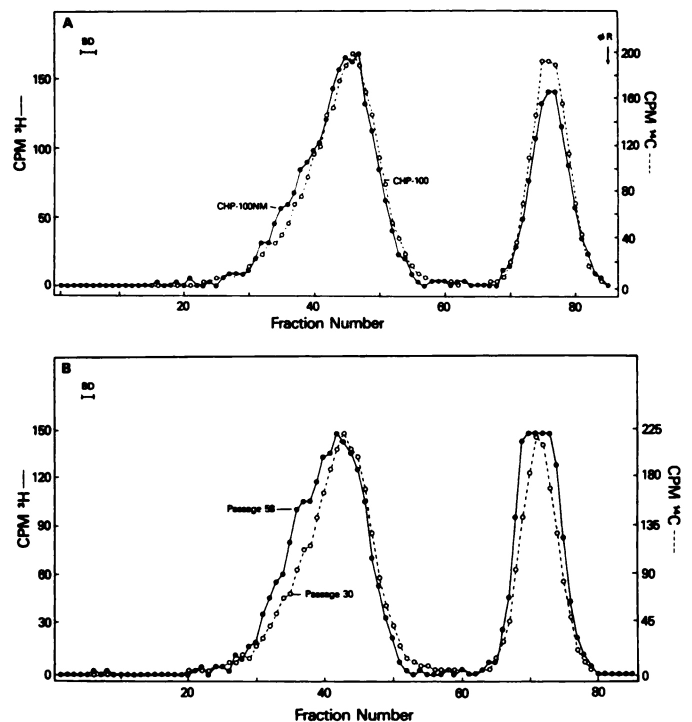

Chart 4. Chromatography on Sephadex G-50 after Pronase digestion of the fucose-containing trypsinates of (*A*) CHP-100 before (○) and after passage through a nude mouse (●) and (*B*) CHP-100 maintained in culture for 30 (○) or 58 (●) passages. All of the cells were grown in the presence of L-[^{3}H]- or [^{14}C]fucose for 72 hr. See text and Chart 1 for further details. ◐, coinciding points.

transferase and acetylcholinesterase, 166 and 164 pmoles and 848 and 585 pmoles/mg of protein, respectively (Table 1). The morphological and growth characteristics of these cells appeared to be slightly different from other neuroblastoma cell lines (10). Examination of this cell line at passage 30 or later at passage 58 showed in the latter passage a slightly increased proportion of the more rapidly eluting glycopeptides (Fractions 29 to 38; Chart 4*B*). Removal of the more loosely associated glycopeptides from CHP-100 cells by a mild trypsinization, trypsinate A, followed by the isolation of the surface membranes showed that more complex glycopeptides were available at the surface but in perhaps a more cryptic form (Chart 5). Small amounts of the more rapidly migrating glycopeptides beginning with Fraction 20 (Chart 5*B*) appeared to be present in the membranes. In addition, Fractions 3 to 8 (Chart 5*B*) contained fucose incorporated into material not digestable to smaller oligosaccharides by Pronase. The dialyzable material (Fractions 65 to 78) was not present in membrane, although it was present in all trypsinates. This has been seen in all membrane preparations from different cell types thus far examined. However, the presence of the more rapidly migrating material has not been seen to this extent (Chart 5*B*) in nontransformed or normal cell lines (Refs. 1 and 5; M. C. Glick, unpublished observations). A 2nd trypsinization showed a Sephadex G-50 profile similar to the first mild trypsinization (Chart 5*A*).

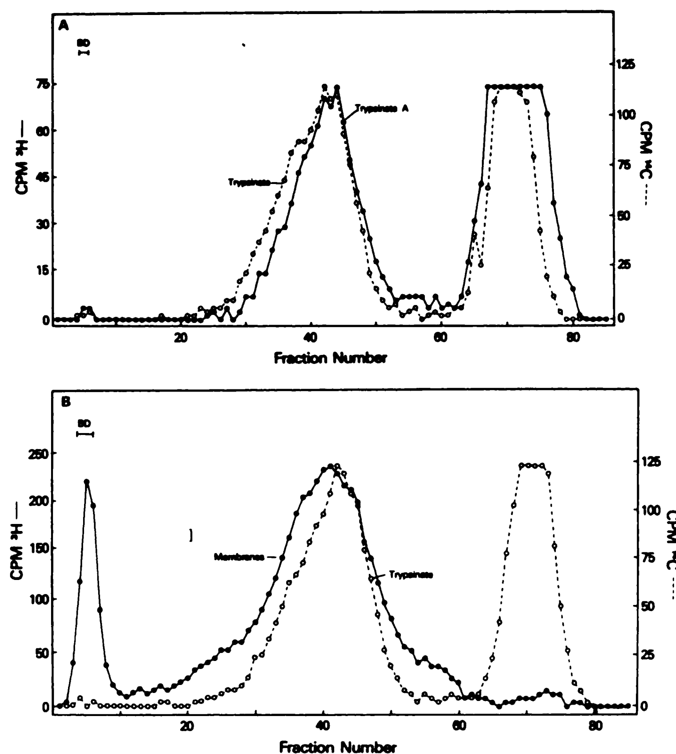

Chart 5. Sephadex G-50 pattern obtained after Pronase digestion of the fucose-containing trypsinate of CHP-100 (○) combined (prior to digestion) with (*A*) trypsinate A of CHP-100 (●) or (*B*) surface membranes of CHP-100 (●). The glycopeptides were made radioactive by growth of the cells in L-[^{3}H]- or [^{14}C]fucose for 72 hr. All details are in legends to Chart 2 and the text.

DISCUSSION

More complex glycopeptides appear to be available at the surface of human neuroblastoma cell lines than on the surface of human skin fibroblasts or brain cells from normal individuals when examined by gel filtration (Charts 2 and 3). The complex glycopeptides are those which elute most rapidly from columns of Sephadex G-50 and may contain longer chained or more highly branched oligosaccharide units (3).

The fact that the surface glycopeptides from the primary neuroblastoma tumors were not identical to those of the neuroblastoma cell lines (Charts 1 and 3) suggests that the cell lines do not completely retain the expression of the original tumor. A variety of surface antigens has been reported for neuronal cells (2) and the expression of any of these could be lost in culture. Alternately, it is possible that the specific cells that survived in the short-term primary culture were not completely representative of the tumor population. Several clonal lines derived from IMR-32 were examined for membrane glycopeptides and all gave identical Sephadex G-50 patterns. The fact that IMR-32, which is a cell line in culture for several years, has glycopeptides that are similar, by these techniques, to those of neuroblastoma cell lines cultured for less than 40 passages implies that the glycoproteins of the neuroblastoma lines are fairly stable.

Previous studies utilizing clones of mouse neuroblastoma (C-1300) and mouse neuroblastoma × rat glioma cell hybrids have shown that a particular group of glycopeptides is more prominent on the surface of cells that have the ability to differentiate morphologically with the formation of axon-like processes (4–6). These glycopeptides are grossly similar to those displayed on the surface of CHP-100. This cell line, CHP-100, derived from a tumor of a 12-year-old patient and diagnosed as consistent with neuroblastoma, did not show the presence of the complex oligosaccharides at the cell surface to the same extent as the other neuroblastoma cell lines (Charts 3 and 4). These cells were passed through nude mice in an effort to determine the tumorigenicity. The cells formed tumors in the nude mice (10) and retained their original levels for the enzymes involved in neurotransmitter synthesis (Table 1). Unfortunately, growth of a tumor in an immunologically suppressed animal is not an absolute criteria for tumorigenicity in humans. The tumor host may be responsible for specific glycopeptides in the tumor cell, since no gross alteration in glycopeptides was seen by passage of this human cell line, CHP-100, into the immunologically deficient mouse (Chart 4*A*). Previous studies with hamster cells have suggested the involvement of the host. That is, a cell line derived from chemically transformed hamster fibroblasts had surface glycopeptides similar to those of normal hamster cells; however, tumors formed by these cells in hamsters had glycopeptides that were more complex and corresponded to other tumor cells (7).

The results of these studies suggest that there are glycopeptides that are chemically distinct on the surface of human tumors. A detailed analysis of the membrane glycoproteins will help to define further the tumor cell and, eventually, it may be possible to separate the array of membrane antigens being reported currently. Indeed, even though the carbohydrates could give an almost unlimited number of specificities, it will not be surprising if cross-reactivities among the various surface antigens are abundant.

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