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Characterization of Mono-, Di-, and Tri-O-Acetylated Sialic Acids on Human Cells

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The presence of mono-, di-, and tri-O-acetylated sialic acids on human cells was demonstrated by using radiochromatographic and chemical techniques. Human melanoma cells and fresh colon tissue were biosynthetically labeled with 6- (^{3}H) glucosamine. Radiolabeled sialic acids were hydrolytically removed from cellular glycoconjugates, purified by ion-exchange chromatography, and separated by paper chromatography on the basis of the number of O-substitutions on each sialic molecule. This analytical technique characterized radiolabeled sialic acids that migrated with the same Rf as synthetic mono-, di-, and tri-O-acetylated ^{14}C-labeled sialic acids. The mono-O-acetylated sialic acids were characterized by their sensitivity to sodium periodate oxidation and a crude mouse liver esterase preparation. The di- and tri-O-acetylated sialic acids were characterized by their resistance to sodium periodate oxidation and sensitivity to the action of crude mouse liver esterase. Chromatographically separated di- and tri-O-acetylated sialic acids from normal human colon tissue were characterized by their respective ion molecular weights by using fast-atom bombardment-mass spectrometry. Using these methods, we chemically characterized mono, di-, and tri-O-acetylated sialic acids expressed on human cells. Aberrant expression of O-acetylated sialic acids was associated with adenocarcinoma of the colon, leading to a nearly complete loss of di- and tri-O-acetylated sialic acids.

Key words: O-acetylation, paper chromatography, fast-atom bombardment mass spectrometry

Sialic acids comprise a family of N- and O- substituted derivatives of neuraminic acid (5-amino-3,5-dideoxy-*D*-glycero-*D*-galacto-nonulosonic acid). A large number of these derivatives enzymatically generated from the parent molecules N-glycolyneuraminic acid (Neu5Gc) and N-acetylneuraminic acid (Neu5Ac) through O-subtitutions at the 4, 7, 8, and 9 positions have been found in nature [1,2] (see Fig 1). A variety of biological interactions have been attributed to the effects of sialic acids [1], including ones owed to negative charge, their influence on macromolecular structure [1], their ability to mask underlying and adjacent antigens [3,4], their antigenicity per se [5], and their role as

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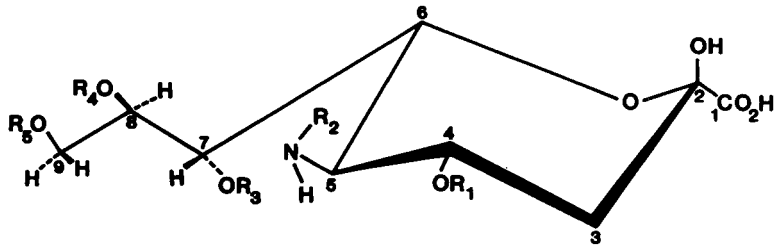

Fig. 1. The parent sialic acid molecule in the chair conformation. The individual carbons are numbered 1 through 9. Sialic acids are glycosidically linked at the anomeric C-2 position to galactose, N-acetylgalactosamine, N-acetylglucosamine, or sialic acid. R_2 indicates substitutions of the NH_2 group and R_1, R_3, R_4, and R_5 represent O-substitutions, which can be acetylated.

components of receptors [1]. In malignant disease, there are reports of increased levels of free and lipid-bound sialic acid in serum [6–9], and altered sialic acid expression has been implicated in invasion [10] and metastasis [11]. Unfortunately, few of these studies have determined the molecular species of sialic acids involved in these interactions.

O-acetylated sialic acids have been described in components of human tissues and fluids, including healthy and diseased colonic epithelial mucin [1,12–14], small intestine [15], B lymphocytes [16], brain gangliosides [17], bile [18], and in trace amounts in serum [19]. Cheresh and colleagues [20,21] have reported the generation of a tumor-associated antigen on human melanoma cells by an O-acetyltransferase that acetylated the C-9 position of the terminal Neu5Ac residue [22] on the disialoganglioside G_{D3}. Although the presence of mono-O-acetylated sialic acids has been confirmed by chemical analysis, the presence of di- and tri-O-acetylated sialic acids on human tissues was only inferred from histochemical and indirect chemical analyses.

The purpose of this investigation was to determine the chemical nature of the mono-, di-, and tri-O-substituents expressed on sialic acids from normal and neoplastic human cells. In previous studies, chemical characterization of O-acetylated sialic acid molecules required large numbers (10^8 to 10^{10}) of purified cells, limiting the range of human cells and tissues that could be studied. Alternatively, we have studied the expression of these molecules on human cells by characterizing mono-, di-, and tri-O-acetylated biosynthetically labeled sialic acids on fresh and cultured normal and malignant cells of human origin by paper chromatography. The migrations of these biosynthetically labeled sialic acids correlated with the migrations of synthesized O-acetylated sialic acids. In this report we present chemical evidence for the expression of mono-, di-, and tri-O-acetylated substituents on sialic acid molecules from human cells.

MATERIALS AND METHODS

Cell Lines and Patient Samples

The HT-29 colon carcinoma lines R and F were maintained as separate cell lines, originating from the same source. The HT-29 (lung) line, isolated from a lung metastatic lesion, the HT-29 (liver) line, isolated from a liver metastatic lesion in nude mice, HC 84, CL 3.5, and FI 81 fetal intestinal cells, and colonic fibroblast cell lines (4014, 4961, and HCF20) were maintained as monolayer cultures in Eagle's minimum essential me-

dium (MEM) (M.A. Bioproducts, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS) (Irvine Scientific, Santa Ana, CA), sodium pyruvate, nonessential amino acids, *L*-glutamine, and a twofold vitamin solution (GiBCO, Grand Island, NY). The HC 1410 and HC 1544 cell lines were derived from liver metastases of patients with primary rectal carcinoma, and HC 8589 was derived from a primary rectal carcinoma. These cell lines were established in culture after sc growth of the tumor in nude mice. The HC 2998 was established in vitro directly from surgically resected adenocarcinoma of the colon. These cell lines were maintained in Ham's F12 (M.A. Bioproducts) medium supplemented with 10% fetal bovine serum (FBS), *L*-glutamine, 5 μg/ml insulin, and 2 μg/ml transferrin (Sigma Chemical Co., St. Louis, MO). Isoenzyme and karyotype analysis verified the human origin of tumor lines. All of the experiments were performed within 20 passages of their establishment in vitro.

The human melanoma cell line PHL was originally established by Dr. W. A. Cassel of Emory University, Atlanta, GA. PHL cells (passage 78) were cultured in Leibowitz L-15 medium (GiBCO) containing 10% FBS in normal atmosphere at 37°C.

Fresh normal colon tissue was obtained from trauma patients who underwent surgery at the University of Texas Medical School at Houston. Specimens were also obtained from patients who underwent surgical resection of primary colorectal carcinomas or hepatic metastases at the University of Texas M. D. Anderson Hospital and Tumor Institute at Houston. All tissues were obtained in accordance with institutional and federal guidelines.

Metabolic Labeling of Sialic Acid Molecules

Cell lines and fresh colon samples were cultured in medium containing (6-^{3}H)*D*-glucosamine hydrochloride (specific activity, 45 Ci/mmol ICN Pharmaceuticals, Irvine, CA) at a concentration of 10 μCi/ml at 37°C in a humidified 5% CO_2 atmosphere in 75-cm^2 tissue culture flasks. PHL cells were cultured until confluent (3–4 days) and harvested by gentle scraping. Colon cells were seeded at 2×10^6 cells/ml, cultured for 3 days, and harvested by gentle scraping. Fresh tissues yielded 1,000–2,000 cpm/10^6 cells as purified sialic acids, while established cell lines yielded between 20,000 and 30,000 cpm/10^6 cells as purified sialic acids.

Hydrolytic Release and Purification of Sialic Acids

Sialic acids were purified from PHL and colon cells according to a modified procedure originally described by Varki and Diaz [23]. In brief, biosynthetically labeled sialic acids of washed, pelleted cells (10^6–10^7) were released by mild-acid hydrolysis in 3 ml of 2 M acetic acid (pH 2.5) at 80°C for 3 hr. After cooling on ice, insoluble material was removed by centrifugation. The supernatant fraction was passed through a 1-ml AG502-X8 (200–400 mesh, hydrogen form; Bio-Rad, Richmond, CA) cation exchange column and washed with 4 ml of water. The column effluent was lyophilized, resuspended in 3 ml of 0.01 M sodium formate (pH 5.5), and applied to a 1-ml AG3-X4A (100–200 mesh, formate form, Bio Rad) anion-exchange column equilibrated in 0.01 M sodium formate (pH 5.5). The column material was washed with 8 ml of 0.01 M formic acid, and then sialic acids were eluted with 10 ml of 1 M formic acid and lyophilized. The lyophilized material was resuspended in water and stored at −20°C before identification by paper chromatography.

Paper Chromatographic Analysis

Biosynthetically labeled sialic acids from PHL and colon cells were identified by ascending paper chromatographic analysis in a solvent system of n-butanol:n-propanol:0.1 N HCl (1:2:1, v/v) [24]. Prior to analysis, Whatman 3-MM paper (Fisher Scientific, Houston, TX) was washed sequentially with 0.1 N hydrochloric acid, distilled water, and chloroform:methanol (2:1, v/v). Carrier Neu5Ac (5μg) containing 2.5 nCi of ^{14}C-labeled Neu5Ac (specific activity, 244 mCi/mmol; Amersham; Arlington Heights, IL) was added to each sample as an internal standard. The chromatogram was developed for 6 hr (solvent migration, 20 cm), air dried at room temperature, and cut sequentially into 0.2-cm strips (1 cm wide) beginning at the origin. Each strip was placed in a 5-ml liquid scintillation vial and soaked in 100 μl of water overnight. Four milliliters of liquid scintillation cocktail (Formula 963, New England Nuclear, Boston, MA) was added to each sample and assayed for levels of ^{3}H and ^{14}C radioactivity. Migration profiles were generated by plotting radioactivity against the migration on the paper chromatogram. Biosynthetically radiolabeled carbohydrates other than sialic acids had slower rates of migration than did sialic acids. Relative percentages of the separated sialic acids were determined by integrating each peak curve to obtain the amount of ^{3}H radioactive material in each peak.

Synthetic O-Acetylation of Neu5Ac

Paper chromatographic standards of mono-, di-, and tri-O-acetylated sialic acids were prepared by per-O-acetylation of Neu5Ac to form N-acetyl-4,7,8,9-tetra-O-acetylneuraminic acid (Neu4,5,7,8,9Ac$_5$), followed by partial de-O-acetylation with mild acid and base. In brief, 500 μg of Neu5Ac (Sigma) containing 0.5 μCi of (^{14}C)-radiolabeled Neu5Ac was resuspended in 200 μl of dry pyridine:acetic anhydride (1:1) and incubated overnight at room temperature. The acetylated material was dried under a stream of nitrogen, resuspended in water, and lyophilized. An aliquot of this material was treated with 2 N acetic acid at 80°C for 3 hr, lyophilized, treated with 0.1 N ammonium hydroxide at 25°C for 1 hr, and lyophilized. This partially de-O-acetylated material was resuspended in water and characterized by paper chromatography.

Chemical Treatment of Sialic Acids

To cleave unsubstituted vicinal hydroxyl groups, we incubated biosynthetically labeled sialic acids in 1 ml of 10 mM sodium periodate on ice. After 30 min, excess periodate was consumed by addition of a tenfold molar excess of glycerol. The sialic acid material was repurified by anion-exchange chromatography and characterized by paper chromatography. To hydrolyze acetyl groups, we diluted radiolabeled sialic acids in 2 ml of 2 N ammonium hydroxide (or 2 ml of water as a control) and heated them at 60°C for 2 hr. After treatment, each preparation was repurified by anion-exchange chromatography and characterized by paper chromatography.

Treatment of Sialic Acids With a Crude Mouse Liver Extract

Sterile liver tissue from a BALB/c mouse was pressed through a 50-mesh stainless-steel screen and washed three times in Hanks' balanced salt solution (HBSS) (GiBCO). The cell pellet was resuspended in 5 volumes of 1% NP-40 (Sigma) in Tris-saline [25] and incubated on ice for 20 min. The insoluble material was removed by centrifugation at 16,000g for 10 min. Radiolabeled sialic acids from PHL melanoma cells were diluted

in 2 ml of the above extract containing 1 mM phenylmethylsulfonylfluoride (Fisher Scientific) and 1 % aprotinin (Sigma) and incubated at 37°C for 16 hr in a sterile polypropylene tube. An identical preparation, without the addition of liver tissue, served as a control. After treatment, each sample was repurified by anion-exchange chromatography and characterized by paper chromatography as described above. The recoveries of the radiolabeled sialic acids from the control and treated fractions after treatment and repurification were 45 and 47%, respectively.

Fast Atom Bombardment–Mass Spectral (FAB-MS) Analysis

Cryopreserved nonradiolabeled normal colon tissue (5×10^9 cells) was pressed through a 50-mesh stainless-steel screen and washed three times in HBSS prior to release and purification of the associated sialic acids as described above. The purified sialic acids were separated by paper chromatography, and material from the region corresponding to the migration of biosynthetically radiolabeled di- and tri-O-acetylated sialic acids (from the same tissue) was eluted in water and lyophilized. Material eluted from the paper directly adjacent to the region described above served as a control. Each sample was resuspended in glycerol:water (1:1,v/v) and 5 μl was placed on the probe plate. Positive-ion FAB-MS spectra were recorded on a Kratos MS50 RF mass spectrometer (acceleration voltage, 8 kV) with a 3,000-ppm sweep and 3.0-sec scan time. The FAB gun used xenon (emission current, 0.15 mA; acceleration voltage, 8 kV). The glycerol served as a matrix for the sample material and an internal standard. Mass equivalents in daltons were determined by standardizing the spectrophotometer with triazine and counting signal peaks representing single mass units. Analysis was confined to the 185–500 region of the mass spectrum, since we were only interested in identifying the monosaccharides released from the human colon cells.

RESULTS

The migration profile of O-acetylated sialic acids was standardized using synthetic (^{14}C)-labeled O-acetylated sialic acids after partial de-O-acetylation of Neu4,5,7,8,9Ac$_5$. Figure 2 represents the migration of these O-acetylated sialic acids expressed as a ratio of their migration divided by the migration of Neu5Ac. The Neu5Ac peak is labeled as 1.0, the mono-O-acetylated sialic acid peak as 1.24, the di-O-acetylated sialic acid peak as 1.40, the tri-O-acetylated sialic acid peak as 1.64, and the tetra-O-acetylated peak as 1.80. The migration of all radiolabeled sialic acid material was found to vary no more than ± 0.02 units from the recorded peak values shown in Figure 2.

To confirm that the synthetic O-acetylated sialic acids migrating at the 1.64 peak were isomers of tri-O-acetylated sialic acid, sialic acid material was eluted from the region for FAB-MS analysis. From this material, a positive ion molecular weight peak of 436 mass units was identified as the molecular weight of tri-O-acetylated sialic acid (data not shown).

The migration profile of radiolabeled sialic acids released from human PHL melanoma cells is shown in Figure 3A. The migration profile of these biosynthetically labeled sialic acids mimicked the migration ratios of synthetic Neu5Ac and O-acetylated sialic acids shown in Figure 2. The O-acetylated sialic acids released from PHL melanoma cells were characterized further by periodate oxidation. Biosynthesis of sialic acid from 6-(^{3}H) glucosamine produces molecules labeled at the C-9 position. The loss of radioactivity after periodate oxidation identifies only those sialic acid molecules with unsub-

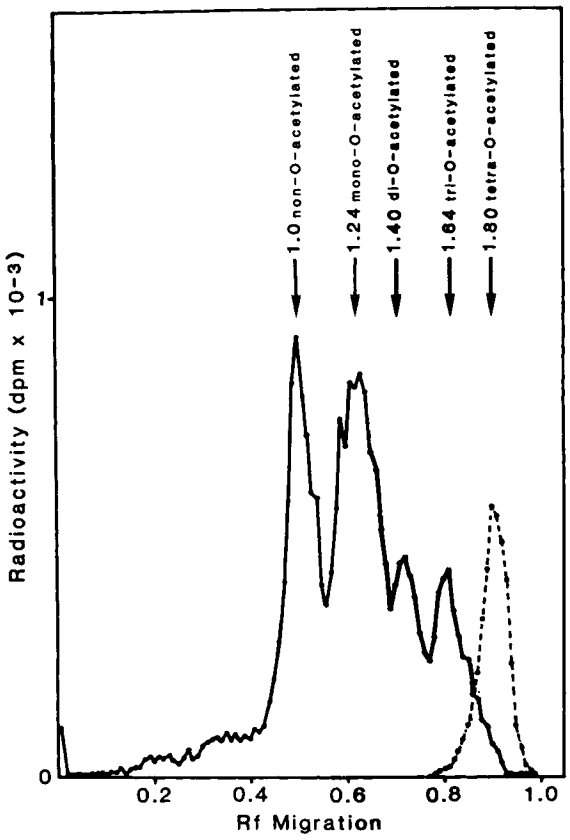

Fig. 2. The migration of synthesized O-acetylated sialic acids by paper chromatography. The paper chromatographic migration profile of each O-acetylated species of sialic acid was established by using chemically synthesized ^{14}C-labeled O-acetylated sialic acid derivatives. Tetra-O-acetylated sialic acid (Neu4,5,7,8,9Ac$_5$) was synthesized and partially de-O-acetylated with 2 N acetic acid at 80°C for 3 hr followed by 0.1 N NH_4 OH at 25°C for 1 hr to yield Neu5Ac, mono-, di-, and tri-O-acetylated sialic acid molecules. The dotted line represents the migration of Neu4,5,7,8,9,Ac$_5$ before treatment and the solid line after treatment with mild acid and alkali. The arrows delineating the peak values for the migration of radiolabeled sialic acids indicate migration ratio values relative to the migration of Neu5Ac.

stituted hydroxyls at C-7 and C-8 or C-8 and C-9 [26]. Substitution on these hydroxyls prevent oxidation and subsequent loss of the radiolabel at the C-9 position. Figure 3B demonstrates greater than 75% reduction of the amount of radioactive material migrating at 1.0 (non-O-acetylated Neu5Ac) and 1.24 (mono-O-acetylated Neu5Ac) but a less-than-10% decrease of radioactive material migrating with peak values of 1.40 (di-O-acetylated Neu5Ac) and 1.64 (tri-O-acetylated Neu5Ac).

Mild alkali treatment of sialic acids has been reported to remove acetyl groups from mono-O-acetylated sialic acid molecules [20,23]. The relative percentages of radiolabeled sialic acids from PHL human melanoma cells after treatment with 2 N ammoniun hydroxide are shown in Table I. This treatment decreased the relative amount of the tri-O-acetylated sialic acids by 44% compared with the starting material control. The total percentage of O-acetylated sialic acids, however, decreased only 1.9%. Liver

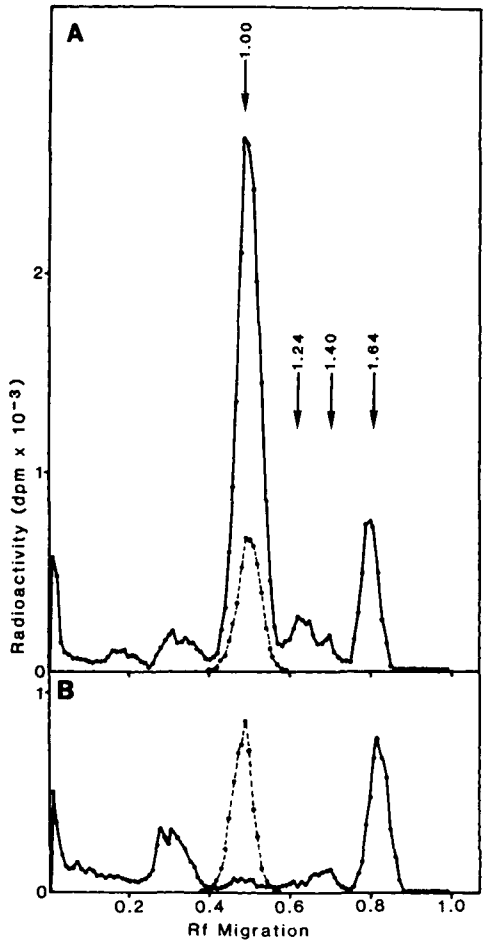

Fig. 3. Sodium periodate treatment of radiolabeled sialic acids released from PHL melanoma cells. The paper chromatographic migration profiles of biosynthetically radiolabeled sialic acid molecules released from human PHL melanoma cells. Sialic acids were treated with (**B**) and without (**A**) 10 mM sodium periodate on ice for 30 min and then repurified prior to paper chromatographic analysis, as described in the discussion of experimental procedures. The solid line represents the biosynthetically labeled material expressed on PHL melanoma cells, and the dotted line represents the migration of the (^{14}C) Neu5Ac internal standard. The arrows represent migration ratio values relative to the migration of Neu5Ac, as described in Figure 2.

tissue has been reported to contain a sialic-acid-specific esterase capable of removing O-acetyl groups [27]. An extract prepared from mouse liver caused a nearly complete removal of all O-acetyl groups compared with the control (Table I). In the control material (incubated in extraction buffer lacking liver tissue) spontaneous elimination of O-acetyl groups from the tri-O-acetylated sialic acid material occurred as reported previously [23]. However, the total percentage of O-acetylated sialic acids decreased only 2.9% from the percentage in the starting control material.

Sialic acid material released and purified from unlabeled, cryopreserved human

TABLE I. Mild Alkali and Esterase Treatment of Sialic Acids Released From PHL Melanoma Cells*

	Migration relative to Neu5Ac (percentages of total sialic acids)				
	1.00	1.24	1.40	1.64	Total O-Ac
Untreated material	72.7	7.8	3.2	16.3	27.3
Water (60°C for 2 hr)	72.3	9.2	3.1	15.4	27.7
2 N ammonium hydroxide (60°C for 2 hr)	74.6	1.30	1.5	10.9	25.4
Extraction buffer (37°C for 16 hr)	75.6	15.5	3.4	5.5	24.4
Crude liver esterase (37°C for 16 hr)	99.3	0.7	0	0	0.7

*Biosynthetically radiolabeled sialic acids expressed on PHL melanoma cells were released, purified, and identified by paper chromatography as described in the discussion of experimental procedures. The relative percentages of non (1.00)-, mono (1.24)-, di (1.64)-, and tri (1.64)-O-acetylated sialic acids expressed on the "untreated material" were determined before and after chemical or enzyme treatment. The total amount of O-acetylation before and after each treatment (total O-Ac) was calculated by subtracting the percentage of Neu5Ac (1.00) from 100%. Fractions of the "untreated material" were treated with 2 N NH_4OH or liver esterase as described in the discussion of experimental procedures.

colon cells migrating on paper to the region corresponding to the migration of biosynthetically radiolabeled di- and tri-O-acetylated sialic acids from the same tissue was analyzed by using FAB-MS. Ion molecular weight peaks generated in the positive-ion mode that were not present in the paper control (paper directly adjacent to the migration of di- and tri-O-substituted sialic acids) are shown in Table II. By this subtractive analysis, molecular ions of 394 and 436 were the only masses detected, corresponding to the ion molecular weights of di-O-acetylated sialic acid and tri-O-acetylated sialic acids, respectively. The 416 and 458 peaks correspond to di- and tri-O-acetylated sialic acids, respectively, each complexed with a sodium ion, while the 185, 277, and 369 peaks correspond to the di-, tri-, and tetra-glycerol peaks, respectively.

Biosynthetically radiolabeled sialic acids expressed on normal and malignant colon cells (of primary and metastatic origin) were separated and identified by paper chromatography. Changes in the expression of tri-O-acetylated sialic acid in normal, "uninvolved" (the excised end of colon tissue resected for carcinoma), "adjacent" (the macroscopically normal mucosa resected adjacent to the frank colon carcinoma), and adenocarcinoma tissues of the colon are shown in Figure 4. Figure 5 summarizes the relative expression of non-, mono-, di-, and tri-O-acetylated sialic acids on normal colon, "uninvolved" tumor mucosa, colonic adenocarcinoma, and hepatic adenocarcinoma derived from colon primaries. In the normal colon (Fig. 5A), the levels of tri-O-acetylated sialic acids was quite high, but decreased with a subsequent rise in the level of mono-O-acetylated sialic acid in the "uninvolved" mucoa of patients with adenocarcinoma of the colon (Fig. 5B). The adenocarcinoma tissues of primary (colon) or secondary (liver) origin all expressed low levels of mono-, di-, and tri-O-acetylated sialic acids (Fig. 5C–F) whether they were fresh or tissue culture established.

TABLE II. FAB Mass Spectra of Di- and Tri-O-Acetylated Sialic Acids Purified From Normal Human Colon Cells*

FAB signal (m/z)	Chemical derivation and molecular configuration	
93	Glycerol	$M + H^+$
185	Glycerol	$(M)_2 + H^+$
277	Glycerol	$(M)_3 + H^+$
369	Glycerol	$(M)_3 + H^+$
394	Di-O-AcNeu5Ac	$M + H^+$
416	Di-O-AcNeu5Ac(Na^+)	$M + Na^+ + H$
436	Tri-O-AcNeu5Ac	$M + H^+$
458	Tri-O-AcNeu5Ac(Na^+)	$M + Na^+ + H^+$

*Di- and tri-O-acetylated sialic acids expressed on normal human colon cells were eluted from their corresponding region on the paper chromatograph (shown in Fig. 2) as described in the Materials and Methods to yield a FAB-MS positive-ion fragmentation pattern.

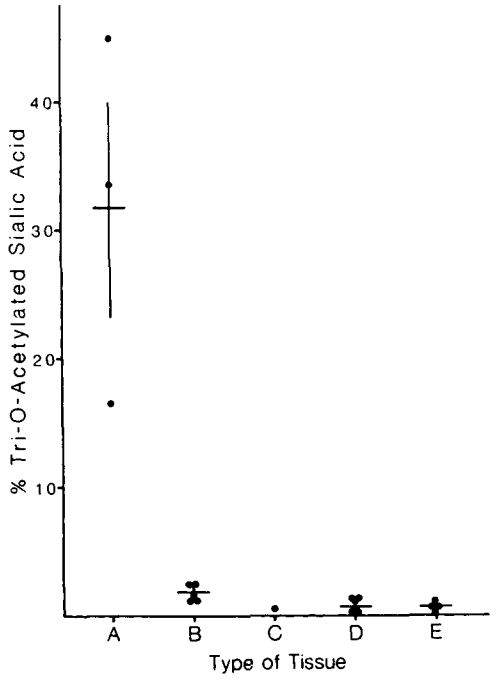

Fig. 4. A comparison of the amounts of tri-O-acetylated sialic acids on colon tissues. The relative amounts of tri-O-acetylated sialic acids on normal colon mucosa (**A**), uninvolved colonic mucosa distant from a colon adenocarcinoma (**B**), colonic mucosa adjacent to a colon adenocarcinoma (**C**), adenocarcinoma of the colon (**D**), and hepatic metastasis from colon primary (**E**) are represented as individual cases. For each tissue type, the mean of the percentage of tri-O-acetylated sialic acids (horizontal line) and standard error (vertical line) are represented.

DISCUSSION

We have presented chemical evidence that normal and malignant human cells express mono-, di-, and tri-O-acetylated sialic acids. To date, only mono-O-acetylated sialic acids have been chemically identified on human cells [1,16–22], while the expres-

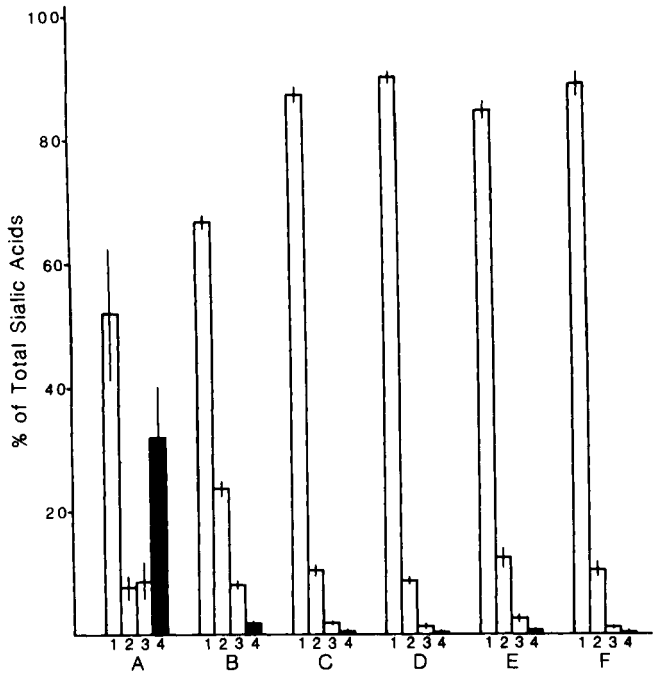

Fig. 5. A summary of non- and O-acetylated sialic expression in human colon. The relative amounts of non (1)-, mono (2)-, di (3)-, and tri (4)-O-acetylated sialic acids expressed on normal colon mucosa (**A**), fresh "uninvolved" colonic mucosa (**B**), fresh colonic adenocarcinoma (**C**), tissue culture established colonic adenocarcinoma (**D**), fresh hepatic adenocarcinoma derived from colon primaries (**E**), and tissue culture established metastatic adenoarcinomas derived from colon adenocarcinoma primaries (**F**). The error bars for each species of sialic acid (1–4) represent the standard error around the mean. Each species of sialic acid was identified by their respective migrations on Whatman 3MM paper, relative to the migration of Neu5Ac.

sion of di- and tri-O-acetylated sialic acids has only been inferred from histochemical and indirect chemical analysis [1,12–14]. As far as we know, this is the first report that indicates the presence of di- or tri-O-acetylated sialic acids on human cells using analytical chemical techniques. There was no evidence of N-glycolylneuraminic acid expression in these tissues above 0.1% of the total sialic acids. Furthermore, the expression of mono-, di-, and tri-O-acetylated sialic acids on human cells is not restricted to the two cell types described in this report. These molecules are expressed on freshly purified normal and malignant populations of human hematopoietic cells, colon, liver, spleen, and kidney tissues as well as other cultured melanoma cells and fibroblasts [28].

The migration of non- and O-acetylated sialic acid derivatives in the paper chromatographic system described in this report are similar to the results of Schauer [1] and others [16] using thin-layer cellulose plates. The heterogeneity within the peaks of migrating mono-, di-, and tri-O-acetylated sialic acids is a result of the random manner in which mild alkali removes O-acetyl groups from per-O-acetylated sialic acid (Fig. 2) [1].

When biosynthetically labeled sialic acids expressed on PHL melanoma cells were treated with sodium periodate, the tritium label at the C-9 position was cleaved from

sialic acids unsubstituted at C-7 and C-8, or C-8 and C-9, rendering them undetectable. Thus the loss of the radioactive peak at 1.24 confirms the mono-O-substituted nature of the sialic acids migrating to that position, while the resistance of the 1.40 and 1.64 radiolabeled peaks is consistent with the presence of di- and tri-O-substituted sialic acid molecules. These results suggest that the O-substitutions on these sialic acid molecules are located on C-7, C-8, or C-9 positions and not on C-4 (see Fig. 1).

To determine the chemical nature of the O-substitutions on these sialic acids released from human cells, we treated the radiolabeled sialic acids from PHL melanoma cells with mild alkali, a commonly used method to remove acetyl groups from O-substituted sialic acids. The relative amount of tri-O-acetylated sialic acids migrating at 1.64 was reduced after mild alkali treatment, but the total amount of O-acetylation remained the same (Table I). Although we were initially surprised by this result, Diaz and Varki [29] have also reported the presence of alkali-insensitive molecules in murine erythroleukemia cells, with nearly the same Rf migration as the O-substituted sialic acids that we have described. Enzymatic treatment of these radiolabeled sialic acids from PHL melanoma cells with a crude preparation of liver esterase resulted in complete removal of the O-substituted molecules.

Di- and tri-O-substituted sialic acids purified from unlabeled, cryopreserved normal colon cells, demonstrating the same mild alkali-insensitive characteristics as the sialic acids from PHL melanoma cells (data not shown), were isolated by paper chromatography and identified by their positive ion molecular weight as di- and tri-O-acetylated sialic acid peaks by using FAB-MS (Table II). This analysis confirmed that the O-substituted molecules were acetyl groups. Unfortunately, the FAB-MS analysis yielded ion molecular weight signals without evident of mass fragmentation, making identification of the positions of O-substitution impossible.

Many histochemical studies [30–32] have suggested that the sialic acids of normal colonic mucosa are predominantly di- and tri-O-substituted and that the loss of O-acetylation on sialic acid molecules expressed in human colon is a sensitive indicator of early malignant change. The results of our chemical studies support these findings, demonstrating lower levels of O-acetylation in the colon carcinomas than in normal colon mucosa (Fig. 5) and differences between the ratios of O-acetylated sialic acids expressed on "uninvolved" tumor mucosa and normal mucosa (Fig. 5). However, the association of tumor formation with the reduction of tri-O-acetylated sialic acids appears to be restricted to colonic epithelial tissue (Table I).

The use of biosynthetic labeling to quantitate relative amounts of non-, mono-, di-, and tri-O-acetylated sialic acids enabled the characterization of significant differences in the expression of these O-acetylated sialic acids on normal and malignant human tissues. However, these differences may not accurately represent the levels of these sialic acid molecules in the natural tissue state: only a direct measurement of the absolute chemical levels, such as by FAB-MS, in fresh tissues could provide such information. Differences were observed in the expression of non- and O-acetylated sialic acids on normal, "uninvolved" tumor mucosa and adenocarcinoma of the colon (Fig. 5).

The relative amount of tri-O-acetylated sialic acid expressed on these tissues appeared to be a sensitive indicator of premalignant change (Fig. 4). These findings support histochemical studies by Greaves and colleagues [31], who observed a significant difference in the histochemical staining pattern of O-acetylation of sialic acids in normal, "transitional" mucosa, benign adenomas, and adenocarcinoma of the colon. We suggest

that these abnormal patterns of O-acetylated sialic acids represent a premalignant field defect, rather than a local secondary effect of tumor growth. Reduced levels of O-acetylation of sialic acids in cases of ulcerative colitis [30] and metaplastic polyps [33] support this suggestion.

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