Comparison of O-Linked Carbohydrate Chains in MUC-1 Mucin from Normal Breast Epithelial Cell Lines and Breast Carcinoma Cell Lines

DEMONSTRATION OF SIMPLER AND FEWER GLYCAN CHAINS IN TUMOR CELLS*

(Received for publication, August 2, 1996, and in revised form, October 9, 1996)

Kenneth O. Lloyd‡§, Joy Burchell¶, Valery Kudryashov‡, Beatrice W. T. Yin†, and Joyce Taylor-Papadimitriou¶

From the Immunology Program, Memorial Sloan-Kettering Cancer Center, New York, New York 10021 and the Imperial Cancer Research Fund, Lincoln’s Inn Fields, London WC2A 3PX, United Kingdom

MUC-1 mucin is considered to be aberrantly glycosylated in breast, ovary, and other carcinomas in comparison with mucin from corresponding normal tissues. In order to clarify these differences in glycosylation, we have compared the O-linked carbohydrate chains from MUC-1 immunoprecipitated from [3H]GlcN-labeled breast epithelial cell lines (MMSV1–1, MTSV1–7, and HB-2) derived from cells cultured from human milk, with three breast cancer cell lines (MCF-7, BT-20, and T47D). Analysis by high pH anion chromatography showed that the normal cell lines had a higher ratio of GlcN/GalN and more complex oligosaccharide profiles than the cancer cell lines. Structural analyses were carried out on the oligosaccharides from MTSV1–7 and T47D MUC-1, and the following structures were proposed. MUC-1 from T47D had rather a simple glycosylation pattern, with NeuAcα2-3Galβ1-3GalNAc-ol, Galβ1-3GalNAc-ol, and GalNAc-ol predominating; in contrast, MUC-1 from MTSV1–7 had more complex structures, including a number of disialo, core 2 species, i.e. NeuAcα2-3Galβ1-4GlcNAcβ1-6Galβ1-3GalNAc-ol and NeuAcα2-3Galβ1-4GlcNAcβ1-6[NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-3GalNAc-ol. Double-labeling experiments with [3H]GlcN and 14C-aminoacids and analysis of GalNAc or GalNAc-ol/protein ratios in MUC-1 showed that there was also a significant difference in the degree of glycosylation of the mucin between the two cell types. We conclude that MUC-1 from breast cancer cell lines has simpler, and fewer, carbohydrate chains than MUC-1 from normal breast epithelial cells, and that these differences, combined or separately, explain the differential tumor specificity of some MUC-1 antibodies and T cells.

*This work was supported in part by National Institutes of Health Grants CA-52477 and CA-08748. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§To whom correspondence should be addressed: Memorial Sloan-Kettering Center, 1275 York Ave., New York, NY 10021. Tel.: 212-639-2257; Fax: 212-717-3379.

†The abbreviations used are: mAb, monoclonal antibody; HPAEC, high pH anion exchange chromatography; GalNAc-ol, N-acetylgalactosaminitol; NeuAcα(C7), 9-acetamido-3,5-dideoxy-l-arabinoh-2-heptulosonic acid; g.u., glucose units; NDV, Newcastle disease virus; FBS, fetal bovine serum; DMEM, Dulbecco’s modified Eagle’s medium; Mes, 2-(N-morpholino)ethanesulfonic acid.

This paper is available on line at http://www-jbc.stanford.edu/jbc/33325
carbohydrate chains were subsequently analyzed by various high performance liquid chromatography and gel filtration procedures and sequential enzyme and chemical treatments. The results show that MUC-1 samples from the three breast cancer lines examined have similar, but not identical, glycosylation patterns and that these differ significantly from the more complex glycosylation patterns of normal epithelial mucins. The data also indicate that like normal breast tissue, the milk-derived cell lines retain the ability to extend O-glycans via the core 2 β1-6GlcNAc transferase pathway.

EXPERIMENTAL PROCEDURES

Cell Culture

The non-malignant cell lines MMSV1–1 and MTSV1–7, and HB2 cells (14) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum supplemented with 10 μg/ml insulin and 5 μg/ml hydrocortisone. Breast cancer cell lines BT20 (15) were grown in minimal essential medium with 10% FBS and 10 μg/ml insulin, T47D (16) in DMEM plus 10% FBS, and MCF-7 (17) in DMEM with 10% FBS and 10 μg/ml insulin.

Radiolabeling and Immunoprecipitation

Cells were grown to 80% confluence and washed with DMEM containing only 0.45 mg/ml glucose. The cells were incubated at 37 °C for 1–2 h in this medium containing 5% FBS and then labeled for 18 h at 37 °C with 100 μCi/ml of [3H]glucosamine hydrochloride (20–40 Ci/mmol, Amersham International). After washing twice with cold phosphate-buffered saline, cells were lysed by incubating with buffer (20 mM EDTA, 1% Triton X-100, 1% deoxycholate, 25 mM leupeptin, 0.625 mM phenylmethylsulfonyl fluoride, and 10 μg/ml aproitin) on ice for 15 min. Ghosts were then scraped and membranes removed by spinning in a microcentrifuge for 10 min at 4 °C. Lysates were immunoprecipitated with CT-1 polyclonal rabbit antiserum (directed to the cytoplasmic tail of MUC1) or normal rabbit serum and protein A-agarose as described previously (18).

For double-labeling experiments, MTSV1–7 and T47D cells were cultured in complete DMEM and 10% FBS containing [3H]GlcN (25 μCi/ml) and [3C]U-labeled amino acid mixture (12.5 μCi/ml; DuPont NEN) for 2 or 3 days at 37 °C. Cells were lysed and the extract used for immunoprecipitation experiments. In order to lower the [%] back-ground counts, lysates were first cleared with normal mouse serum (2 μl/100 μl of lysate) and protein A-agarose. Double immunoprecipitations were then carried out as follows: the immunoprecipitate with mAb 139H2 or normal mouse serum was extracted in 2% SDS, and the extract diluted to 0.1% SDS and precipitated with mAb 139H2 or normal mouse serum as described above. The final immunoprecipitates were extracted with 2% SDS, and aliquots were counted for 3H and 4C in a Beckman scintillation counter and analyzed for radiolabeled sugars as described below.

Sugar Analysis

[3H]GlcN-labeled MUC-1 was eluted from the immunoprecipitates with 2% SDS in H2O at 37 °C for 3 h. Aliquots were then hydrolyzed in 2 or 4 N trifluoroacetic acid at 100 °C for 3 h for hexosamine analysis. After evaporation in a Speed Vac (Savant Instruments Co., Holbrook, NY), the sample was dissolved in 30 μl of H2O containing deoxygalacto-side (1.0 μg), galactosamine (1.0 μg), and glucosamine (1.0 μg). The sample (25 μl) was analyzed by HPAEC as described below (Program 1). Aliquots were also hydrolyzed in 0.1 N HCl at 80 °C for 1 h for sialic acid analysis. After evaporation in a Speed Vac, the sample was dissolved in 30 μl of H2O containing deoxygalactose (1.0 μg) and NeuAc (1.0 μg). The sample was analyzed by HPAEC as described below (Program 2).

Release of O-Linked Oligosaccharides

Radioimmunoprecipitates (on protein A-agarose beads) were treated directly with 0.1 M NaOH, 1 M NaBH4, (19, 20) for 2–3 days at room temperature. After careful neutralization with acetic acid (2 N), the sample was passed over a column of Dowex 50 H+ (1.0 ml) and the eluate evaporated to dryness in a rotary evaporator at 35 °C. The remaining boric acid was removed by evaporation in methanol. The sample was finally dissolved in H2O (200–500 μl) for subsequent analysis.

High Performance Anion Exchange Chromatography (HPAEC)

HPAEC separation of sugars and oligosaccharides was carried out in a BioLC system ( Dionex Corp., Sunnyvale, CA) fitted with a pulsed amperometric detector. Different columns, solvents, and gradients were used for the analysis and separation of sugars and neutral and acidic oligosaccharides as follows.

Analysis of Hexosamines (Program 1)—Glucosamine and galactosamine were separated on a Carbopak PA1 column (4 × 250 mm) by isocratic elution with 0.01 M NaOH at 1.0 ml/min (21). For radiolabeled samples, fractions (0.25 ml) were collected directly into scintillation vials and counted in a liquid scintillation fluid (5 ml; National Diagnostics, Atlanta, GA) using a scintillation counter (Cobra, Beckman Instruments Co., Fullerton, CA).

Analysis of Sialic Acids and Separation of Reduced Oligosaccharides (Program 2)—For sialic acid and reduced oligosaccharide separations, the samples were analyzed on a CarboPak PA1 column (4 × 250 mm) using a gradient from 0.2 M NaOH to 0.2 M NaOH + 0.25 M sodium acetate at 1.0 ml/min over 30 min (22). For radioactive samples, fractions (0.25 ml) were collected directly in scintillation vials and counted in Liquiscint (5 ml) after neutralizing with 1 N HCl and adding H2O2 (250 μl).

In addition to the elution times of a series of reduced oligosaccharides listed in Ref. 22, the elution times under these conditions of the following new standard oligosaccharides were determined: NeuAcα2→3Galβ1→3GalNAc-ol (8.5 min), Galβ1→3NeuAcα2→GalNAc-ol (9.2 min). NeuAcα2→3Galβ1→4GlcNAcβ1→6 (NeuAcα2→3Galβ1→3GalNAc-ol (19.5 min), and Galβ1→4GlcNAcβ1→6Galβ1→3 GalNAc-ol (3.6 min). Because of inter-run variations in this system, the elution times of unknown oligosaccharides were directly compared with standard compounds.

Separation of Acidic Reduced Oligosaccharides by Ion Exchange Chromatography (Program 3)—Reduced oligosaccharides were also separated under non-alkaline conditions on a CarboPak PA1 column (4 × 250 mm) by elution with 0.01 M pyridine-acetic acid, pH 5.1, buffer for 5 min followed by a gradient from 0.01 M pyridine-acetic acid, pH 5.1, to 0.5 M pyridine-acetic acid, pH 5.1, over 30 min at 1.0 ml/min. For radioactive samples, fractions (0.25 ml) were collected, without post-column addition of NaOH, and aliquots counted directly in Liquiscint (5 ml). Radioactive peaks were pooled, the pyridine-acetic acid removed by evaporation, and the samples used for further analysis.

Separation of N-Acetylgalactosaminiloligosaccharides and Reduced Disaccharides (Program 4)—N-Acetylgalactosaminiloligosaccharides and small reduced oligosaccharides were analyzed on a CarboPak MA-1 column (4 × 250 mm) with a gradient from 0.07 M NaOH to 0.7 M NaOH over 30 min at 0.4 ml/min. For radioactive samples, fractions (0.25 ml) were collected, without post-column addition of NaOH, and aliquots counted directly in Liquiscint (5 ml). After neutralizing with 1 N HCl, this column and program was also used to identify N-acetylgalactosaminiloligosaccharides after hydrolysis and re-N-acetylation as described by Karlsson and Hansson (23).

Gel Filtration and Enzyme Sequence Treatment of Oligosaccharides

Reduced oligosaccharides were separated and analyzed by gel filtration, as modified from the procedure of Kobata and co-workers (24), on a Bio-Gel P4 (200–400-mesh, Bio-Rad) column (1.4 × 100 cm) by elution in 0.1 m pyridine-acetic acid buffer, pH 5.5, at room temperature. Fractions (1.0 ml) were collected, and aliquots were counted for radioactivity in Liquiscint. The column was calibrated with various sugars and reduced oligosaccharides, including glucose, N-acetylgalacosaminiloligosaccharides, Galβ1→3GalNAc-ol, NeuAcα2→3Galβ1→4GlcNAcβ1→6 (NeuAcα2→3Galβ1→3GalNAc-ol, and Galβ1→4GlcNAcβ1→6 (Galβ1→3GalNAc-ol, and the sizes of the oligosaccharides were calculated in glucose units (g.u.) as defined by these authors. Under our chromatographic conditions hexoses correspond to 1 g.u., N-acetylgalactosaminiloligosaccharides to 2 g.u., N-acetylgalactosaminiloligosaccharides to 3.0 g.u. Reisolated oligosaccharides were treated with glycosidases under the following conditions: (i) Vibrio cholerae sialidase (Calbiochem), 10 millunits/50 μl 100 mM sodium acetate, pH 5.5, with 10 mM CaC2O4; (ii) Newcastle disease virus sialidase (Oxford Glycosystems, Rosedale, NY); and (iii) jack bean β-galactosidase (Sigma), 20 millunits/50 μl in 100 mM citrate-phosphate buffer, pH 5.5; (iv) Streptococcus pneumoniae β-galactosidase (Oxford Glycosystems), 2 millunits/50 μl in citrate-phosphate, pH 6.0; (v) jack bean β-N-acetylgalactosaminidase (Sigma), 0.5 units/50 μl citrate-phosphate, pH 5.0; and (vi) Bacteriodes fragilisendo-β-galactosi-dase, 1 millunit/50 μl in sodium acetate, pH 5.8. All incubations were conditioned with glycosidase as described in the original reference.
carried out at 37 °C for 16–24 h. Sialic acid was also removed from oligosaccharides by mild hydrolysis in 0.1 N HCl at 80 °C for 1 h.

Periodate Oxidation

The position and degree of substitution on the GalNAc-ol residue (core structure) was determined as follows. Reduced oligosaccharides were treated with dilute sodium periodate (1 mM in phosphate-buffered saline, pH 7.0) under conditions in which only sugar alcohols (GalNAc-ol) and the side chain of NeuAc were oxidized (25, 26) and reanalyzed by Bio-Gel P4 chromatography. Following this reaction the side chain of NeuAc has lost radioactivity (from C-9), but the resulting heptulosonic acid still corresponds to about 5 g.u. on Bio-Gel P4 chromatography. The substituted two and four carbon aldehydes resulting from the cleavage of GalNAc-ol between C-4 and C-5 in core 2 and core 4 structures (see "Results") contribute 1.0 and 2.0 g.u., respectively. These data were derived by applying the procedure to core 1 (Galβ1→3GalNAc-ol) and core 2 (NeuAca2→3Galβ1→4GlcNAcβ1→6 [NeuAca2→3Galβ1→3] GalNAc-ol radioactive standards. Oligosaccharides were also oxidized with stronger periodate (0.1 M) and then reduced and hydrolyzed to cleave after the oxidized sugar residue according to the procedure of Smith and co-workers (27).

RESULTS

Radioimmunoprecipitation of MUC-1 from Cell Lines—Immunoprecipitation of [3H]GlcN-labeled cell extracts with CT-1 antiserum and analysis by SDS-polyacrylamide gel electrophoresis and autoradiography showed that specific high molecular weight species were precipitated from the normal epithelial cell line (MTSV1–7) and three breast cancer cell lines (Fig. 1). CT-1 (18) is directed to the terminal amino acids of the cytoplasmic tail, which is not glycosylated, so that all glycoforms are precipitated by this antiserum. Differences observed in the number and apparent sizes of the product from the various cell lines were observed; they are due to polymorphisms in the numbers of tandem-repeat units (3, 4). Immunoprecipitation of [3H]Fuc-labeled MTSV1–7 cell extracts with anti-CT-1 did not yield any specific components (data not shown).

Hexosamine and Sialic Acid Analysis of Radiolabeled MUC-1 from Normal and Breast Cancer Cell Lines—Analysis of the hexosamine content of [3H]GlcN-labeled MUC-1 immunoprecipitates (CT-1 antibody) by acid hydrolysis and HPAEC analysis showed that two components corresponding to GalN and GlcN could be identified (Fig. 2). The ratio of radiolabeled GlcN/GalN varied quite considerably among the different cell lines (Table I). All three normal cell lines had a high GlcN/GalN ratio, whereas in the three breast cancer cell lines the ratio was much lower. In MUC-1 from the breast cancer cell line T47D, GalN was the only hexosamine detected (Fig. 2C and Table I). These variations could not be ascribed to different patterns of metabolism of [3H]GlcN by the various cultured cell lines, as the GlcN/GalN ratio in total cell lysates from the five cell lines was relatively constant at 4:1. These results indicate that GlcNAc-containing oligosaccharides should predominate in the normal breast epithelium MUC-1, whereas in breast cancer MUC-1 has fewer GlcNAc-containing structures. T47D MUC-1 was different from MUC-1 precipitated from the other cell lines in having only GalNAc-containing oligosaccharides. Radiolabeled NeuAc was identified in MUC-1 from the three normal

Fig. 1. Immunoprecipitation of MUC-1 from [3H]GlcN-labeled cell lines analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. CT-1, rabbit anti-MUC-1 antiserum; NRS, normal rabbit serum.

Fig. 2. HPAEC analysis of radiolabeled hexosamine and sialic acid content of MUC-1 immunoprecipitates. A, MTSV1–7 hexosamines; B, MTSV1–7 sialic acid; C, T47D hexosamines; D, T47D sialic acid. ■, CT-1 antibody immunoprecipitates; □, normal rabbit serum immunoprecipitates. The samples were analyzed on a Dionex PA-1 column using Program 1.
and three breast cancer cell lines (Fig. 2, B and D; Table I).

While this method does not provide a strictly quantitative measure of GlcNAc/GalNAc/NeuAc ratios because of the unequal metabolic redistribution of the $^3$H label from $[^3$H]GlcN into the different sugars, it does give a semi-quantitative way of determining the hexosamine and sialic acid composition of radiolabeled antigens. Moreover, radiolabel levels in the different oligosaccharides derived by enzyme treatment of the MUC-1-derived oligosaccharides as described below indicated that the radioactivity was distributed among GalNAc, GlcNAc, and NeuAc at a ratio of approximately 1.0:1.0:0.75.

O-Linked Oligosaccharides of MUC-1 from Normal Breast Cancer Cell Lines and Breast Cancer Cell Lines Analyzed by HPAEC—The HPAEC chromatographic profiles of the reduced oligosaccharides released from the MUC-1 immunoprecipitated with CT-1 antibody from two normal breast epithelial cell lines (MTSV1–7 and HB2) by alkaline borohydride treatment were very similar to each other (Fig. 3A shows the MTSV1–7 result). Peak 4, eluting in the disialo-oligosaccharide region, predominated. Oligosaccharide profiles from MUC-1 derived from the three breast cancer cell lines (Fig. 3, B–D) differed from these patterns in having less of the disialo peak and larger proportions of monosialo (peak 2) and/or neutral (peak 1) species. The profile of MCF-7 MUC-1 most closely resembled the profile of MUC-1 from the normal epithelial cell lines in that the disialo species (peak 4) was a major peak (Fig. 3B). BT-20 MUC-1 had less of peak 4 and more monosialo species (Fig. 3C). T47D MUC-1 yielded the simplest profile, with the neutral and monosialo peaks predominating (Fig. 3D). MUC-1 immunoprecipitates using mAb 139H2 gave oligosaccharide profiles similar to those derived using CT-1 antiserum (data not shown). These results are consistent with the hexosamine analysis data (Table I), which showed that the normal breast cell lines differed from the breast cancer cell lines in having a high proportion of GlcNAc. From these results it would also be predicted that the disialo peak 4 contains mainly oligosaccharides rich in GlcNAc. Correspondingly, peaks 1 and 2 from T47D MUC-1 oligosaccharides would be predicted to contain only GalNAc (GalNAc-ol after reduction).

Structural Analysis of MUC-1 Oligosaccharides from MTSV1–7 Normal Epithelial Cell Line—The radiolabeled reduced oligosaccharides from MTSV1–7 MUC-1 were separated on a preparative scale by ion-chromatography on a Dionex PA1 column with a pyridine-acetate buffer (Fig. 4A). A complex pattern of peaks was observed with neutral (1) and disialo-oligosaccharides (4a, b, and c) components predominating. Recomn chromatography of components 4a, b and c showed that they corresponded to peak 4 in the HPAEC chromatogram shown in Fig. 3A, and were identified as disialo-oligosaccharides. This result illustrates the superior resolving power of the PA-1 column in the ion-exchange mode. The major peaks (M-1, -4a, -4b, and -4c) were isolated and analyzed further.

Neutral Oligosaccharides—The neutral fraction (M-1) from the ion-exchange column represented 18% of the applied radioactivity. Further fractionation on a Bio-Gel P4 column yielded one minor peak (M-1a) eluting at 6 g.u. and a major peak

| Cell line | GalNAc | GlcNAc | NeuAc | GlcNAc/GalNAc |
|-----------|--------|--------|-------|--------------|
| Normal breast epithelial cell lines | | | | |
| MTSV1–7 | 23.4 | 52.4 | 24.0 | 2.24 |
| HB-2 | 21.0 | 48.7 | 30.3 | 2.32 |
| MMSV-1 | 18.7 | 61.3 | 20.0 | 3.28 |
| Breast cancer cell lines | | | | |
| MCF-7 | 26.4 | 40.0 | 33.7 | 1.52 |
| BT-20 | 44.3 | 21.6 | 40.0 | 0.49 |
| T47D | 68.3 | 0 | 40.6 | Only GalNAc |

FIG. 3. HPAEC analysis of radiolabeled reduced oligosaccharides from MUC-1. A, MTSV1–7; B, MCF-7; C, BT-20; D, T47D. Peaks 1, 2, and 4 are discussed under "Results." Peak 3 is from NeuAc. The samples were analyzed on a Dionex PA-1 column using Program 2.
using Program 3. 

TheseparationswerecarriedoutonaDionexPA-1column -4a, -4b, -4c, T-1, T-2, and T-4c are discussed under "Results.". Peak 3 is from NeuAc. The separations were carried out on a Dionex PA-1 column using Program 3.

(M-1b) eluting at 4 g.u. The major peak (M-1b) was shown after acid hydrolysis and HPAEC analysis to contain only radioactive GlcNAc-ol and no GlcNAc or GalNAc. Oligosaccharide M-1b was further analyzed on a MA-1 column and shown to elute with Galβ1→3GalNAc-ol. The minor oligosaccharide (M-1a) was not examined further.

Oligosaccharide M-4a—This reduced disialo-oligosaccharide eluted on a Bio-Gel P4 column at a position corresponding to 20 glucose units (Fig. 5A). Sugar analysis (Programs 1 and 2) after acid hydrolysis showed the presence of radioactive GlcNAc, GalNAc-ol, and NeuAc. Partial acid hydrolysis resulted in the loss of 2 NeuAc residues, as did treatment with NDV neuraminidase, thus showing the presence of two radioactive peaks (Fig. 6A) and the absence of core 4 structure in oligosaccharide M-4a, as shown in Fig. 8.

Sugar analysis by HPAEC on a PA1 column (Programs 1 and 2) showed after acid hydrolysis in this component (data not shown). This disialo-oligosaccharide eluted with NeuAc2→3Galβ1→4GlcNAcβ1→6(NeuAc2→3Galβ1→4GlcNAcβ1→6Galβ1→3)GalNAc-ol in ion-exchange, HPAEC, and Bio-Gel P4 chromatography. Mild acid hydrolysis or treatment with V. cholerae neuraminidase released NeuAc and an oligosaccharide eluting with Galβ1→4GlcNAcβ1→6Galβ1→3GalNAc-ol on HPAEC chromatography (Program 2) and Bio-Gel P4 chromatography. On the Bio-Gel P4 column, M-4b eluted at a position corresponding to 17 g.u. before desialylation (Fig. 9) and 7 g.u. after desialylation, indicating the presence of two NeuAc units. NeuAc was also totally removed by digestion with NDV neuraminidase, thus showing the presence of two NeuAc2→3Gal linkages. Further digestion with diploccal β-galactosidase resulted in the loss of one hexose unit and a component eluting with GlcNAcβ1→6Galβ1→3GalNAc-ol on Bio-Gel P4 chromatography (Fig. 9C). Further hydrolysis with β-hexosaminidase gave two radioactive peaks corresponding to Galβ1→3GalNAc-ol and GalNAc (Fig. 9D). Treatment of oligosaccharide M-4b with mild sodium periodate as described above resulted in the formation of only two radioactive peaks (Fig. 6C). The larger compound eluted at a position corresponding to NeuAc(C7)α2→3Galβ1→4GlcNAcβ1→1CH2-CHO. The later eluting peak probably corresponds to radioactive formaldehyde derived from NeuAc. Partial acid hydrolysis of the compound eluting at the position of 9 g.u. resulted in a shift in the Bio-Gel P4 chromatogram corresponding to the loss of one non-radioactive, NeuAc(C7) residue and an oligosaccharide eluting at 4.0 g.u., corresponding to Galβ1→4GlcNAcβ1→6Galβ1→3GalNAc-ol on Bio-Gel P4 chromatography (Fig. 6D). This product was shown by acid hydrolysis and HPAEC analysis to still contain GlcNAc. This result is consistent with the presence of a GalNAc-containing core 2 (Fig. 7; n = 0) and the absence of core 4 structure in oligosaccharide M-4b, as the latter species of this size would have afforded three radioactive peaks by mild periodate treatment (Fig. 7). Core 1 (n = 1) or core 3 structures would also have yielded two radioactive peaks after mild periodate treatment, but these structures would have given different products following glycosidase treatments. From these data we conclude that oligosaccharide M-4b is probably the disialo, core 2 species shown in Fig. 8.
Oligosaccharide M-4c—This reduced oligosaccharide eluted with NeuAc\(^{a2,3}3\text{Gal(NeuAc}\(^{a2,3}6\)GalNAc-ol by HPAEC and by gel filtration on a Bio-Gel P4 column. Sugar analysis by HPAEC on PA1 (Program 2) and MA1 (Program 4) columns showed that this component contained radiolabeled GalNAc-ol and NeuAc but no GlcNAc or GalNAc (data not shown). The NeuAc/GalNAc-ol ratio was twice that in oligosaccharide T-2. Mild acid hydrolysis or \textit{V. cholerae} neuraminidase treatment resulted in a peak on Bio-Gel P4 chromatography corresponding to the liberation of two units of NeuAc. HPAEC showed that this peak contained a compound eluting with Gal\(^{\beta1,3}\text{GalNAc-ol as well as the NeuAc. Digestion with NDV neuraminidase resulted in a product eluting with Gal\(^{\beta1,3}\text{GlcNAc-ol on HPAEC. These data indicate that M-4c is NeuAc}\(^{a2,3}3\text{Gal(GlcNAc}\(^{a2,3}6\)GalNAc-ol, a structure found in many mucins (Fig. 8).}

Structural Analysis of MUC-1 Oligosaccharides from T47D Breast Cancer Cell Line—The radiolabeled reduced oligosaccharides released from T47D MUC-1 immunoprecipitates (CT-1 antibody) were separated on a preparative scale by ion exchange chromatography on a Dionex PA1 column by elution with a gradient of pyridine-acetate buffer (Fig. 4B). A neutral
peak (T-1) and a monosialo compound (T-2) were isolated and analyzed further. A minor disialo peak (T-4c) was not studied. Rechromatography showed that these corresponded to peaks 1 and 2 in the HPAEC profile (Fig. 3). Sugar analysis showed that T-1 and T-2 contained no radioactive GlcN or GalN but that both contained N-acetylgalactosaminitol and T-2 contained NeuAc (data not shown). Peak T-1 was further analyzed on a Dionex MA 1 column (Program 4) without hydrolysis, and components eluting with GalNAc-ol (T-1a) and Galb133GalNAc-ol (T-1b) were identified.

The monosialo peak (T-2) eluted with NeuAco23Galb13GalNAc-ol (10.8 min) and not with Galb133GalNAc-ol (11.4 min) or disialooligosaccharides (24 min) on HPAEC (Fig. 10A). The compound was hydrolyzed by either mild hydrolysis or V. cholerae neuraminidase to a neutral compound eluting with Galb133GalNAc-ol in the two cell lines was 3.6. It was therefore concluded that MTSV1–7 contained about 3.5 times as much GalNAc as T47D when compared on an equalized 14C basis. As normal breast epithelium and breast cancer MUC-1 have identical tandem repeat sequences (28), and therefore similar amino acid compositions for their peptide cores, this is a reasonable method of comparison. Even though one of the alleles for T47D mucin is smaller than the alleles of MTSV1–7 this would not account for the different ratios. After reduction with OH−BH4−, all the GalNAc was converted to GalNAc-ol showing that it was all linked to peptide and the normalized ratio of GalNAc-ol in the two cell lines was 3.6. It was therefore concluded that MTSV1–7 contained about 3.5 times as much carbohydrate chains as T47D MUC-1. It seems unlikely that this difference could be accounted for by a difference in the uptake or metabolism of [3H]GlcN and 14C-aminoacids, as the 3H/14C ratio in the total cell lysates (representing all the glycoproteins of the cells) was similar in the two cell lines (5.2 for MTSV1–7 and 3.3 for T47D), whereas the MUC-1 immunoprecipitates were highly enriched in 3H and the ratios were different from each other (3H/14C ratio of 281 for MTSV1–7 and 27 for T47D).

Comparison of the Degree of Glycosylation of MUC-1 Peptide in Normal Breast and Breast Cancer Cell Lines—To estimate the degree of substitution of the peptide moiety of MUC-1 from MTSV1–7 and T47D, a double labeling experiment was carried out using [3H]GlcN to label the carbohydrate chains and a 14C-aminoacid mixture to label the peptide backbone. Comparison of 3H/14C ratios in the MUC-1 immunoprecipitates allowed a quantitative comparison of radiolabel incorporated into carbohydrate and protein in MUC-1 from the two cell lines. Analysis of 3H-labeled GlcN and GalN by HPAEC gave a comparison of the GalNAc content, as normalized to protein, and sugar analysis after OH−BH4− treatment determined what proportion of the GalNAc had been converted to GalNAc-ol. The results (Table II) showed that MTSV1–7 had a much higher sugar content than T47D when normalized to protein as measured by 14C-aminoacid content. Furthermore, MTSV1–7 MUC-1 contained 3.4 more GalNAc than T47D when compared on an equalized 14C basis. As normal breast epithelium and breast cancer MUC-1 have identical tandem repeat sequences (28), and therefore similar amino acid compositions for their peptide cores, this is a reasonable method of comparison. Even though one of the alleles for T47D mucin is smaller than the alleles of MTSV1–7 this would not account for the different ratios. After reduction with OH−BH4−, all the GalNAc was converted to GalNAc-ol showing that it was all linked to peptide and the normalized ratio of GalNAc-ol in the two cell lines was 3.6. It was therefore concluded that MTSV1–7 contained about 3.5 times as much carbohydrate chains as T47D MUC-1. It seems unlikely that this difference could be accounted for by a difference in the uptake or metabolism of [3H]GlcN and 14C-aminoacids, as the 3H/14C ratio in the total cell lysates (representing all the glycoproteins of the cells) was similar in the two cell lines (5.2 for MTSV1–7 and 3.3 for T47D), whereas the MUC-1 immunoprecipitates were highly enriched in 3H and the ratios were different from each other (3H/14C ratio of 281 for MTSV1–7 and 27 for T47D).

FIG. 7. Scheme showing probable effect of mild periodate oxidation on core 1, 2, 3, and 4 structures. Radioactive atoms or residues are shown in bold type. R" indicates a radiolabeled NeuAc-containing structure and R" the corresponding non-radioactive species after oxidation.

FIG. 8. Proposed structures of reduced oligosaccharides isolated from T47D and MTSV1–7 MUC-1 and the yield of each species as % of total radioactivity.
in nude mice (14). Moreover, in collagen gels these cell lines form organized structures characteristic of normal mammary epithelial cells and unlike breast carcinoma cells (29). Particularly pertinent to this study is the fact that these cell lines do not react with mAb SM3 (12, 30), which was raised to the peptide core of the MUC-1 mucin and shows selective reactivity with breast cancer specimens and not with normal breast epithelium in immunohistological analysis (8). In this study we show that these normal epithelial cell lines differ significantly from breast cancer cell lines in the glycosylation characteristics of their MUC-1 mucin molecules. The results support the contention that the breast epithelial cell lines afford a good model system in which to study the transformation events resulting in the generation of breast cancer. An important parameter in the design of these experiments was the use of polyclonal CT-1 antiserum or mAb 139H2 for the immunoprecipitation of MUC-1 from the normal and cancer cell lines. CT-1 antiserum is directed against the cytoplasmic tail of MUC-1 and will immunoprecipitate all species of MUC-1 irrespective of their glycosylation characteristics. mAb 139H2 is less sensitive than most anti-MUC-1 mAbs to the degree of glycosylation of the tandem repeat sequences (12, 30, 31).

The glycosylation patterns of MUC-1 from the three breast cancer cell lines studied (MCF-7, BT-20, and T47D) were similar to each other, but each showed distinctive features as determined by analyzing the alkaline borohydride-released reduced oligosaccharides by HPAEC. MUC-1 from the normal epithelial cell lines differed from the cancer cell lines in having a preponderance of disialylated chains. Among the cancer cell lines, the MCF-7 pattern most closely resembled the normal epithelial cell line patterns, with a substantial amount of disialo-oligosaccharides being detected (Fig. 3B). In many respects MCF-7 cells represent the most differentiated of the breast cancer cell lines, showing polarity and apical expression of the mucin and as such could be closer in phenotype to the normal epithelial cells. T47D showed the most disparate pattern with simple neutral mono- and disaccharides and a monosialooligosaccharide predominating (Fig. 3D), while BT-20 exhibited a glycosylation pattern intermediate between the other two cell lines (Fig. 3C).

These differences could also be detected by a simple experimental approach in which the ratio of $^3$H-labeled GlcNAc/GalN in MUC-1 immunoprecipitates was determined by HPAEC under alkaline conditions (Fig. 2). Using this approach, it was demonstrated that only $^3$HGalN was detected in MUC-1 from...
T47D cells whereas [3H]GlcN, as well as [3H]GalN, was detected in the other two cancer cell lines. In contrast, MUC-1 from the three normal epithelial cell lines had [3H]GlcN as the predominant hexosamine. [3H]-N-Acetylmuramidase acid was detected in MUC-1 from all the breast cancer and breast epithelium cell lines studied. This technique provides a convenient method for the initial analysis of the types of glycan chains present in glycoproteins and can be extended to glycoproteins radiolabeled with other sugars, e.g. [3H]Glc, to provide a more comprehensive compositional analysis.\(^2\)

A more detailed analysis of the O-linked chains from one of the normal epithelial cell lines (MTSV1–7) showed that the sugar chains from MUC-1 from this cell line were longer and more complex than those from the breast cancer cell line T47D (Fig. 8). In MUC-1 from MTSV1–7 cells, two major core 2 disialohexa- and octa-oligosaccharides (M-4a and -4b) were detected. Neutral oligosaccharides, but few monosialo species, were present (Fig. 3). In contrast, MUC-1 from T47D cancer cells contained no core 2 species. GalNac and simple oligosaccharides (Galβ1→3GalNac-ol and NeuAco2→3Galβ1→3GalNac-ol) were the major chains detected (Fig. 8), consistent with the absence of GlcNac determined by sugar analysis (Table I). Interestingly, NeuAco2→6GalNac-ol, corresponding to the sialyl-Tn epitope, was not detected.

In studies analyzing the O-glycans attached to milk mucins, Hanisch and colleagues found several core 2 structures in both neutral and acidic fractions (10, 11). Our data show that sialylated core 2 structures were added to the MUC1 mucin produced by MTSV1–7 cells, indicating that this cell line retains a basic glycosylation pattern (chain extension via the core 2 GlcNac transferase) similar to that used by normal breast epithelial cells in vivo. The major difference between the structures added by MTSV1–7 cells to MUC1 and those added to the milk mucins was the presence of long polylactosamine side chains, some of which contained an unusual GlcNac-1,6Gal linkage, in the milk mucin (10, 11). The difference in chain length may reflect the difference between secreted mucin (in milk) and cell-associated mucin (on MTSV1–7 cells). In a recent study Hanisch and co-workers (32) compared the glycosylation characteristics of mucin from skim milk and milk fat membrane glycoprotein with mucin from T47D and MDA-MB231 cell lines and a tumor sample. Although these authors did not specifically study MUC-1 mucin, they also showed shorter carbohydrate chains in the major mucin fraction from T47D cells in comparison with the other sources and demonstrated the presence of NeuAco2→3Galβ1→3GalNac as the major glycan chain in this cell line.

In another study analyzing the glycosylation of the MUC1 mucin found in milk and produced by the breast cancer cell line BT20, Hull and colleagues (9) used the DF3 antibody to purify the glycoprotein for analysis. The major structure found in the milk mucin was Galβ1→4GlcNac(Galβ1→3)GalNac-ol, with smaller amounts of mono-sialylated core 2 structures. In the BT20 cell line, the major O-glycans were Galβ1→3GalNac-ol (the T epitope) and mono sialylated derivatives of this disaccharide. The DF3 antibody is directed to an epitope contained in the tandem repeat region of MUC1 where sites for O-glycosylation are found, and the reactive epitope can be masked by longer or more complex O-glycans. Thus only some glycoforms of the MUC1 mucin would be isolated, and this could explain the presence of only short O-glycans in the mucin purified from milk. It could also explain the lack of core 2 structures in the mucin purified by DF3 from BT20 cells. Although we did not analyze the oligosaccharide attached to MUC1 from BT20 in detail, we did find GlcNac to be present when all the glycoforms were precipitated by the CT-1 antisera and would therefore have expected a core 2 structure to be present. Alternatively the lack of core 2 structures reported by Hull et al. (9) could reflect a difference in the pattern of glycosylation in two clones of BT20 cells.

The consensus from these studies is that normal breast epithelial MUC-1 and milk glycoproteins have predominantly branched core 2 structures with varying proportions of sialylated polylactosamine or lactosamine substituents linked β1→6 to the GalNac residue. Breast cancer, and possibly some other epithelial tumors, express MUC-1 with simpler core 1 chains. Consistent with these conclusions are results from a recent study by Brockhausen et al. (33) showing that whereas normal breast epithelial cells express core 2 GlcNac-transferase activity, mRNA and enzyme activity is either absent or present at reduced levels in the same three breast cancer cell lines that we studied. The increased activity of CMP-NeuAc:Galβ1→3GalNac-2,3 sialyltransferase found by these investigators in the three cancer cell lines is also consistent with the high amount of NeuAco2→3Galβ1→3GalNac-ol (oligosaccharide T-2) found in this study in the T47D cancer cell line. Interestingly, these findings in epithelial tumors are the inverse of the situation in lymphocytes and their tumors in which the mucin-like leukosialin from leukemia cells has the more branched core 2 structures and normal unactivated T cells has simpler structures (34). In carcinomas, glycosylation characteristics seem to be tissue-related. Thus, in a pancreatic cancer cell line SW1990, Ho et al. (35) demonstrated, using immunological approaches, that MUC-1 probably carries sialyl-Le\(^a\) and sialyl-Le\(^b\) structures, whereas we did not find fucosylated structures in breast MUC-1. Similarly, Capon et al. (36) showed that colonic cancer cell line CL-16E (a clone of the HT29 line) produces mucins bearing core 4 (GlcNacβ1→6[GlcNacβ1→3]GalNac) chains, as well as the core 1 and 2 found in breast cancer cell lines.

As discussed above, it has been suggested that the specificity of certain anti-peptide mAbs (e.g. SM3) for tumors as compared to normal tissues derives from the fact that the peptide core of MUC-1 in cancer cells is more accessible to antibody than in normal cells because of underglycosylation of the cancer mucins. By showing that cancer cell MUC-1 has simpler and less

\(^2\) K. O. Lloyd, unpublished data.
branched carbohydrate chains than the normal epithelium MUC-1, our data strongly support this mechanism. However, the data also show that the degree of glycosylation of the peptide backbone, i.e. the number of serine and/or threonine residues substituted with glycan chains, is less in T47D. This finding may also explain the tumor specificity of mAb SM3, as well as human antibodies (37) and mouse monoclonal antibodies (38) that recognize serine and/or threonine-containing epitopes that are outside the immunodominant PDTRP region, and would be expected to be glycosylated in normal cells.

Acknowledgments—We thank Dr. Minoru Fukuda (La Jolla Research Foundation) and Dr. Inka Brockhausen (Hospital for Sick Children, Toronto) for providing core 2 standard compounds and Dr. John Hilkens (Netherlands Cancer Institute, Amsterdam) for mAb 139H2. We also thank Claudette Bryant for skillful secretarial assistance.

REFERENCES
1. Strous, G. J., and Dekker, J. (1992) CRC Crit. Rev. Biochem. Mol. Biol. 27, 57–99
2. Carlstedt, I., Sheehan, J. K., Corfield, A. P., and Gallagher, J. T. (1985) Essays Biochem. 20, 40–76
3. Gendler, S. J., Spicer, A. P., Lalani, E.-N., Duhig, T., Peat, N., Burchell, J., Pemberton, L., Boswell, M., and Taylor-Papadimitriou, J. (1991) Am. Rev. Respir. Dis. 144, S42-S47
4. Gendler, S. J., and Spicer, A. P. (1995) Annu. Rev. Physiol. 57, 607–634
5. Taylor-Papadimitriou, J., Buijs, F., and Ligtenberg, M. (1989) in Methods in Carbohydrate Chemistry (Whistler, R. L., ed) pp. 361–370, Academic Press, New York
6. Taylor-Papadimitriou, J., Stewart, L., Burchell, J., and Beverley, P. (1993) Thorax 48, 215–216
7. Taylor-Papadimitriou, J., Stewart, L., Burchell, J., and Beverley, P. (1993) Thorax 48, 215–216
8. Taylor-Papadimitriou, J., Stewart, L., Burchell, J., and Beverley, P. (1993) Thorax 48, 215–216
9. Taylor-Papadimitriou, J., Stewart, L., Burchell, J., and Beverley, P. (1993) Thorax 48, 215–216
10. Taylor-Papadimitriou, J., Stewart, L., Burchell, J., and Beverley, P. (1993) Thorax 48, 215–216
11. Taylor-Papadimitriou, J., Stewart, L., Burchell, J., and Beverley, P. (1993) Thorax 48, 215–216
12. Taylor-Papadimitriou, J., Stewart, L., Burchell, J., and Beverley, P. (1993) Thorax 48, 215–216
13. Taylor-Papadimitriou, J., Stewart, L., Burchell, J., and Beverley, P. (1993) Thorax 48, 215–216
14. Taylor-Papadimitriou, J., Stewart, L., Burchell, J., and Beverley, P. (1993) Thorax 48, 215–216
Comparison of $O$-Linked Carbohydrate Chains in MUC-1 Mucin from Normal Breast Epithelial Cell Lines and Breast Carcinoma Cell Lines: DEMONSTRATION OF SIMPLER AND FEWER GLYCAN CHAINS IN TUMOR CELLS
Kenneth O. Lloyd, Joy Burchell, Valery Kudryashov, Beatrice W. T. Yin and Joyce Taylor-Papadimitriou

J. Biol. Chem. 1996, 271:33325-33334.
doi: 10.1074/jbc.271.52.33325

Access the most updated version of this article at http://www.jbc.org/content/271/52/33325

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 36 references, 9 of which can be accessed free at http://www.jbc.org/content/271/52/33325.full.html#ref-list-1