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Benzyl-*N*-acetyl-α-D-galactosaminide inhibits the sialylation and the secretion of mucins by a mucin secreting HT-29 cell subpopulation

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We have analysed the mucins synthesized by the HT-29 MTX cell subpopulation, derived from the HT-29 human colon carcinoma cells through a selective pressure with methotrexate (Lesuffleur *et al.*, 1990, *Cancer Res* **50**: 6334–43), in the presence of benzyl-*N*-acetyl-α-galactosaminide (GalNAcα-*O*-benzyl), which is a potential competitive inhibitor of the β1,3-galactosyltransferase that synthesizes the T-antigen. The main observation was a 13-fold decrease in the sialic acid content of mucins after 24 h of exposure to 5 mM GalNAcα-*O*-benzyl. This effect was accompanied by an increased reactivity of these mucins to peanut lectin, testifying to the higher amount of T-antigen. The second observation was a decrease in the secretion of the mucins by GalNAcα-*O*-benzyl treated cells. The decrease in mucin sialylation was achieved through the *in situ* β-galactosylation of GalNAcα-*O*-benzyl into Galβ1-3GalNAcα-*O*-benzyl, which acts as a competitive substrate of Galβ1-3GalNAc α2,3-sialyltransferase, as shown by the intracellular accumulation of NeuAcα2-3Galβ1-3GalNAcα-*O*-benzyl in treated cells.

Keywords: HT-29 cells; mucins; aryl-glycosides; *O*-glycosylation; sialyltransferases

Abbreviations: BSM, bovine submaxillary mucin; MTX, methotrexate; PBS, sodium phosphate 10 mM, NaCl 0.15 M, pH 7.4 buffer; *p*Np, *p*-nitrophenol; TBS, Tris/HCl 10 mM, NaCl 0.15 M, pH 7.4 buffer.

Enzymes: CMP-NeuAc: Galβ1-3/4GlcNAc α2,3-sialyltransferase, ST3(*N*), EC 2.4.99.6; CMP-NeuAc: Galβ1-4GlcNAc α2,6-sialyltransferase, ST6(*N*), EC 2.4.99.1; CMP-NeuAc: Galβ1-3GalNAc α2,3-sialyltransferase, ST3(*O*), EC 2.4.99.4; CMP-NeuAc: R-GalNAcα1-*O*-Ser α2,6-sialyltransferase, ST6(*O*)-I, EC 2.4.99.3; CMP-NeuAc: NeuAcα2-3Galβ1-3GalNAc α2,6-sialyltransferase, ST6(*O*)-II, EC 2.4.99.7; UDP-GlcNAc: Galβ1-3GalNAc-*R* (GlcNAc to GalNAc) β1,6-*N*-acetylglucosaminyltransferase, EC 2.4.1.102; UDP-GlcNAc: GalNAcα-*R* β1,3-*N*-acetylglucosaminyltransferase, EC 2.4.1.147; UDP-Gal: GalNAc-*R* β1,3-galactosyltransferase, EC 2.4.1.122.

Introduction

Mucins are the main components of the mucus overlaying respiratory, digestive or genital epithelia. Structural alterations and changes in the secretion of these molecules have been well documented in various pathological conditions and diseases, such as malignancies, cystic fibrosis, gastric ulcer and bronchitis [1, 2].

Malignant transformation of colonic cells leads to alterations of the glycan moiety of secreted mucins, which express 'shorter', more sialylated and more sulfated structures [3, 4]. These structural changes were suggested to be connected with the metastatic behaviour of colonic tumour cells, and the research on these *O*-linked oligosaccharides recently intensified due to the demonstration of the role of such glycans in interactions with recognition molecules [5].

The analysis of the biosynthesis and composition of

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mucins are facilitated by the availability of mucin-secreting cell cultures. The HT-29 human colon carcinoma cell line is heterogeneous and in the postconfluent state, consists of more than 95% undifferentiated cells and a small proportion of differentiated mucin-secreting and absorptive cells [6–8]. Differentiated populations of either absorptive or mucin-secreting phenotype were obtained under various conditions of metabolic stress. A stably differentiated mucin-secreting clonal cell line (Cl.16E) was derived from HT-29 cells after sodium butyrate treatment [7] and homogeneous mucin-secreting populations were obtained by adaptation of HT-29 cells to different concentrations of methotrexate (MTX) [8]. Antibodies raised against Cl.16E mucins stain goblet cells in both normal colonic and normal gastric mucosa [9]. The small proportion of mucin-secreting cells present in parental HT-29 cultures expresses mucins of either gastric or colonic immunospecificity, whereas HT-29 MTX cells synthesize mainly gastric-type mucins [8, 10]. Recently, the pattern of mucin gene expression was defined in the different HT-29 cell sub-populations, and HT-29 MTX cells express mainly *MUC1*, *MUC2*, *MUC3* and *MUC5C* genes [11]. However, little is known about the structure of the oligosaccharide chains of HT-29 MTX mucins.

The use of aryl-glycosides of GalNAc as competitive inhibitors of the elongation of the *O*-glycan chains recently appeared as a useful way to investigate the structure-function relationships of these molecules. A number of investigators have used these compounds to study mucin or mucin-type glycoprotein biosynthesis [12–14] and it is interesting to elucidate the point(s) of competition of these molecules in the normal cellular glycosylation pathway.

We have investigated the effect of GalNAcα-*O*-benzyl on the glycosylation pattern of mucins secreted by HT-29 MTX cells and, in this paper, we show that this compound plainly competes with the sialylation of the HT-29 MTX mucins via an *in vitro* β1,3-galactosylation, and that the observed lack in sialylation is accompanied with a decreased secretion of mucin molecules.

Materials and methods

MATERIALS

All reagents were of analytical grade. CMP-[^{14}C]-Neu5Ac (9.61 GBq mmol^{-1}), [^{3}H]-threonine (1 TBq mmol^{-1}) and [^{3}H]-Gal (1.48 TBq mmol^{-1}) were purchased from Amersham Labs (UK). Unlabelled CMP-Neu5Ac, 2,3-dehydro-2-deoxy-Neu5Ac, fetuin, Galβ1-3GalNAcα-*p*Np, Galβ1-3GalNAcα-*O*-benzyl, GalNAcα-*O*-benzyl, bovine submaxillary mucin and sialidase from *Clostridium perfringens* were purchased from Sigma Chemical Co. (USA). *N*-acetyllactosamine (Galβ1-4GlcNAc) was from Calbiochem (CA, USA). Digoxigenin conjugate of peanut (*Arachis hypogaea*) agglutinin (PNA-dig), anti-digoxigenin Fab fragments conjugated with alkaline phosphatase, 5-bromo-4-chloro-3-indolyl-phosphate (X-phosphate), 4-nitro blue tetrazolium chloride (NBT), porcine liver Galβ1-3GalNAc α2,3-sialyltransferase were from Boehringer Mannheim (Germany). The anti-Tn (Tn-5; [15]) and anti-Thomsen-Friedenreich (BM 22.19; [16]) antibodies were the gift of Dr Danuta Dus (Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Wroclaw, Poland).

CELL CULTURE AND METABOLIC LABELLING

HT-29 MTX cells derived from parental HT-29 cells by adaptation to 10^{-5} M methotrexate were obtained from Dr Thécla Lesuffleur (INSERM U-178, Villejuif, France). Cells were grown in Dulbecco's modified Eagle's minimal essential medium (Eurobio, Paris, France), supplemented with 10% heat-inactivated (30 min, 56 °C) fetal calf serum. Cells were seeded at 1.5×10^6 cells in 75 cm^2 flasks or at 0.25×10^6 cells in six well culture plates and cultured at 37 °C in 10% CO_2:90% air atmosphere. The cell viability was checked by Trypan blue exclusion. The medium was changed daily and cells were studied in the late post-confluent period (21 days after seeding), when all cells display a mucin-secreting phenotype [8]. For metabolic labelling, cells were cultured as described above until day 20 and were then labelled either with [^{3}H]-threonine (1.48 Mbq ml^{-1}) for 24 h in threonine-free Dulbecco's minimal essential medium (Institut Jacques Boy, Reims, France) or with [^{3}H]-galactose (37 Mbq ml^{-1}) for up to 72 h in low glucose Dulbecco's minimal essential/H-16 medium (Gibco, Gaithesburg, MD). After [^{3}H]-threonine labelling, the medium was collected and cells were lysed in a RIPA buffer (10 mM Tris-HCl pH 8.0, 10 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% phenylmethylsulfonyl fluoride, 1 mM EDTA). The effect of GalNAcα-*O*-benzyl on mucin biosynthesis was examined by addition at day 20 of 5 mM GalNAcα-*O*-benzyl in the medium simultaneously with the radioactive precursor.

PURIFICATION AND BIOCHEMICAL CHARACTERIZATION OF MUCINS FROM HT-29 MTX CELLS

Mucins from HT-29 MTX cells were purified by ultracentrifugation and Anion Exchange High Performance Liquid Chromatography (AE-HPLC) according to Huet *et al.* [17]. Briefly, 24 h after the addition of 5 mM of GalNAcα-*O*-benzyl to the culture medium of differentiated HT-29 MTX cells cultured for 20 days, cells were rinsed twice with PBS, scraped with a rubber policeman, and directly lysed by ultrasonication in PBS. Cell extracts were collected after centrifugation for 5 min at 1000 $\times$ **g** at 4 °C. After adding cesium bromide (0.42 g ml^{-1}), cell lysates were ultracentrifuged (200 000 $\times$ **g** for 72 h) using a Beckman 70.1 Ti rotor [18]. Mucin-containing fractions

were then further purified by AE-HPLC using a TSK DEAE 5PW (7.5 mm × 75 mm) Spherogel column (Beckman). The system was equilibrated with 5 mM sodium/potassium phosphate buffer, pH 6.0, at a flow rate of 0.8 ml min^{-1}. A total of 2 mg protein was injected. Elution was carried out in the same buffer using a NaCl gradient (0–1 M). Eluates were collected as 0.8 ml fractions and analysed for absorbance at 280 nm, orcinol reactivity, and dimethylmethylene blue reactivity [19]. The mucin fraction was collected [17] and sugar analysis was carried out by gas-liquid chromatography of trimethylsilyl derivatives of methyl glycosides formed by methanolysis in 1.5 M HCl in methanol at 80 °C for 24 h [20]. To determine sulfate content, samples were hydrolysed in 1 M HCl for 5 h at 100 °C and sulfate was determined by AE-HPLC [21]. All compositional analyses were performed twice to examine the reproducibility of the results.

ELECTROPHORESIS AND WESTERN BLOTTING

SDS-PAGE was performed on 2–5% gradient polyacrylamide gels [22], under reducing conditions using 50 μg of protein per lane. For autoradiography, gels were fixed in 40% ethanol: 10% glycerol: 10% acetic acid (by vol), soaked in Amplify (Amersham, UK) for 20 min, dried on Whatman paper, and processed for fluorography. For Western blotting, after separation by SDS-PAGE, proteins were transferred to a nitrocellulose membrane as described by Vaessen *et al.* [23]. The membranes were successively treated with polyvinylpyrolidone (2% in TBS), incubated with PNA-dig (2 μg ml^{-1} in TBS). Then, the nitrocellulose membranes were incubated for 1 h with anti-digoxigenin alcaline phosphatase-labelled Fab fragments (1 μg ml^{-1} in TBS). After washing, labelled glycoproteins were revealed by NBT/X-phosphate staining.

ENZYME-LINKED IMMUNOABSORBENT ASSAY (ELISA)

Mucins were plated overnight at 4 °C on 96-well Maxisorp Immunoplates. Unbound sites were blocked with 4% defatted milk in PBS. After washing with PBS-Tween-20 0.05% (six times), the Tn-5 (1:1000) and BM 22.19 (1:1000) antibodies were added and left for 2 h at 37 °C. After washing (six times), peroxidase-conjugated anti-mouse antibody was applied for 1 h at 37 °C. Plates were washed and developed using *o*-phenylenediamine (1 mg ml^{-1}) in 0.1 M phosphate-citrate buffer, pH 5.5, with hydrogen peroxide 0.03%. The reaction was terminated with 1 M HCl, and the optical density was measured at 492 nm.

SIALYLTRANSFERASE (ST) ASSAYS

Preparation of cell extracts

Cells were lysed at 0 °C with 10 mM sodium cacodylate buffer pH 6.5, containing 1% Triton X-100, 20% glycerol, 0.5 mM dithiotreitol, and 5 mM $MnCl_2$ (0.5 ml per 75 cm^2 flask). After a 10 min incubation under continuous stirring, cell homogenates were centrifuged at 10 000 × **g** for 15 min and the supernatants were used for enzymatic assays. Protein concentration was determined according to the method described by Peterson [24] using bovine serum albumin (BSA) as standard.

The activity of five sialyltransferases: ST3(*N*), ST6(*N*), ST3(*O*), ST6(*O*)-I and ST6(*O*)-II were measured with total cell homogenates as enzyme source with respect to the substrate specificity of the different enzymes.

ST3(N) and ST6(N) (N-acetyllactosamine as acceptor substrate)

Cell extracts (40 μl) were carried to a final volume of 120 μl with 0.1 M sodium cacodylate pH 6.5, 1% Triton X-100, 0.2 M galactose (as inhibitor of β-galactosidase), 1 mM 2,3-dehydro-2-deoxy-Neu5Ac (as inhibitor of sialidases), 52.9 μM CMP-[^{14}C]-Neu5Ac (0.58 GBq $mmol^{-1}$, 3.67 kBq per 120 μl), containing 1 mM of *N*-acetyllactosamine as exogenous substrate. After 2 h of incubation at 37 °C, separation of NeuAcα2-3Galβ1-4GlcNAc isomer from the corresponding NeuAcα2-6 isomer was achieved by HPLC [25].

ST3(O) (Galβ1-3GalNAcα-pNp as acceptor substrate)

Incubations were performed under the conditions described above using 40 μl of cell extract and Galβ1-3GalNAcα-*p*Np (1 mM final concentration). After 1 h of incubation at 37 °C, the reactions were stopped by adding 1 volume of ethanol; samples were centrifuged at 3000 × **g** for 5 min and supernatants were directly processed for descending paper chromatography in ethyl acetate:pyridine:water (10:4:3, by vol) [26].

ST6(O)-I (asialo-BSM as acceptor substrate)

Incubations were performed under the conditions described above using 40 μl of cell extract and 250 μg of asialo-BSM. After 2 h of incubation at 37 °C, the reactions were stopped by adding 1 ml of ice-cold phosphotungstic acid (5% in 2 M HCl). The precipitate was collected on glass fibre filters, washed extensively with 5% trichloroacetic acid, distilled water and ethanol, and processed for scintillation counting [27].

ST6(O)-II (fetuin as acceptor substrate)

Assays were performed under the conditions described for ST6(*O*)-I using 40 μl of cell extract and 250 μg of fetuin [25].

BIOCHEMICAL CHARACTERIZATION OF GALNACα-*O*-BENZYL PROCESSING IN HT-29 MTX CELLS

After 5, 24, and 48 h of metabolic labelling with [^{3}H]-galactose of control and GalNAcα-*O*-benzyl-treated cells cultured in six-well culture plates, the medium was collected and after washing, cells were directly lysed by

ultrasonication in water. Cell extracts were collected after centrifugation at 13 500 × **g** for 15 min. Cell extracts were filtrated on 0.20 μm nitrocellulose filtration units before HPLC fractionation. Reverse phase high performance liquid chromatography was carried out on a C-18 column (Lichrosorb RP-18, Merck, Germany). The system was equilibrated in water at a flow rate of 1 ml min^{-1}. One hundred μl of cell extract was injected, eluted isocratically for 10 min in water and then with an acetonitrile/water gradient moving from 100% of water to 80% acetonitrile/20% water in 30 min. 1 ml fractions were collected and processed from scintillation counting. Desialylation of purified peak II was performed in a final volume of 100 μl with 50 mU ml^{-1} of sialidase from *Clostridium perfringens* in 50 mM citrate buffer pH 6.0, 0.9% NaCl, 0.1% $CaCl_2$ for 1 h at 37 °C prior to injection. [^{14}C]-NeuAcα2-3Galβ1-3GalNAcα-*O*-benzyl was prepared by incubation of Galβ1-3GalNAcα-*O*-benzyl with porcine liver Galβ1-3GalNAc α2,3-sialyltransferase and CMP-[^{14}C]-NeuAc.

Results

Comparative analysis of mucins from control and GalNAcα-O-benzyl treated HT-29 MTX cells

The effect of GalNAcα-*O*-benzyl upon the biosynthesis and secretion of HT-29 MTX mucins was first studied through metabolic labelling with [^{3}H]-threonine over 24 h. Incorporation of [^{3}H]-threonine was determined in the medium and cell lysate of control cells and GalNAcα-*O*-benzyl-treated cells after removing of free [^{3}H]-threonine by ultrafiltration in the presence of cold threonine. After the treatment with GalNAcα-*O*-benzyl, the cell-associated labelling increased about 1.6-fold whereas the secreted radioactivity decreased 5.4-fold (Fig. 1). The electrophoretic profiles of both cell lysates and culture media from [^{3}H]-threonine-labelled control and GalNAcα-*O*-benzyl-treated cells are shown in Fig. 2. After fractionation by SDS-PAGE (2–5% gradient gel) and autoradiography, a major band was observed in each cell lysate, the migration of which appeared to decrease after treatment with GalNAcα-*O*-benzyl. SDS-PAGE analysis of the culture medium from GalNAcα-*O*-benzyl-treated cultures revealed a faint smear with a mobility similar to that of the corresponding cell lysate. This pattern contrasted with the discrete band observed in culture medium from control HT-29 MTX cells.

Mucins from control cells and from cells cultured for 24 h in the presence of GalNAcα-*O*-benzyl were isolated by ultracentrifugation through a CsBr gradient and preparative AE-HPLC [17] and analysed for carbohydrate and sulfate composition (Table 1). Carbohydrate analysis of control HT-29 MTX cell mucins revealed mainly GalNAc, Gal, GlcNAc and sialic acid. The sialic acid content was very high (1.3 molar ratio to GalNAc) whereas the sulfate content was very low (0.1 molar ratio to GalNAc). As indicated in Table 1, the GalNAcα-*O*-benzyl treatment led to a striking decrease in the sialic acid content which was 13-fold lower in comparison to the amount of sialic acid in control HT-29 MTX mucins.

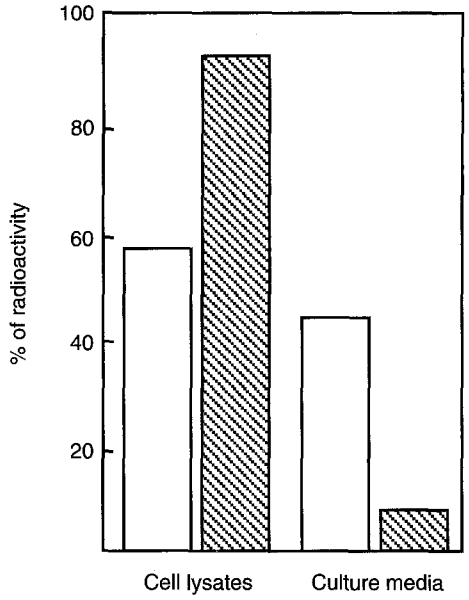

Figure 1. Measurement of the [^{3}H]-threonine-labelling over 24 h in the cell lysates and culture media of control HT-29 MTX cells (open bars) and HT-29 MTX cells treated with 5 mM of GalNAcα-*O*-benzyl (hatched bars). The results are expressed in percentage of total radioactivity incorporated in proteins for each culture.

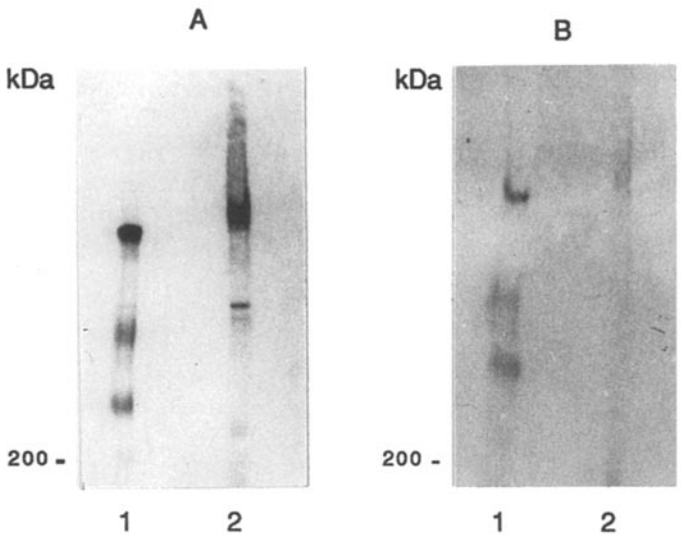

Figure 2. SDS-PAGE autoradiogram of HT-29 MTX cell lysates (A) and culture media (B) after labelling with [^{3}H]-threonine. Lane 1, control HT-29 MTX cells; lane 2, HT-29 MTX cells cultured in the presence of 5 mM of GalNAcα-*O*-benzyl for 24 h. Equal amounts (50 μg) of protein were loaded per lane. The origin is at the top of the figure.

Table 1. Carbohydrate composition and sulfate content of HT-29 MTX HPLC purified mucin fraction from control and GalNAcα-*O*-benzyl treated cells.

	Mucins from control HT-29 MTX cells	*Mucins from GalNAcα-O-benzyl treated HT-29 MTX cells*
Galactose	1.4	1.4
GalNAc	1	1
GlcNAc	0.6	0.5
Sialic acid	1.3	0.1
Sulfate	0.1	0.2

All results are expressed as molar ratios to GalNAc.

On the other hand, the relative amount of other monosaccharides (e.g. Gal, GlcNAc) as well as sulfate content were quite similar to control.

To further study the effect of GalNAcα-*O*-benzyl on the biosynthesis of mucins by HT-29 MTX cells, their reactivity with a panel of mAbs and lectins was examined by Enzyme-Linked Immunoabsorbent Assay (Fig. 3) or by Western blotting (Fig. 4) on purified mucin fractions. GalNAcα-*O*-benzyl treatment resulted in an increase in the expression of Tn-antigen and chiefly T-antigen as detected with mAb Tn-5 (Tn-antigen), or mAb BM 22.19 and PNA (T-antigen). Immunoassays with the anti-Tn antibody showed that the Tn reactivity of mucins increased two-fold after treatment by GalNAcα-*O*-benzyl. No binding of anti-T mAb was observed on HT-29 MTX mucins in the absence of treatment with GalNAcα-*O*-benzyl whereas a significant binding appeared on the mucins of treated cells (Fig. 3). Western blotting with PNA also showed a great increase in the T antigen reactivity of mucins obtained after GalNAcα-*O*-benzyl treatment (Fig. 4).

Biochemical characterization of GalNAcα-O-benzyl processing in HT-29 MTX cells

As described above, the main alterations induced in the glycan moiety of mucins purified from HT-29 MTX cells treated with 5 mM of GalNAcα-*O*-benzyl appeared to be a dramatic decrease in sialic acid content and, concomi-

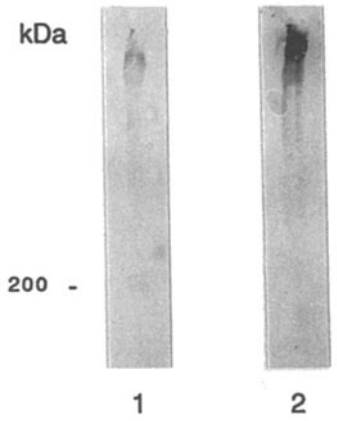

Figure 4. Western blotting analysis. Mucins purified from control (1) and GalNAcα-*O*-benzyl treated (2) HT-29 MTX cells were separated by 2–5% SDS polyacrylamide gel electrophoresis and blotted onto nitrocellulose. T-antigen was revealed with digoxigenin-labelled PNA. The origin is at the top of the figure.

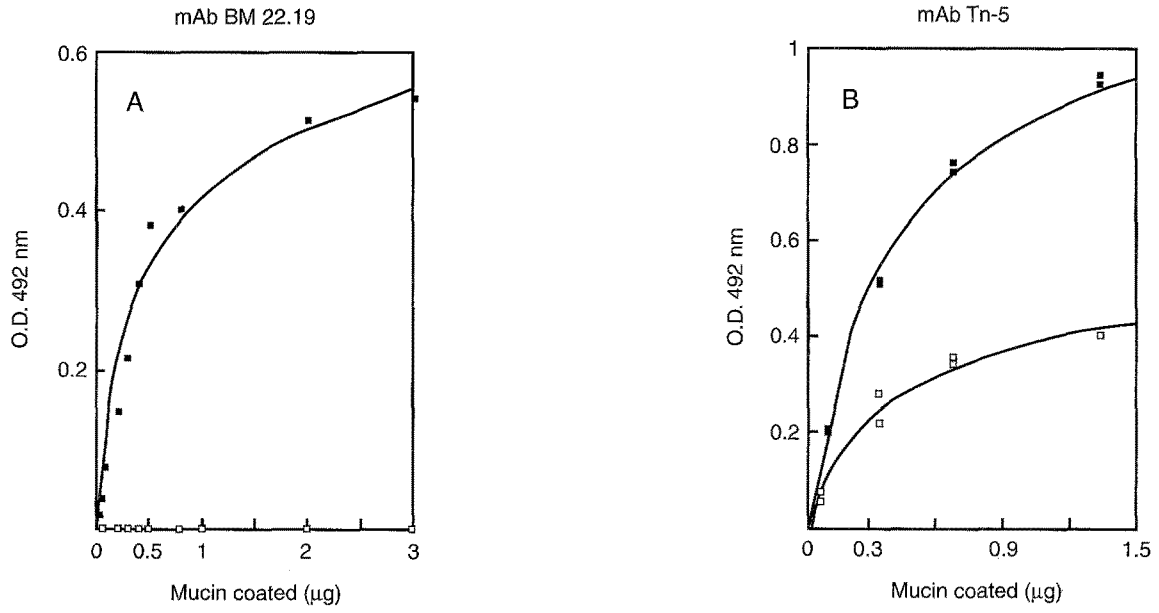

Figure 3. Enzyme-linked immunoabsorbent assays of purified mucins from control (open squares), and from GalNAcα-*O*-benzyl treated cells (black squares) with mAb BM 22.19 detecting T-antigen (A), and with mAb Tn-5 detecting Tn-antigen (B).

tantly, the highly increased expression of the T-antigen. This result suggested that the GalNAcα-*O*-benzyl treatment essentially affected the transfer of sialic acid onto mucin glycans. We have therefore investigated the level of expression of five sialyltransferase activities in mucin-secreting postconfluent HT-29 MTX cells (21 days after seeding). The results of enzymatic assays, summarized in Table 2, clearly showed that the highest transfer of sialic acid was observed onto Galβ1-3GalNAcα-*p*Np, which is a specific substrate for the Galβ1-3GalNAc α2,3-sialyltransferase (ST3(*O*)). This latter result tended to show that GalNAcα-*O*-benzyl could be converted into Galβ1-3GalNAcα-*O*-benzyl inside the cells, as the Galβ1-3GalNAcα-*O*-benzyl is also a suitable substrate for ST3(*O*) and could compete *in vivo* with the sialylation of HT-29 MTX mucins. In order to research this latter compound and its putative sialylated derivative in treated cells, we cultured HT-29 MTX cells in the presence of 5 mM of GalNAcα-*O*-benzyl together with [^{3}H]-Gal for up to 48 h. The cell lysates collected after 5, 24, and 48 h of incubation were analysed by reverse phase HPLC. Figure 5 shows the occurrence of several radioactive compounds which were retained on the C-18 column and eluted by increasing the percentage of acetonitrile. These compounds, specifically detected in treated cells, accumulated throughout the period of incubation. Peak I (at 23 min) was shown to comigrate with the [^{14}C]-labelled standard Galβ1-3GalNAcα-*O*-benzyl. Peak II (at 12 min) comigrated with the [^{14}C]-labelled NeuAcα2-3Galβ1-3GalNAcα-*O*-benzyl used as an internal standard. The presence of Galβ1-3GalNAcα-*O*-benzyl in HT-29 MTX treated cell homogenates was also confirmed by an *in vitro* assay using purified porcine liver ST3(*O*) as enzyme source and a heat-denatured HT-29 MTX treated cell extract as an acceptor substrate source [17]. To assess the identity of peak II as the product of sialylation of Galβ1-3GalNAcα-*O*-benzyl, peak II was collected, desialylated using *Clostridium perfringens* sialidase, and further analysed by reverse phase HPLC. As shown in Figure 6, after sialidase treatment, peak II (at 12 min) shifted to peak I (at 23 min).

Discussion

The sugar derivative GalNAcα-*O*-benzyl behaves *in vitro* as a competitive inhibitor for the substitution of GalNAc residues O-linked to the apomucin core and competitively inhibits the galactosyltransferase that synthesizes the disaccharide Galβ1-3GalNAc [12]. When this compound was added in the culture medium of cell producing mucins, it was found that mucins expressed a higher level of Tn-antigen (GalNAcα-*O*-Ser), but unexpectedly also expressed a higher level of T-antigen (Galβ1-3GalNAcα-*O*-Ser) [13]. In a recent study, it was shown that the inhibition of elongation of O-linked GalNAc in epitectin from H.Ep.2 cells by 4 mM GalNAcα-*O*-benzyl was only partial [14].

In this study, we analysed the effect of GalNAcα-*O*-benzyl treatment upon a subpopulation of HT-29 mucin-secreting cells, which synthesize highly sialylated mucins [17] with a gastric like immunoreactivity [8] at late confluence. The differentiation process of HT-29 MTX cells starts immediately before confluence, which occurs 7 days after seeding, and is complete 21 days after seeding [8]. HT-29 MTX cells were therefore cultured in standard conditions until day 20 and were then treated with 5 mM GalNAcα-*O*-benzyl for 24 h. The mucins of treated cells were purified by ultracentrifugation and investigated for sugar composition and immunoreactivity with lectins and mAbs specifically recognizing the Tn- or T-antigen. The sugar composition of the mucins produced by GalNAcα-*O*-benzyl treated cells revealed a marked inhibition in the incorporation of sialic acid, while the other sugars did not appear significantly affected as examined in molar ratio to GalNAc. The sialic acid level in the mucins of GalNAcα-*O*-benzyl treated cells was found to be 13-fold lower in comparison to the sialic acid level in control mucins. Besides, the mucins of GalNAcα-*O*-benzyl treated cells showed a slight increase in the expression of Tn-antigen and a marked increase in the expression of T-antigen. No significant expression of sialyl-Tn antigen was observed either in the mucins of control cells or in the mucins of treated cells (data not

Table 2. Sialyltransferase activities in late postconfluent HT-29 MTX cell cultures.

Enzyme	*EC number*	*Product*[1]	*Sialyltransferase activity* (nmol mg^{-1} h^{-1})
ST6(*N*)	EC 2.4.99.1	**NeuAcα2-6**Galβ1-4GlcNAc	< 0.02
ST3(*N*)	EC 2.4.99.6	**NeuAcα2-3**Galβ1-4GlcNAc	0.73 ± 0.22
ST3(*O*)	EC 2.4.99.4	**NeuAcα2-3**Galβ1-3GalNAcα-*p*Np	14.58 ± 5.38
ST6(*O*)-I	EC 2.4.99.3	**NeuAcα2-6**GalNAcα1-*O*-Ser	1.18 ± 0.16
ST6(*O*)-II	EC 2.4.99.7	NeuAcα2-3Galβ1-3GalNAcα1-*O*-Ser / **NeuAcα2-6**	0.15 ± 0.04

Sialyltransferase activities were assayed in triplicate using cell homogenates as enzyme source prepared as described in Materials and methods section.
[1]The sialic acid residue transfered is in bold characters.

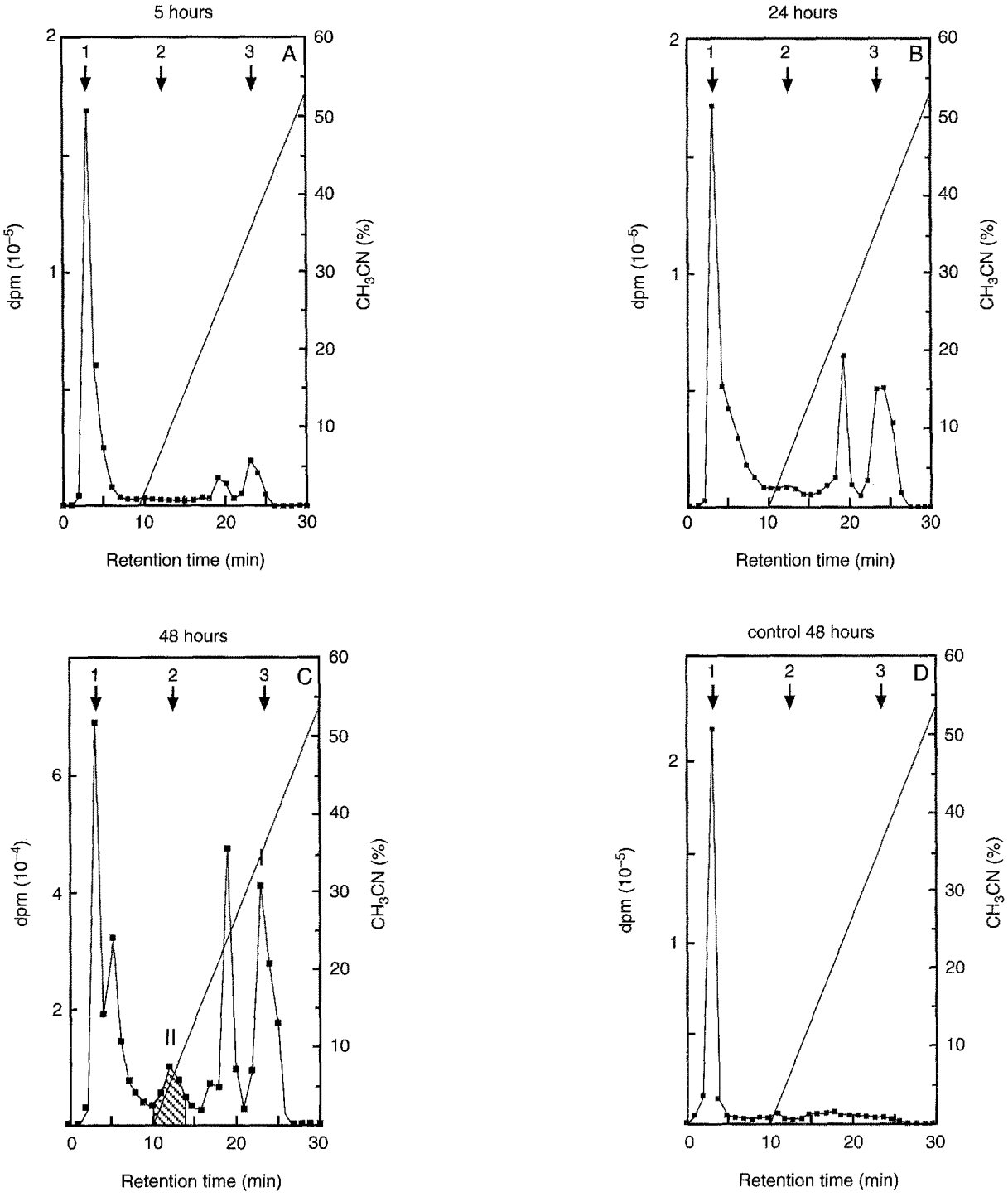

Figure 5. Reverse phase HPLC of cell extracts from control and GalNAcα-*O*-benzyl treated cells after metabolic labelling with [^{3}H]-Gal. The incorporation of [^{3}H]-Gal was examined after 5 h (A), 24 h (B), and 48 h (C) of exposure to 5 mM of GalNAcα-*O*-benzyl. The HPLC profile of control cells (D) is only shown for 48 h of incubation with [^{3}H]-Gal. The retention times of standards are indicated: 1, Gal; 2, NeuAcα2-3Galβ1-3GalNAcα-*O*-benzyl; 3, Galβ1-3GalNAcα-*O*-benzyl. The hatched area indicates the collected peak II. The peak eluting at about 19 min. could not be identified.

shown). The effect upon the biosynthesis and secretion of mucins was also seen through a continuous labelling with [^{3}H]-threonine, which was predominantly incorporated into apomucins. Cell lysates and culture media were quantified by scintillation counting and were analysed by 2–5% gradient SDS-PAGE. The changes in mucin glycosylation after GalNAcα-*O*-benzyl treatment were accompanied: (i) by a decrease in the percentage of

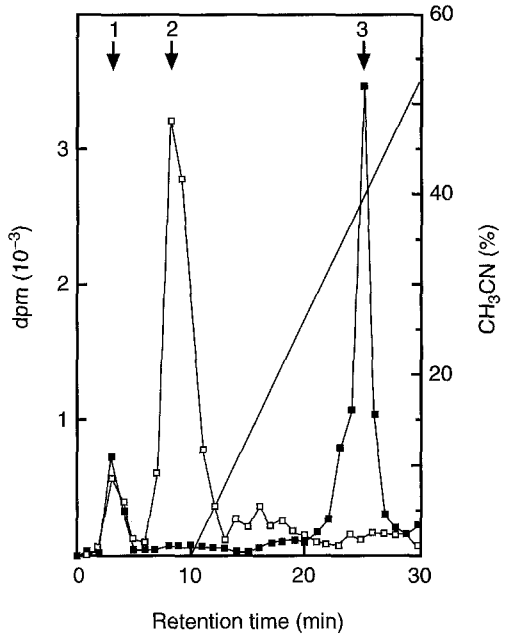

Figure 6. Reverse phase HPLC of peak II before (□) and after (■) treatment with *Clostridium perfringens* sialidase. The retention times of standards are indicated: 1, Gal; 2, NeuAcα2-3Galβ1-3GalNAcα-*O*-benzyl; 3, Galβ1-3GalNAcα-*O*-benzyl.

secreted [^{3}H]-threonine labelled compounds; and (ii) by a lower electrophoretic mobility by SDS-PAGE, which is probably related to the decreased sialic acid content. Consequently, it might be suggested that incompletely sialylated HT-29 MTX mucins cannot be efficiently secreted.

The increased expression of Tn-antigen in the mucins of treated cells is probably relevant to the competition between GalNAcα-*O*-benzyl molecules and *O*-linked GalNAc residues of apomucins for elongation by further glycosyltransferases, either the UDP-GlcNAc: GalNAcα-*R* β1,3-*N*-acetylglucosaminyltransferase (EC 2.4.1.147) or the UDP-Gal: GalNAc-*R* β1,3-galactosyltransferase (EC 2.4.1.122). However, the competitive effect towards both glycosyltransferases appeared poorly effective as we found no significant change in the incorporation of both GlcNAc and Gal residues in mucins after GalNAcα-*O*-benzyl treatment. This discrepancy could be explained by, at least, two different hypotheses. Either the affinity of these glycosyltransferases would be higher for the GalNAc residues linked to apomucins than for GalNAcα-*O*-benzyl. For example, the β1,3-galactosyltansferase can transfer Gal to free GalNAc or to different glycosides of GalNAc but the K_m for these acceptors (100–200 mM) is much higher than for mucins (about 100 μM) [28]. A second hypothesis would be that the β1,3-galactosyltransferase and β1,3-N-acetylglucosaminyltransferase activities are sufficient to transfer Gal and GlcNAc respectively onto both GalNAc residues linked to apomucins and GalNAcα-*O*-benzyl. This second hypothesis could explain the rapid accumulation of Galβ1-3GalNAcα-*O*-benzyl in treated cells as we demonstrated by metabolic labelling with [^{3}H]-Gal. As discussed previously, the main effect of GalNAcα-*O*-benzyl treatment is an increased expression of T-antigen resulting from a defect in its sialylation. Three different sialyltransferases participate in the sialylation of T-antigen: (i) the CMP-NeuAc: Galβ1-3GalNAc α2,3-sialyltransferase (ST3(*O*)) which achieves the synthesis of sialylα3-T antigen; (ii) the CMP-NeuAc: R-GalNAcα1-Ser α2,6-sialyltransferase (ST6(*O*)-I) where R can be H-, or Galβ1-3-, or NeuAcα2-3Galβ1-3- and which is responsible for the biosynthesis of sialyl-Tn antigen and of sialylα6-T antigen; and (iii) the CMP-NeuAc: NeuAcα2-3Galβ1-3GalNAc α2,6-sialyltransferase (ST6(*O*)-II) which required the trisaccharide to synthesize the tetrasaccharide NeuAcα2-3Galβ1-3[NeuAcα2-6]GalNAc.

These three sialyltransferases are expressed in HT-29 MTX cells (Table 2) and it is clear that ST3(*O*) is the main sialyltransferase activity detected in these cells. This finding was also observed in a previous work from Dall'Olio *et al.* [29] and is also in agreement with the fact that NeuAcα2-3Galβ1-3GalNAc is one of the major carbohydrate chains of the mucins synthesized by HT-29 MTX cells (unpublished observation) as well as of the mucins from HT-29-derived Cl.16E clone [30].

Since GalNAcα-*O*-benzyl treatment of HT-29 MTX cells for 24 h does not modify the expression of ST3(*O*) (data not shown), one possible hypothesis to explain the decreased sialylation of T-antigen in HT-29 MTX mucins after GalNAcα-*O*-benzyl treatment was the *in situ* generation of the disaccharide Galβ1-3GalNAcα-*O*-benzyl, which would behave as an efficient competitive substrate for the α2,3-sialylation of the T-antigen by ST3(*O*) [13]. Therefore, we investigated the intracellular metabolism of GalNAcα-*O*-benzyl in HT-29 MTX by culturing the cells in the presence of [^{3}H]-Gal together with GalNAcα-*O*-benzyl. By reverse phase HPLC, we characterized several hydrophobic [^{3}H]-Gal labelled compounds which accumulated in a time-dependent manner inside the cells. One of these compounds was identified as [^{3}H]-Galβ1-3GalNAcα-*O*-benzyl (peak I), and a second as NeuAcα2-3-[^{3}H]-Galβ1-3GalNAcα-*O*-benzyl (peak II). This finding clearly indicates that: (i) GalNAcα-*O*-benzyl is converted *in situ* to Galβ1-3GalNAcα-*O*-benzyl; and (ii) this latter compound serves as an acceptor substrate for ST3(*O*). This accumulation of NeuAcα2-3Galβ1-3GalNAcα-*O*-benzyl allowed us to propose that the decrease in the sialylation of mucins produced by HT-29 MTX cells in the presence of GalNAcα-*O*-benzyl is due to a competition between Galβ1-3GalNAc sequences linked to apomucins and Galβ1-3GalNAcα-*O*-benzyl for ST3(*O*). However,

Galβ1-3GalNAcα-*O*-benzyl is also a potential substrate for Galβ1-3GalNAc-R β1,6-*N*-acetylglucosaminyltransferase (EC 2.4.1.102) and sulfotransferase, and our findings indicated that the incorporation of GlcNAc and sulfate was unaffected in mucins of HT-29 MTX cells. This differential effect of GalNAcα-*O*-benzyl treatment on the incorporation of sialic acid and GlcNAc to core 1 could depend on the apparent affinity of the corresponding enzymes for Galβ1-3GalNAcα-*O*-benzyl. Indeed, this benzyl-disaccharide is known to be a very good acceptor substrate for ST3(*O*). For example, the apparent K_m of rat ST3(*O*) [26] or of human placenta ST3(*O*) [31] for Galβ1-3GalNAcα-*O*-benzyl is about 30 μM and the enzyme from acute myeloïd leukaemia cells exhibits an apparent K_m equal to 0.4 mM [32]. By comparison, this benzyl-disaccharide is a poorer acceptor substrate for the β1,6-*N*-acetylglucosaminyltransferase according to the apparent K_m values (1.1–2.0 mM) observed for the enzymes from different mammalian sources [32].

The finding that the increase in Tn-antigen is less than the increase in T-antigen suggests that the competitive inhibition of β1,3-galactosyltransferase, which is the primary effect of GalNAcα-*O*-benzyl in *in vitro* assays [12], is incomplete over 24 h of GalNAcα-*O*-benzyl treatment of cultured HT-29 MTX cells, as previously observed in HM7 colon cancer cells [13]. However, further investigations as a function of time and dose of GalNAcα-*O*-benzyl will help to determine if a more complete inhibition of mucin glycosylation can be obtained *in vivo*.

In conclusion, Galβ1-3GalNAcα-*O*-benzyl formed *in situ* thus appears to behave as a specific efficient competitor for the ST3(*O*) in HT-29 MTX cells. This data might indicate an intracellular accumulation of Galβ1-3GalNAcα-*O*-benzyl in the same sub-compartment as the one of ST3(*O*). Our results also suggest that the ST3(*O*) activity is insufficient to transfer sialic acid to both Galβ1-3GalNAcα-*O*-benzyl and T-antigens linked to the apomucins, although ST3(*O*) is the main sialyltransferase activity expressed in HT-29 MTX cells. As a consequence of the GalNAcα-*O*-benzyl treatment, the synthesized mucins which fail to be sialylated, cannot be thoroughly secreted in the extracellular medium. It might be suggested that this impairment in the secretion process results in part from the lack of sialic acid. Therefore, GalNAcα-*O*-benzyl treatment might be a useful tool for studying the sialylation process and the biological effect of its products.

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References

1. Bhavanandan VP (1991) *Glycobiology* **1**: 493–503.
2. Rose MC (1992) *Am Physiol Soc* **263**: L413–17.
3. Kurosaka A, Nakajima H, Funakoshi I, Matsuyama M, Nagayo T, Yamashina I (1983) *J Biol Chem* **258**: 11594–98.
4. Kim YS (1992) *J Cell Biochem Suppl* **16G**: 91–96.
5. Varki A (1993) *Glycobiology* **3**: 97–130.
6. Pinto M, Appay MD, Simon-Assmann P, Chevalier G, Drocopoli N, Fogh J, Zweibaum A (1982) *Biol Cell* **44**: 193–96.
7. Augeron C, Laboisse CL (1984) *Cancer Res* **44**: 3961–69.
8. Lesuffleur T, Barbat A, Dussaulx E, Zweibaum A (1990) *Cancer Res* **50**: 6334–43.
9. Maoret JJ, Font J, Augeron C, Codogno P, Baury C, Aubery M, Laboisse CL (1989) *Biochem J* **258**: 793–99.
10. Lesuffleur T, Kornowski A, Luccioni C, Muleris M, Barbat A, Beaumatin J, Dussaulx E, Dutrillaux B, Zweibaum A (1991) *Int J Cancer* **49**: 721–30.
11. Lesuffleur T, Porchet N, Aubert JP, Swallow D, Gum JR, Kim YS, Real FX, Zweibaum A (1993) *J Cell Sci* **106**: 771–83.
12. Kuan SF, Byrd JC, Basbaum C, Kim YS (1989) *J Biol Chem* **264**: 19271–77.
13. Huang J, Byrd JC, Yoon WH, Kim YS (1992) *Oncol Res* **4**: 507–15.
14. DiIulio NA, Bhavanandan VP (1995) *Glycobiology* **5**: 195–99.
15. Duk M, Steuden I, Dus D, Radzikowski C, Lisowska E (1992) *Glycoconjugate J* **9**: 148–53.
16. Steuden I, Duk M, Czerwinski M, Radzikowski C, Lisowska E (1985) *Glycoconjugate J* **2**: 303–14.
17. Huet G, Kim I, De Bolos C, Lo-Guidice JM, Moreau O, Hemon B, Richet C, Delannoy P, Real FX, Degand P (1995) *J Cell Sci* **108**: 1275–85.
18. Houdret N, Perini JM, Galabert C, Scharfman A, Humbert P, Lamblin G, Roussel P (1986) *Biochim Biophys Acta* **880**: 54–61.
19. De Jong JGN, Wevers RA, Sambeek RLV (1992) *Clin Chem* **38**: 803–7.
20. Lamblin G, Boersma A, Klein A, Roussel P, Van Halbeek H, Vliegenthart JFG (1984) *J Biol Chem* **259**: 9051–58.
21. Lo-Guidice JM, Wieruszeski JM, Lemoine J, Verbert A, Roussel P, Lamblin G (1994) *J Biol Chem* **269**: 18794–813.
22. Laemmli UK (1970) *Nature* **227**: 680–81.
23. Vaessen RTMJ, Kreike J, Groot GSP (1981) *FEBS Lett* **124**: 193–96.
24. Peterson GL (1977) *Anal Biochem* **83**: 346–56.
25. Dall'Olio F, Malagolini N, Serafini-Cessi F (1992) *Biochem Biophy Res Commun* **184**: 1405–10.
26. Delannoy P, Pelczar H, Vandamme V, Verbert A (1993) *Glycoconjugate J* **10**: 91–98.
27. Vandamme V, Cazlaris H, Le Marer N, Laudet V, Lagrou C, Verbert A, Delannoy P (1992) *Biochimie* **74**: 89–100.
28. Schachter H, Brockhausen I (1989) In *Mucus and Related Topics* (Chantler E, Ratcliffe NA, eds) pp. 1–26, Cambridge: Society for Experimental Biology.

29. Dall'Olio F, Malagolini N, Guerrini S, Serafini-Cessi F (1993) *Biochem Biophys Res Commun* **196**: 714–20.
30. Capon C, Laboisse CL, Wieruszeski JM, Maoret JJ, Augeron C, Fournet B (1992) *J Biol Chem* **267**: 19248–57.
31. Joziasse DH, Bergh MLE, ter Hart HGJ, Koppen PL, Hooghwinkel GJM, Van den Eijnden DH (1985) *J Biol Chem* **260**: 4941–51.
32. Kuhns W, Rutz V, Paulsen H, Matta KL, Baker MA, Barner M, Granovsky M, Brockhausen I (1993) *Glycoconjugate J* **10**: 381–94.