The Breast Tumor-associated Epitope Defined by Monoclonal Antibody 3E1.2 Is an O-linked Mucin Carbohydrate Containing N-Glycolylneuraminic Acid

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ABSTRACT

The breast cancer-associated epitope (mammary serum antigen or MSA) defined by monoclonal antibody (Mab) 3E1.2 is a neuraminidase-sensitive carbohydrate expressed on MUC-1-encoded molecules. However, the reactivity of Mab 3E1.2 is also reduced by protease treatment of the mucin, which suggests that 3E1.2 binds to multimers of the sialylated carbohydrate in a protein conformation-dependent manner. The common N-acetyl derivative of neuraminic acid (5-acetylneuraminic acid) is not involved in the epitope, since lectins specific for 5-acetylneuraminic acid (linked to GalNAc or Gal) are nonreactive with MSA-positive molecules. However, the N-glycolyl derivative, 5-glycolyneuraminic acid (NeuSGc), forms a major part of the epitope since both free NeuSGc and porcine stomach mucin (90% neuraminic acid as NeuSGc) inhibit the binding of Mab 3E1.2, while bovine or ovine submaxillary mucins, fetuin, bovine gangliosides, and other carbohydrates do not. Indeed, the presence of NeuSGc on human tumor mucin was confirmed by electrospray mass spectrometry. NeuSGc is attached to an O-linked carbohydrate, since the expression of MSA by MCF-7 breast cancer cells is inhibited by the O-glycosylation inhibitor phenyl-N-acetyl-o-D-galactosaminide, but not by the N-glycosylation inhibitor tunicamycin, and the epitope is removed by treatment with O-glycase but not N-glycanase F, endoglycosidase F, or endoglycosidase H, which are specific for N-linked glycans. This is likely to be a core glycan since 3E1.2 reacts after treatment of the mucin with trifluoromethanesulfonic acid, which removes most backbone and peripheral carbohydrates. Treatment with galactosidase or N-acetyl glucosaminidase enhances the binding of Mab 3E1.2, indicating that the NeuSGc is not attached to galactose or N-acetyl galactosaminyl. Furthermore, the susceptibility of MSA to treatment with Arthrobacter aereaefaciens neuraminidase [which is specific for (2-6)-linked NeuNAc] and the loss in reactivity of GalNAc-specific lectins after periodate oxidation [(2-3)-linked but not (2-6)-linked NeuNAc] indicate that the NeuSGc may be attached (2-6) to peptide-linked GalNAc. These results show that MSA is a NeuSGc-containing O-linked core glycan, which represents a unique tumor-associated epitope not previously identified on human mucins.

INTRODUCTION

Murine monoclonal antibody 3E1.2, produced by immunization with a human breast carcinoma, has selective reactivity with >90% breast carcinoma tissues and a limited reactivity with normal breast tissue and other normal secretory epithelium (1) but not with the human milk fat globule membrane (2). The antigen defined by 3E1.2 (MSA)(2) has also been detected in serum, with elevated levels in a high proportion of patients with breast cancer (3). Studies have shown that levels of MSA are useful for monitoring patients with breast cancer; the antibody has also been used to localize metastases in axillary lymph nodes in patients with breast cancer by immunoscintigraphy (4).

MSA has been shown to be expressed on high-molecular-weight mucins found in serum and tumors from breast cancer patients (2) and in the ascites of ovarian cancer patients (5). The epitope is a neuraminidase-sensitive carbohydrate structure present on the MUC-1 core protein (2, 6). To characterize the antibody-reactive epitope, we have used MSA from human tumor ascsites and the MCF-7 and ZR75-1 breast tumor cell lines to investigate the effects of glycosylation inhibitors, endoglycosidases, proteases, sialated glycoproteins, and sialic acid analogues on Mab 3E1.2 binding. In addition, Mab 3E1.2 and lectin reactivity was examined after different deglycosylation techniques. The studies indicate that 3E1.2 reacts with a unique NeuSGc-containing epitope not defined by other antibodies.

MATERIALS AND METHODS

Monoclonal Antibodies

Mabs 3E1.2 and other anti-MUC-1 Mabs OM-1, BC2, and BC3 were described previously (1, 7, 8). Mabs were purified from ascitic fluid, which was clarified by centrifugation at 300 × g for 10 min to remove cells and subsequently at 30,000 × g for 10 min to remove lipid. The purification of 3E1.2 (IgM) and OM-1 (IgM) was described previously (6). Mab BC3 (IgM) was purified by precipitation with 8% polyethylene glycol (9) followed by chromatography on Sephacryl S300-HR (Pharmacia, Upsalla, Sweden). Mab BC2 (IgG) was purified on protein A Sepharose CL-4B using a modified buffer system which gives increased binding of this Ig subclass (10). Mab concentration was estimated by absorbance at 280 nm (A280) using values for E1%1cm of 13.5 for IgG and 11.85 for IgM (11). Mabs were >95% pure, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and staining with Coomassie blue R250. The activity of Mabs was determined by indirect ELISA using assay plates coated with the MSA from the breast carcinoma cell line ZR75-1 (described below).

Cell Culture Techniques

Human tumor cell lines were maintained in Iscoves medium (Pacific Diagnostics, Australia) supplemented with l-glutamine (2 mS), penicillin (100 units/ml), streptomycin (100 μg/ml) (CSL, Melbourne, Australia), and 8% (v/v) fetal calf serum (Flow Labs, Australia). The ZR75-1 and MCF-7 breast carcinoma cell lines are a source of MSA, with the antigen secreted into the culture medium of MCF-7 but not ZR75-1 cells. Antigen was released from ZR75-1 cells by homogenization, freeze-thawing 5 times with liquid nitrogen, and centrifugation at 30,000 × g for 30 min, with the supernatant used in the MSA antigen ELISA. In some experiments using the MCF-7 cell line, 0.5 μg/ml (w/v) or 5 μg/ml (w/v) tunicamycin (Sigma, St. Louis, MO) or 0.5, 1.0, or 2.5 mM PAGal (Sigma) was added to the culture medium to inhibit N- or O-linked glycosylation (12, 13). The supernatant was collected after 72 h and tested for lactate dehydrogenase activity, MUC-1 core protein, and MSA activity.

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2 The abbreviations used are: MSA, mammary serum antigen; Gal, galactose; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine; NeuNAc, N-acetylneuraminic acid; Neu5Ac, 5-acetylneuraminic acid; Neu5Gc, 5-glycolyneuraminic acid; PAGal, phenyl-N-acetyl-o-D-galactosaminide; LDH, lactate dehydrogenase; RT, room temperature; TS, Trit-NaCl, pH 7.5, containing 0.9% NaCl; TST, TS containing 0.05% Tween 20; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; TFSMS, trifluoromethanesulfonic acid; SNA, Sambucus nigra agglutinin; MAA, Maackia amurensis agglutinin; WGA, wheat germ agglutinin; BI, Bandeiraea simplicifolia; Endo F, endoglycosidase F; Endo H, endoglycosidase H.
Purification of Mucin from Human Ascites

Ascites fluid was collected from a patient with a moderately differentiated uterine endometrium carcinoma, stage IV, clarified by centrifugation at 10,000 × g for 10 min at 4°C, and mucin was purified by chromatography on Sephacryl S300-HR (6).

LDH Assay

Cell cytotoxicity was determined by the release of intracellular LDH. LDH activity was determined by the conversion of lactate to pyruvate in the presence of NAD (14). Culture supernatants (0.2 ml) were incubated for 6 min at RT with 0.8 ml substrate solution (40 mg NAD, 640 μl 2-amino-2-methylpropion-1-ol, 100 μl d,L-lactic acid in 10 ml distilled water). The production of NADH was determined by absorbance at 340 nm.

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cases, samples were pretreated with neuraminidase for 2 h. Control incubations were performed as above, and the reaction was stopped by boiling for 5 min after the addition of 20% (w/v) sodium dodecyl sulfate to a final 1% (w/v).

Glycopeptidase F, Endo F, and Endo H. To remove N-linked glycans, TFMSA-treated or untreated mucin in 100 ml 0.25 mM sodium phosphate, pH 7.4, 10 mM EDTA, 0.02% (w/v) sodium azide, 10 mM 2-mercaptoethanol, 0.1% (v/v) sodium dodecyl sulfate, and 0.6% (v/v) Nonidet P40 was treated with 5 ml of 200 units/ml glycopeptidase F (Boehringer Mannheim) overnight at 37°C. Endo F and Endo H (Boehringer Mannheim) were used similarly, except that 0.25 mM sodium acetate, pH 6.5, was used for Endo F (50 units/ml), while 0.05 mM sodium acetate, pH 5.5, was used for Endo H (1 unit/ml). Control incubations were performed and reactions were stopped as described above.

Galactosidase. TFMSA-treated mucin was dialyzed into 0.1 M sodium phosphate, pH 6.5, or 0.1 M sodium phosphate, pH 7.3, containing 3 mM MgCl2 and treated with an equal volume of 100 units/ml Escherichia coli α- or β-galactosidase (Sigma) for 6 h at 37°C. Control incubations were performed with buffer in place of galactosidase or mucin.

N-Acetylglucosaminidase. TFMSA-treated mucin was dialyzed into 0.1 M citrate-phosphate buffer, pH 5.0, and treated with an equal volume of 0.56 unit/ml Canavalia ensiformis β-N-acetylglucosaminidase (Sigma) for 6 h at 37°C. Control incubations were performed as described above.

Periodate Oxidation. Samples were first bound to nitrocellulose as described for dot blotting and treated with 50 mM sodium metaperiodate (Sigma) in 50 mM sodium acetate buffer, pH 5.5, for 30 min at RT. The nitrocellulose was washed five times with water (2 min/wash), and 0.2 mg/ml (w/v) aqueous sodium borohydride (Merck, Darmstadt, Germany) was added for 30 min at RT. The washing steps above were repeated, and in some cases the sample was then treated with 0.1 unit/ml V. cholerae neuraminidase (Calbiochem) in 50 mM sodium acetate, pH 5.5, for 1 h at 37°C. Antigen was visualized as described for dot blotting. Control incubations were done with 50 mM sodium acetate, pH 5.5, in place of periodate, borohydride, or neuraminidase.

Protease Digestion of Mucins. Purified mucin or TFMSA-treated mucin, previously dialyzed into 0.1 M Tris-HCl, pH 8.0, containing 0.02% (w/v) NaN3, was incubated overnight at 37°C with an equal volume of either 25 mg/ml (w/v) trypsin (type XIII from bovine pancreas, tosyl lysine chloromethyl ketone treated), chymotrypsin, or pronase E (type III from papaya latex), or used as supplied.

The reaction was stopped by boiling for 10 min. After centrifugation, the supernatant was retained. Control incubations were also performed in the absence of either mucin or protease.

Electrospray Mass Spectrometry of Sialic Acids

Native or TFMSA-treated endometrial tumor mucin was treated with V. cholerae neuraminidase as described, except that mucin and neuraminidase had been dialyzed previously into 20 mM ammonium acetate, pH 5.5. Liberated neuraminic acid, which was separated from mucin and neuraminidase by filtration on an Ultrafree-MC 10,000-molecular-weight cutoff membrane (Millipore, Bedford, MA), was analyzed by electrospray mass spectrometry. A Sciex API 115 triple quadrupole mass spectrometer (Thornhill, Ontario, Canada) was used with a pneumatically assisted electrospray (ionspray) interface was used. A syringe infusion pump (Harvard Apparatus, South Natick, MA) was used to inject sample solutions directly into an ionspray interface at typical flow rates of 2-5 μl/min. The interface sprayer was operated at a negative potential of 4 kV. For comparison, 100 pmol/μl Neu5Ac and Neu5Gc (Sigma) were also run.

RESULTS

Reactivity of Mabs and Lectins with Native, HF-treated, and TFMSA-treated Mucin Purified from Human Endometrial Cancer Ascites

Since 3E1.2 recognizes a carbohydrate epitope on native and TFMSA-treated mucin but not HF-treated mucin (5), the reactivities of lectins with native and deglycosylated mucin were compared, because this gave an indication of which saccharides may be involved in Mab binding and the extent of deglycosylation with different techniques. This was of particular benefit with HF- and TFMSA-treated mucin, since only a small portion of the carbohydrate remained. Both treatments remove the majority of O-linked glycans and some N-linked glycans but not the same glycans, with TFMSA treatment being less severe (18-23). Comparison of the carbohydrates remaining in TFMSA-treated mucin (3E1.2 positive) and HF-treated mucin (3E1.2 negative) may indicate which carbohydrates are part of the 3E1.2 epitope. It is important to note that many lectins have a loose specificity, and these results may not be definitive.

Backbone and Peripheral Glycans. The binding of lectins by Western blotting is shown in Fig. 1, while the results of indirect ELISA are summarized in Table 1. The loss in reactivity of the sialic acid-binding lectins SNA and AMA and the fucose-binding asparagus pea lectin after TFMSA or HF treatment (Fig. 1 and Table 1) suggests that peripheral saccharides have been removed by the treatment. The binding of WGA, which also binds to sialic acid, is enhanced by TFMSA treatment (Fig. 1 and Table 1), since this lectin also binds to GlcNAc and GalNAc located in the core carbohydrate region (24-26). Similarly, neuraminidase treatment led to the total loss of SNA and AMA binding, but only partial loss of WGA binding due to the GlcNAc and GalNAc remaining in the molecule. The decreased reactivity of coral tree lectin (Fig. 1 and Table 1) suggests that many terminal residues attached via Gal(1-4)GlcNAc on N-linked glycans and type 2 backbone chains on O-linked glycans have also been released. Similarly, the decrease in the binding of tomato lectin (Fig. 1 and Table 1) suggests trimers and tetramers of GlcNAc do not remain, and the decreased reactivity of peanut lectin with TFMSA-treated mucin indicates that terminal Gal(1-3)GalNAc has been removed. The loss in reactivity of garden pea, concanavalin A, lentil, and Phaseolus vulgaris-E lectins after treatment with TFMSA or HF (Fig. 1 and Table 1) suggests that most mannose-containing backbone structures on N-linked glycans have also been removed.

Core Glycans. Since most of the backbone and peripheral glycans have been removed by TFMSA treatment and the reactivity with 3E1.2 is still retained, 3E1.2 may react with a core glycan. TFMSA treatment led to an increase in the binding of soybean, WGA, and BS1 lectins (Fig. 1 and Table 1), suggesting the exposure of O-linked core glycans consisting mainly of GlcNAc, with Gal and/or GlcNAc possibly attached, and N-linked GlcNAc monomers and dimers. The increased reactivity of peanut lectin after neuraminidase treatment of TFMSA-treated mucin (Table 1) confirms that the O-linked Gal(1-3)GalNAc core still remains. GlcNAc-GalNAc may not be present, however, since the reactivity of WGA may be due to N-linked GlcNAc monomers or dimers. In fact, the strong reactivity of WGA after neuraminidase treatment of TFMSA-treated mucin (Table 1) indicates that the reactivity is not with NeuNAc. The loss of reactivity of soybean lectin and the decreased reactivity of BS1 (both GlcNAc reactive) suggest that
Fig. 1. Reactivity of lectins with native (a), TFMSA-treated (b), and HF-treated (c) mucin in Western blotting. Samples were prepared as described in "Materials and Methods." *Ordinate*, position of BioRad-prestained high-molecular-weight markers. The reactivities of selected Mabs are given for comparison. L., lectin; Con A, concanavalin A.
most O-linked glycans have been removed after HF treatment (Fig. 1). The strong reactivity of WGA with HF-treated mucin suggests that GlcNAc, mainly present as the N-linked dimer, is the major species remaining. It is unlikely that WGA is reacting with GalNAc or NeuNAc in HF-treated mucin since other GalNAc- or NeuNAc-reactive lectins have reduced or destroyed reactivity. The loss in reactivity of O-glycan-reactive lectins corresponds to a loss in reactivity of 3E1.2 after HF treatment, suggesting that 3E1.2 reacts with an O-linked core glycan containing GalNAc and possibly Gal.

Reactivity of Mabs 3E1.2 and BC2 with Native and TFMSA-treated Mucin after Endoglycosidase and Neuraminidase Treatments

To investigate which carbohydrates were part of MSA, mucin from the ascites of a patient with an endometrial carcinoma was purified by gel filtration chromatography and treated with a variety of agents. Treatment of native (Fig. 2b) or TFMSA-treated mucin (Fig. 3a) with neuraminidase from V. cholerae (Figs. 2c and 3b), C. perfringens, and A. ureafaciens (not shown) led to the loss of 3E1.2 binding in Western blotting, suggesting that NeuNAc is an integral part of the epitope. A control containing neuraminidase only (Fig. 2a) showed no reactivity with 3E1.2, indicating that there was no nonspecific activity. Endoglycosidases did not have a significant effect on the binding of 3E1.2 to native mucin (Fig. 2, d–g). However, 3E1.2 binding to TFMSA-treated mucin (Fig. 3, a, e, and h–i) was significantly decreased by O-glycanase (Figs. 3, d and m) but was not affected by the other endoglycosidases (Fig. 3, f and j–l). These results suggest that the epitope for Mab 3E1.2 lies on an O-linked carbohydrate containing sialic acid or a derivative (NeuNAc). In addition, since galactosidase and N-acetylgalactosaminidase treatments led to an increase in binding in ELISA and dot blotting (Fig. 4), Gal and GlcNAc are not involved, and NeuNAc is possibly attached to a serine- or threonine-linked GalNAc. It was also noted that the binding of the MUC-1 core protein-reactive Mab BC2 to TFMSA-treated mucin (Fig. 3, a and i) was not affected by neuraminidase or O-glycanase treatment (Fig. 3, b–d and m), suggesting that the loss in 3E1.2 reactivity was due to the loss of carbohydrate and not the breakdown of the mucin core protein by contaminant proteases.

Effect of Periodate Treatment on Mab and Lectin Binding

The effect of periodate and neuraminidase treatment on the binding of lectins was tested to indicate any possible linkage between NeuNAc and GalNAc (Fig. 5). Periodate treatment of TFMSA-treated mucin abolished the binding of Mab 3E1.2 (not shown), soybean lectin, BS1 lectin, and RCA40 lectin, while the binding of BC2 (not shown), WGA, and peanut lectin was unchanged (Fig. 5). Neuraminidase treatment of TFMSA-treated mucin after periodate treatment led to a strong reactivity with peanut lectin, while the binding of other Mabs and lectins was unchanged. The loss of reactivity of the GalNAc-specific soybean, BS1, and RCA40 lectins after periodate treatment indicates that if NeuNAc is attached to GalNAc, it is at the C6 position, since GalNAc is resistant to periodate oxidation when NeuNAc is bound at C3 (27).

Inhibition of 3E1.2 Binding to ZR75–1 MSA by Native and TFMSA-treated Endometrial Tumor Mucin: Effect of Protease Treatment of Tumor Mucin

To determine whether Mab binding was dependent on the conformation of the protein backbone, the effect of protease treatment of endometrial tumor mucin on Mab binding was tested (Table 2). The effect was assessed by the ability of untreated or protease-treated endometrial tumor mucin to inhibit the binding of 3E1.2 to ZR75–1-derived MSA. Trypsin and chymotrypsin treatment of native mucin had no significant effect on the inhibition of 3E1.2 binding, while pronase and papain treatment of native mucin gave a significant decrease in inhibitory activity. The susceptibility of TFMSA-treated mucin to trypsin was also tested, since the decreased level of glycosylation exposes more of the protein core (as judged by the increased binding of Mab BC2). Trypsin treatment of TFMSA-treated mucin led to the loss of inhibitory activity (Table 2). Since neuraminidase was shown previously to destroy Mab binding in Western blotting, the effect in the inhibition assay was tested for comparison with the proteases used. Neuraminidase treatment led to the loss of inhibition in the ELISA (Table 2). Silver staining showed that extensive proteolysis had occurred with all proteases, with high-molecular-weight bands (Mr > 200,000) in untreated samples and a collection of low-molecular-weight bands (Mr < 5,000) in proteolyzed samples (not shown).
Fig. 3. Effect of neuraminidase and endoglycosidases on 3E1.2 and BC2 binding to TFMSA-treated mucin in Western blotting. Numbers, left, position of BioRad prestained standards. Samples were incubated as described in “Materials and Methods.” a, TFMSA-treated mucin, no neuraminidase or O-glycanase; b, TFMSA-treated mucin plus neuraminidase; c, TFMSA-treated mucin plus neuraminidase plus O-glycanase; d, TFMSA-treated mucin plus O-glycanase; e, TFMSA-treated mucin, no Endo H; f, TFMSA-treated mucin plus Endo H; g, TFMSA-treated mucin, no N-glycosidase F; h, TFMSA-treated mucin, no endoglycosidase F or N-glycanase F; i, TFMSA-treated mucin plus N-glycosidase F plus endoglycosidase F; j, TFMSA-treated mucin plus N-glycosidase F plus endoglycosidase F; k, TFMSA-treated mucin plus O-glycanase. When no enzyme or mucin was included, the buffer in which these were dissolved was added in their place to serve as controls. Enzyme controls (enzyme, no mucin) were negative in all cases, as were mucin samples that were treated with A. ureafaciens or C. perfringens neuraminidase (not shown).

Fig. 4. Effect of galactosidase and N-acetyl glucosaminidase on 3E1.2 binding to TFMSA-treated mucin in dot blotting and indirect ELISA. Samples were prepared as described in “Materials and Methods.” A, TFMSA-treated mucin; B, TFMSA-treated mucin plus α-galactosidase; C, TFMSA-treated mucin plus β-galactosidase; D, TFMSA-treated mucin plus N-acetyl glucosaminidase. The net absorbance at 405 nm in the indirect ELISA is also shown.

Fig. 5. Effect of periodate and neuraminidase treatment on the reactivity of biotinylated lectins with TFMSA-treated mucin. Samples were prepared as described in “Materials and Methods.” A, TFMSA-treated mucin; B, TFMSA-treated mucin plus neuraminidase; C, TFMSA-treated mucin plus periodate; D, TFMSA-treated mucin plus periodate plus neuraminidase. PNA, peanut lectin; SOY, soybean lectin; RCA60, R. communis M, 60,000 lectin. The specificities of lectins are shown in Table 1.
Inhibition of 3E1.2 Binding and Lectin Binding to ZR75-1-derived MSA by Glycoproteins

Since NeuNAc is an essential part of the 3E1.2 epitope, the reactivity of Mab 3E1.2 with NeuNAc-containing glycoproteins was tested. Porcine mucin (90% of NeuNAc as Neu5Gc) inhibited the binding of 3E1.2 to MSA in a ZR75-1 cell extract (Table 4), while bovine mucin (mainly 9-acetyl neuraminic acid), fetuin (mainly Neu5Ac), ovine mucin (>98% of glycans as Neu5Ac O(2-6)GalNAc), asialofetuin (<1% NeuNAc), and human transferrin had no effect (not shown). By comparison, the binding of Mab BC3 was not affected significantly by any of the glycoproteins tested (Table 4), while the binding of the NeuNAc-specific lectins SNA and MAA was inhibited strongly by fetuin but not asialofetuin (not shown). Bovine and porcine mucins also inhibited SNA binding weakly, while bovine mucin but not porcine mucin gave strong inhibition of MAA binding (not shown). These results suggest that Neu5Gc may be the NeuNAc derivative involved in 3E1.2 binding. Furthermore, the majority of Neu5Gc-containing oligosaccharides isolated from porcine mucins have Neu5Gc attached to GalNAc via an O(2-6) bond (28, 29), suggesting that this may be the structure with which 3E1.2 is reacting.

Table 2 Inhibition of 3E1.2 binding to ZR75-1-derived MSA by native and TFMSA-treated endometrial tumor mucin: effect of protease treatment of tumor mucin

<table>
<thead>
<tr>
<th>Proteas</th>
<th>Mucin alone (±SD)</th>
<th>Mucin + protease (±SD)</th>
<th>Protease alone (±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native mucin</td>
<td>94.0 ± 0.7</td>
<td>97.0 ± 1.0</td>
<td>8.5 ± 3.0</td>
</tr>
<tr>
<td>Trypsin</td>
<td>97.5 ± 0.1</td>
<td>93.2 ± 0.5</td>
<td>13.9 ± 1.9</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>94.2 ± 0.2</td>
<td>78.7 ± 3.3</td>
<td>0.8 ± 1.1</td>
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<tr>
<td>Pronase</td>
<td>95.2 ± 1.3</td>
<td>53.8 ± 4.4</td>
<td>4.1 ± 5.1</td>
</tr>
<tr>
<td>Neuraminidase*</td>
<td>94.6 ± 0.4</td>
<td>2.9 ± 0.1</td>
<td>5.7 ± 3.2</td>
</tr>
<tr>
<td>TFMSA-treated mucin</td>
<td>47.9 ± 0.3</td>
<td>71.7 ± 0.1</td>
<td>8.5 ± 3.0</td>
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* Samples were used at 1/50 in the MSA ELISA as described in "Materials and Methods." n = 2 for all samples.

Inhibition of 3E1.2 Binding to MSA by Neu5Ac and Neu5Gc

Since the inhibition of 3E1.2 by porcine mucin suggested a role of Neu5Gc in Mab binding, the effect of free Neu5Gc and Neu5Ac on the binding of 3E1.2 to MSA produced by the ZR75-1 breast cancer cell line was tested. The binding of Mab 3E1.2 to a ZR75-1 cell extract was inhibited by Neu5Gc but not by Neu5Ac, while the binding of Mabs BC3 and OM-1 was not inhibited by either derivative (Table 5). All Mabs were inhibited by ascites taken from a patient with advanced ovarian cancer (used as a positive control; not shown). Similar results were obtained when Mabs were used at the same concentration rather than at the concentration that gave the same absorbance (not shown). Other sugars tested showed no inhibition (lactose, sucrose, d-glucuronic acid lactone, GlcNAc, GalNAc, Gal, etc.).

Table 3 Effect of glycosylation inhibitors on in vitro mucin production by MCF-7 Breast Carcinoma Cell Line

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BC2-BC3 assay (A410 ± SD)</th>
<th>MSA assay (% inhibition ± SD)</th>
<th>LDH assay (A140)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tunicamycin (µg/ml)</td>
<td>0.255 ± 0.092</td>
<td>72 ± 5</td>
<td>0.153</td>
</tr>
<tr>
<td>0.5</td>
<td>0.151 ± 0.020</td>
<td>64 ± 7</td>
<td>0.135</td>
</tr>
<tr>
<td>5.0</td>
<td>0.112 ± 0.014</td>
<td>60 ± 6</td>
<td>0.134</td>
</tr>
<tr>
<td>PAGal (mM)</td>
<td>0.255 ± 0.092</td>
<td>72 ± 5</td>
<td>0.153</td>
</tr>
<tr>
<td>0.5</td>
<td>0.612 ± 0.007</td>
<td>36 ± 2</td>
<td>0.137</td>
</tr>
<tr>
<td>1.0</td>
<td>0.976 ± 0.022</td>
<td>38 ± 4</td>
<td>0.136</td>
</tr>
<tr>
<td>2.5</td>
<td>1.315 ± 0.008</td>
<td>15 ± 1</td>
<td>0.143</td>
</tr>
</tbody>
</table>

* All samples concentrated 10-fold in an Ultrafree-MC 10,000-molecular weight cutoff cartridge (Millipore) before assay.

In LDH assay, tissue culture media gave A140 = 0.130. Duplicates were pooled and assayed.

n = 4 for tunicamycin and control incubations; n = 2 for PAGal incubations.

Table 4 Inhibition of 3E1.2 binding to ZR75-1-derived MSA by porcine mucin

<table>
<thead>
<tr>
<th>Mab*</th>
<th>Glycoprotein concentration (µg/ml)</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>3E1.2</td>
<td>14</td>
<td>80</td>
</tr>
<tr>
<td>BC3</td>
<td>10</td>
<td>11</td>
</tr>
</tbody>
</table>

* Mabs used at 0.5 µg/ml.

BC3 bovine submaxillary mucin, ovine submaxillary mucin, fetal calf fetuin and asialofetuin, and human transferrin showed no inhibition of 3E1.2 or BC3 binding at any of the concentrations above. With no inhibitor, A410 nm values were 1.247 (3E1.2) and 0.808 (BC3).

Table 5 Inhibition of 3E1.2, OM-1, and BC3 binding to ZR75-1-derived MSA by Neu5Gc and Neu5Ac

<table>
<thead>
<tr>
<th>Mab*</th>
<th>Inhibitor concentration (µg/ml)</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>3E1.2</td>
<td>125</td>
<td>250</td>
</tr>
<tr>
<td>Neu5Gc</td>
<td>15</td>
<td>29</td>
</tr>
<tr>
<td>OM-1</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>BC3</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Neu5Ac</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>OM-1</td>
<td>14</td>
<td>16</td>
</tr>
<tr>
<td>BC3</td>
<td>3</td>
<td>5</td>
</tr>
</tbody>
</table>

* With no inhibitor, A410 nm values were 0.923 (3E1.2), 0.882 (OM-1), and 0.915 (BC3).
mannose, melibiose, bovine brain gangliosides, disialogangliosides GD_{1a}, trisialogangliosides GT_{1b}, N-acetyl neuramin-lactose from bovine colostrum and human milk, N-acetyl lactosamine, neuraminic acid-β-methylglycoside, keratin from human skin, and keratin sulfate from bovine cornea; Sigma). The inhibition by Neu5Gc but not other derivatives suggests that Neu5Gc is a major part of the epitope.

Electrospray Mass Spectrometry

Mass spectrometry was used to confirm the presence of Neu5Gc in human endometrial tumor mucin. Standard Neu5Ac and Neu5Gc gave mass peaks at \( M_0 \), 324,000 and 308,000, respectively, which correspond to the molecular weight minus 1 of each (Fig. 6, a and b). In addition, minor peaks corresponding to trifluoroacetic acid adducts of the pseudomolecular ions were also seen at \( M_0 \), 438,000 and 422,000, respectively (Fig. 6, a and b). Peaks of lower \( m/z \) values (with the exception of the trifluoroacetic acid anion at \( M_0 \), 113,000) are fragment ions. The molecular ions of both species were observed in native mucin (Fig. 6c), while only those of Neu5Gc were observed in TFMSA-treated mucin (Fig. 6d), confirming the presence of Neu5Gc in MSA-positive molecules.

DISCUSSION

In these studies we have shown that the reactivity of Mab 3E1.2, which defines the breast tumor-associated MSA, is sensitive to treatment with different neuraminidases, O-glycanase, periodate, and various proteases but is not adversely affected by treatment with galactosidase, N-acetylglucosaminidase, or endoglycosidases which remove N-linked glycans. In addition, the production of MSA by the MCF-7 breast tumor cell line is inhibited in a dose-dependent manner by the O-glycosylation inhibitor PAGal but not the N-glycosylation inhibitor tunicamycin, confirming the reactivity of this Mab with an O-linked glycan. Furthermore, porcine stomach mucin (>90% sialic acid as the N-glycolyl derivative) and free N-glycolyneuraminic acid inhibit the binding of 3E1.2 to MSA from the ZR75–1 breast tumor cell line, supporting our contention that Neu5Gc is a major part of the epitope. The presence of Neu5Gc in human tumor mucin was confirmed by electrospray mass spectrometry.

Since the reactivity of this Mab with both untreated and TFMSA-treated mucin is destroyed by neuraminidase treatment, sialic acid or a derivative (NeuNAc) is an essential part of the MSA epitope defined by this Mab. More than 25 derivatives of NeuNAc have been reported to occur naturally, and these may be attached to a variety of residues by a number of

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Fig. 6. Electrospray mass spectrometry of neuraminidase-liberated sialic acid. Samples were prepared and analyzed as described in "Materials and Methods." Spectra: A, standard Neu5Ac; B, standard Neu5Gc; C, sialic acid fraction from neuraminidase treatment of native mucin; and D, sialic acid fraction from neuraminidase treatment of TFMSA-treated mucin.
Mab 3E1.2 recognizes N-glycolylneuraminic acid on mucins

linkages. The most common derivative found in humans is N-acetylgalactosaminic acid (Neu5Ac), followed by the 5,9-diacetyl, 7,9-diacyl, 9-O-acetyl, and 2-deoxy-2,3-dideoxy derivatives (30). The O-acetyl derivatives of Neu5Ac are hydrolyzed by neuraminidase at a much slower rate than Neu5Ac or are resistant to neuraminidase treatment (31–33), which suggests that these derivatives are not part of the MSA epitope, which is very sensitive to neuraminidase treatment. In addition, glycoproteins containing high levels of NeuNAc such as fetuin [6% NeuNAc, mainly Neu5Ac α(2–3)Gal or Neu5Ac α(2–6)Gal], bovine submaxillary mucin [10% NeuNAc, mainly 9-acetylgalactosaminic acid α(2–6)Gal or 8,9-diacetylgalactosaminic acid α(2–6)Gal], and ovine submaxillary mucin [98% of the glycans as Neu5Ac α(2–6)GalNAc] do not inhibit 3E1.2 binding to ZR75–1. This indicates that the specificity of Mab 3E1.2 is not for these NeuNAc derivatives, as is the case with sialic acid-binding lectins SNA and MAA [α(2–6)Gal/GalNAc and α(2–3)Gal-linked residues, respectively], which were inhibited by these glycoproteins. The lack of reactivity of the sialic acid-binding lectins SNA and MAA with TFMSA-treated mucin, which is detected by 3E1.2, confirms this.

The reactivity of Mab 3E1.2 with porcine stomach mucin was of particular interest since more than 90% of the NeuNAc in this species is in the form of NeuSGc (35, 36), and most of this is attached to GalNAc (28, 29). Less than 1% of the total NeuNAc found in healthy humans occurs in this form (37). Free NeuSGc inhibited 3E1.2 binding to ZR75–1 while Neu5Ac did not, indicating that Neu5Gc is part of the 3E1.2 epitope, which is overexpressed on human carcinoma-associated mucins. The presence of this derivative on both native and TFMSA-treated endometrial tumor mucin was confirmed by electrospray mass spectrometry. The SNA lectin does not react with the Neu5Gc derivative (38), which would explain the lack of reactivity of this lectin with TFMSA-treated mucin. Similarly, WGA has also been reported to have a low affinity for this derivative (39). Furthermore, V. cholerae neuraminidase hydrolyzes the Neu5Gc and Neu5Ac derivatives at a similar rate (31), which would explain the sensitivity of 3E1.2 binding to this treatment.

Neu5Gc as a component of gangliosides has been discussed as a cancer-associated epitope (Hanganaziu-Deicher antigen) in humans (40–42). In addition, recombinant human glycoproteins expressed in Chinese hamster ovary cells have been shown to contain traces of Neu5Gc α(2–3)Gal as a terminating structure on N-linked glycoproteins (43), but the presence of this derivative on human mucins has not been demonstrated. Furthermore, the quantity of Neu5Gc gangliosides in cancer is said to be extremely low (44). Antibodies to Neu5Gc are found in humans, and the Hanganaziu-Deicher ganglioside antigen is thought to be responsible for the induction of Hanganaziu-Deicher-specific antibodies in patients with various diseases and in patients receiving therapeutic foreign immune serum (44). Many other tumor-associated sialylated carbohydrate structures on mucins and glycolipids which are recognized by Mabs have been described, but none contain the Neu5Gc derivative. For example, those structures recognized by Mabs B7.23 (sialyl-Tn), N19-9 (sialyl-Lewis*), C50 (sialyl-Lacto1), and C5LEX-1 (sialyl-Lewis*) contain the common Neu5Ac derivative (45–48).

In vitro glycosylation inhibitor studies suggest that the epitope for this Mab lies on an O-linked carbohydrate since treatment with PAGal (O-linked glycosylation inhibitor) (13) led to a significant decrease in both 3E1.2 binding (MSA assay) and O-glycosylation (as judged by the exposure of protein epitopes detected in the BC2-BC3 assay). Treatment of MCF-7 breast cancer cells in culture with 2.5 mM PAGal led to a decrease in the inhibition of 3E1.2 binding by culture supernatant from 72% to 15%, while the same treatment led to an increase in absorbance from 0.255 to 1.315 in the MUC-1 core protein (BC2-BC3 assay). Tunicamycin treatment (N-glycosylation inhibitor) (12) led to a small decrease in 3E1.2 binding (from 72% to 60%), but this was likely to be due to decreased synthesis of the core protein, as judged by the BC2-BC3 assay (decrease in absorbance from 0.255 to 0.112). In addition, the glycosidases that are specific for N-linked carbohydrate (glycopeptidase F, Endo F, Endo H) had no effect on 3E1.2 binding. Between them, these glycosidases cleave the majority of high-mannose, hybrid, and complex-type N-linked glycans (49). Surprisingly, O-glycanase, which cleaves at the GalNAc-serine linkage when Gal but not GlcNAc is attached, had no effect on 3E1.2 binding to the native mucin, but this enzyme has lower activity unless the sample is pretreated with neuraminidase (50), which would have destroyed 3E1.2 binding. However, O-glycanase decreased the binding of 3E1.2 to TFMSA-treated mucin since TFMSA-treatment led to the loss of most sialic acid, as indicated by the lack of reactivity of SNA and MAA lectins. The loss of O-linked glycans and 3E1.2 reactivity by HF treatment also suggests that an O-linked glycans involved.

Lectin binding studies indicate that only core carbohydrate structures remained on the TFMSA-treated mucin which was reactive with 3E1.2. The presence of the GalNAc-Gal core glycan was indicated by the reactivity of peanut lectin with TFMSA-treated mucin after neuraminidase treatment, and the loss of binding to 3E1.2 to TFMSA-treated mucin after O-glycanase treatment, which cleaves at Gal(1–3)GalNAc but not GlcNAc(1–3)GalNAc (50). It is unlikely that the Neu5Gc is attached to Gal or GlcNAc, since treatment with galactosidase or N-acetyl glucosaminidase enhanced 3E1.2 binding. In addition, sialic acids bound to internal Gal residues to which GalNAc is linked are resistant to neuraminidase treatment (27), while the epitope on both native and TFMSA-treated mucin is neuraminidase sensitive. Furthermore, the MAA lectin, which did not react with TFMSA-treated mucin or porcine mucin, binds equally to either Neu5Ac α(2–3)Gal or Neu5Gc α(2–3)Gal (51). It is possible that Neu5Gc is attached to GalNAc by an α(2–6) bond (glycolylisialyl-Tn), since most of the Neu5Gc on porcine mucin, which reacted with 3E1.2, is attached to GalNAc by this linkage (28, 29); (b) A. ureafaciens neuraminidase [strong preference for α(2–6) linkage] (27, 35) destroys 3E1.2 binding; (c) the loss of reactivity of the GalNAc lectins after periodate treatment indicates that Neu5Gc is attached to GalNAc at C6, since GalNAc is resistant to periodate oxidation when GalNAc is bound at C3 (27); and (d) results of TFMSA and HF treatments by other groups have shown that all O-linked saccharides except GalNAc are removed (20–22) while 20% of the NeuNAc still remains linked to GalNAc by an α(2–6) bond after less severe treatments with TFMSA (23). However, further work is needed to confirm the structure of this epitope. Neu5Gc α(2–6)GalNAc has been shown to occur at low frequency on bovine submaxillary mucin (52–54). Surprisingly, 3E1.2 binding to MSA was not inhibited by this mucin, although the concentration of this epitope would be much less than on porcine mucin and human tumor mucin, since mass spectrometry indicated Neu5Gc was the major sialic acid derivative present on TFMSA-treated mucin.

Treatment of native mucin with papain and pronase led to a
decrease in the inhibition of 3E1.2 binding by mucin, while trypsin treatment of TFMSA-treated mucin led to a loss of inhibitory activity. Papain and pronase are less specific than trypsin and chymotrypsin and would generally yield smaller peptide fragments due to greater proteolysis, while TFMSA-treated mucin would be more susceptible to proteolysis, since more of the peptide is exposed for cleavage. The loss of 3E1.2 binding after protease treatment of mucin suggests that the peptide backbone may confer multivalency for the epitope or may aid conformationally in the optimal binding of this Mab. Differences in tertiary structure may explain the different reactivity of 3E1.2 toward human MUC-1 tumor mucin and bovine submaxillary mucin, which also contains traces of glycolylsialyl-Tn (52–54). Similarly, Mab MLS 102 has been reported to recognize a “cluster” of sialyl-Tn in mucin-type glycoproteins (29). The sialyl-Tn structure defined by Mabs MLS 102, B72.3, and TKH-2 has been reported to be elevated in tumors (44, 45, 55, 56). However, 3E1.2 is unlikely to react with this structure since (a) 3E1.2 binding is inhibited by Neu5Gc but not Neu5Ac; (b) 3E1.2 binding is inhibited strongly by porcine mucin (most sialic acid as Neu5Gc linked to GalNAc) (31) but not bovine or ovine mucins, while MLS 102 is inhibited strongly by ovine and bovine mucins and only weakly by porcine mucin (55); (c) sialyl-Tn is expressed strongly by intestinal tumors (45, 57) while 3E1.2 reacts strongly with breast tumors and weakly with intestinal tumors (1); and (d) TFMSA-treated mucin (3E1.2-positive) was not detected by SNA lectin, which is specific for this structure (38).

Apart from sialyl-Tn, other sialylated glycans defined by Mabs have been reported to be elevated in tumors. For example, the sialylated Lewis structures defined by Mabs N99–9, C50, and SLEX-1 all contain the Neu5Ac derivative attached to Gal (46–48). In addition, other Mabs have been described that react with neuraminidase-sensitive epitopes of undefined structure (58, 59). However, the epitope described is unique since the presence of Neu5Gc has not been reported to occur on human mucins. Indeed, the lack of reactivity of 3E1.2 with normal mucin expressed on the human milk fat globule membrane (2, 5, 6) and the poor reactivity in the serum of healthy individuals (3) shows that this structure may well be tumor specific. The epitope is not masked by more extensive glycosylation of normal mucin since TFMSA treatment of human milk fat globule membrane does not expose the epitope (6). It is postulated that the exposure of this epitope in tumors could be due to the accumulation of a glycolylsialyl-truncated glycan, possibly caused by the low reactivity of galactose transferase (Tn accumulated) and the concurrent increased activity of sialyltransferase with an increased specificity for Neu5Gc. Additionally, increased activity of the enzyme cytidine 5'-monophosphosphate-Neu5Ac hydrolase (formerly called monooxygenase), which converts Neu5Ac to Neu5Gc (60), could lead to increased levels of the Neu5Gc analogue.

The structure will be confirmed through mass spectrometry and nuclear magnetic resonance of the pure material (61–64) or the use of synthetic carbohydrates (65, 66). This work also leads to the possibility of using a synthetic MSA glycoconjugate as a vaccine for breast and ovarian cancer, as illustrated recently in mice with the TF and Tn antigens (67, 68). These and other studies are now in progress to determine the relevance and diagnostic/therapeutic potential of this novel tumor-associated epitope on mucins.

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The Breast Tumor-associated Epitope Defined by Monoclonal Antibody 3E1.2 Is an O-linked Mucin Carbohydrate Containing N-Glycolylneuraminic Acid

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