Biochemical analysis of a bladder-cancer-associated mucin: structural features and epitope characterization

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Three monoclonal antibodies (mAbs), M344, M300 and M75, were shown to define a unique tumour-associated antigen (TAA) of superficial bladder tumours. The antigenic determinants are expressed on a very-high-molecular-mass component and, in about 50 % of the positive samples, one determinant is also detected on a 62 kDa molecular species, observed only under reducing conditions. The objectives of the present study were to characterize further this TAA by analysing (1) the biochemical nature of the epitopes recognized by the three mAbs, and (2) the biochemical and structural features of the molecule bearing them. The antigenicity was resistant to heat denaturation, trypsin and α -chymotrypsin treatments but highly sensitive to papain and Pronase digestion. NaIO₄ oxidation decreased reactivity to mAbs M344 and M300 but enhanced reactivity to mAb M75. The three determinants were insensitive to β -galactosidase and α -L-fucosidase but were sensitive to *Vibrio cholerae* neuraminidase.

None of the three mAbs reacted with ovine, bovine or porcine submaxillary mucins. Deglycosylation with O-glycosidase or trifluoromethanesulphonic acid completely abolished the reactivity of the mAbs whereas N-glycosidase F deglycosylation had no appreciable effect. The presence on the molecule of cryptic Gal β (1 $\rightarrow$ 3)GalNAc as a major core disaccharide was demonstrated by a heterologous sandwich assay using mAb M75 and peanut agglutinin. Thiol reduction using β -mercaptoethanol increased mobility of the high-molecular-mass component in polyacrylamide gels. We thus conclude that mAbs M344 and M300 react with sialylated carbohydrate epitopes, and mAb M75 reacts with a partially cryptic and periodate-resistant sialylated epitope expressed on a typical secreted high-molecular-mass oligomeric mucin which we named MAUB for mucin antigen of the urinary bladder.

INTRODUCTION

Bladder transitional cell carcinoma is the fifth most common malignancy in Western countries [1,2], and a majority of bladder cancers appear as superficial papillary tumours. We have previously identified three monoclonal antibodies (mAbs), M344, M300 and M75 (M series mAbs), defining a tumour-associated antigen (TAA) expressed on more than 70 % of superficial papillary bladder tumours but on only 15 % of infiltrating bladder cancers [3]. Immunohistochemical studies have shown no reactivity with fetal or adult normal human tissues [3,4] and variable reactivities with a few prostatic, colonic, breast and endometrial cancers [4]. Measurement of this antigen was shown to be useful for the diagnosis [5,6] and prognosis [7–9] of bladder cancer. Preliminary studies showed reactivity of the M series mAbs with a very-high-molecular-mass component and also with a 62 kDa component in about half of the positive samples [3]. By immunoelectron microscopy, the mAbs were shown to react with very large cytoplasmic vacuoles in tumour cells and by Western blotting, with material shed in the culture medium of MGH-U3 bladder tumour cells (Y. Fradet, R. Pankov, A. Bergeron and C. Parent-Vaugeois, unpublished work). The high molecular mass and the secreted nature of the antigen thus suggest that the TAA defined by the M series mAbs may be a mucin-type glycoprotein.

Mucins are heavily glycosylated high-molecular-mass glycoproteins secreted by mucosae and some exocrine glands [10,11]. In their native conformation these molecules exist as extremely large oligomers with molecular masses of more than 10^3 kDa. The present models of mucin oligomerization suggest that the mucin monomers are polymerized in an end-to-end fashion

through disulphide bonds involving cysteine residues found in their N- and C-terminal cysteine-rich domains [10,11]. The central domain of the mucin polypeptide is composed of a variable number of tandem repeats which are rich in serine, threonine and proline residues and may or may not be separated by domains rich in hydrophobic residues [10,12]. The abundance of serine and threonine residues in these domains provides the backbone necessary for the bulk glycosylation of mucins through O-glycosylation of these residues [13]. A recently growing interest in mucins has come from the recognition that they express many tumour-associated epitopes presenting interesting diagnostic, prognostic and eventually immunotherapeutic potential [14–17]. These tumour-associated determinants frequently result from aberrant glycosylation leading to the appearance of new carbohydrate epitopes or to an enhanced accessibility of normally cryptic peptide epitopes [18–20].

The present study aimed to confirm the mucin nature of the TAA recognized by the M series mAbs. We conclude that the antigen is a typical secreted high-molecular-mass oligomeric mucin and that both M300 and M344 mAbs recognize sialylated epitopes, whereas mAb M75 recognizes a sialylated epitope that is partially cryptic and periodate-resistant.

MATERIALS AND METHODS

Antigens and antibodies

Antigen preparations were obtained by extraction of proteins from MGH-U3 cells grown *in vivo* as nude-mouse tumours (T-MGH-U3) with 0.25% *n*-dodecyl β -D-maltoside detergent (Calbiochem, La Jolla, CA, U.S.A.) in 50 mM Tris/HCl, pH 8.0.

Abbreviations used: mAb, monoclonal antibody; TAA, tumour-associated antigen; T-MGH-U3, MGH-U3 cells cultured as nude mouse tumour; OSM, ovine submaxillary mucin; BSM, bovine submaxillary mucin; PSM, porcine submaxillary mucin; ECL, enhanced chemiluminescence; PNA, peanut agglutinin; TBS, Tris-buffered saline; TFMSA, trifluoromethanesulphonic acid.

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containing 150 mM NaCl and 2 mM PMSF, as previously described [21]. For some glycosidase treatments, detergent extractions were instead carried out in 20 mM sodium cacodylate, pH 7.0, containing 10 mM calcium acetate or in 0.1 M sodium phosphate, pH 7.4, in the presence of PMSF. Transitional cell carcinoma Ta and T1 tumour specimens were obtained from the Pathology Department of L'Hôtel-Dieu de Québec and processed as for nude mouse xenografts. Purified ovine submaxillary mucin (OSM) and porcine submaxillary mucin from blood group A⁺ and A⁻ (PSM A⁺ and PSM A⁻) were purchased from Biocarp Chemicals (Cedarlane, Hornby, Ontario, Canada). Purified bovine submaxillary mucin (BSM) was purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.).

mAbs M344, M300 and M75 are all murine IgG₁, obtained by simultaneous immunizations with superficial papillary bladder tumour cells and mouse polyclonal serum against normal urothelium [3]. They were purified from ascites by octanoic acid and (NH₄)₂SO₄ precipitations [22] or by fast preparative liquid chromatography. mAb 19A211, which was also produced in our laboratory, is directed against a sialylated carbohydrate epitope defining another superficial bladder TAA [23]. mAb HMFG-2, against a protein epitope of the mucin-like MUC1 [24], was provided by Dr. Joyce Taylor-Papadimitriou (Imperial Cancer Research Fund, London, U.K.). mAb B72.3, against NeuAc α (2 → 6)GalNAc (sialyl-Tn) [25], and mAb 49H8, recognizing Gal β (1 → 3)GalNAc (T or Thomsen-Friedenreich antigen) [26], were provided by Dr. Jeffrey Schlom (National Institutes of Health, Bethesda, MD, U.S.A.) and Dr. B. Michael Longenecker (Biomira, Edmonton, Alberta, Canada) respectively.

Immunoassays

Double determinant immunoradiometric assays

Double-determinant immunoradiometric assays were carried out as previously described [21], using 50 μ l of hybridoma tissue culture supernatant as the capture antibody and 2×10^5 c.p.m. of ¹²⁵I-labelled M344 mAb [27] as the detecting antibody.

Solid-phase competitions

Poly(vinyl chloride) microwell plates were coated with T-MGH-U3 proteins (5 μ g/well). After blocking with Tris-buffered saline (TBS) containing 5% BSA for 1 h at 37 °C, 2×10^5 c.p.m./well ¹²⁵I-labelled M344 mAb (approx. 50 ng) alone or in the presence of a 20- or 100-fold excess by weight of competing antibody were added and incubation was carried out at 37 °C for 1 h. Wells were then extensively washed with TBS and counted.

Heterologous sandwich assays

Expression of the cryptic core disaccharide Gal β (1 → 3)GalNAc on the antigen recognized by M series mAbs was analysed with *Arachis hypogaea* lectin [peanut agglutinin (PNA)] (Sigma). Briefly, each well of poly(vinyl chloride) microwell plates was coated with goat anti-mouse polyclonal antibody and blocked as described above. Then 50 μ l of hybridoma tissue culture supernatant was added to each well and incubated overnight at 4 °C. The wells were washed with TBS and 5 μ g of partially desialylated (see below) or untreated T-MGH-U3 protein was added and incubated for 3 h at 37 °C. Each well was once again washed and incubated with 2×10^5 c.p.m./well ¹²⁵I-labelled PNA [27] for 1 h at room temperature. After a final TBS wash, each well was counted.

Chemical and physical treatments

Periodate oxidation

T-MGH-U3 protein samples were treated with variable concentrations of NaIO₄ (Fisher Scientific, Montréal, Québec, Canada) in 50 mM sodium acetate, pH 4.5, at 37 °C in the dark. Reactions were stopped by the addition of ethylene glycol to a final concentration of 20 mM.

Reduction and carboxymethylation

T-MGH-U3 proteins in 0.5 M Tris/HCl, pH 8.5, containing 6 M guanidinium chloride were incubated for 2 h at 37 °C in the presence of 10 mM dithiothreitol. Iodoacetamide was added to a final concentration of 20 mM and the solution incubated on ice in the dark for 2 h. The solution was then dialysed against TBS.

Deglycosylation by trifluoromethanesulphonic acid (TFMSA) and β -elimination

Desialylated T-MGH-U3 proteins (see below) were dialysed against water and lyophilized. They were then subjected to solvolysis [28] by dissolving 5 mg of lyophilized proteins in 1.2 ml of a TFMSA/anisole (2:1, v/v) mixture (ICN, St. Laurent, Québec, Canada) and incubating on ice for 2 h with gentle mixing. The reaction was stopped by freezing the solution at -70 °C and by adding 2 vol. of cold (-70 °C) diethyl ether. The solution was extracted with an equal volume of ice-cold 50% pyridine followed by three sequential extractions of the aqueous phase with an equal volume of cold (-70 °C) diethyl ether. The partially deglycosylated proteins (aqueous phase) were dialysed against a large volume of water and then lyophilized before being submitted to a second round of TFMSA/anisole treatment. Further deglycosylation was accomplished by subjecting the partially deglycosylated proteins to reverse β -elimination [29]. Briefly, the treated proteins were oxidized with 50 mM NaIO₄ in 50 mM sodium acetate, pH 4.5, at 37 °C for 30 min. Reaction was stopped by the addition of ethylene glycol to a final concentration of 20 mM.

Enzymic treatments

Proteases

Four different proteases were used at a final concentration of 1 mg/ml. Incubations were performed at 37 °C for 3 h and 24 h, followed by 5 min at 100 °C to destroy protease activity. Enzymes and their buffers were: trypsin and α -chymotrypsin (Sigma) in 40 mM Tris/HCl, pH 8.0, containing 20 mM CaCl₂; papain (Sigma) in 20 mM cysteine/HCl, pH 6.0, containing 5 mM EDTA; and Pronase (Boehringer-Mannheim, Laval, Québec, Canada) in 20 mM Tris/HCl, pH 8.0, containing 10 mM CaCl₂. No enzyme reaction mixtures were used as controls.

Glycosidases

T-MGH-U3 protein extracts (2 mg/ml) in sodium cacodylate or sodium phosphate buffer were boiled for 5 min and then treated with various glycosidases for 3 h at 37 °C: neuraminidase from *V. cholerae* (Calbiochem) 100 m-units/ml in sodium cacodylate buffer; β -galactosidase from *Escherichia coli* (Sigma) and α -L-fucosidase from bovine kidney (Boehringer-Mannheim) 40 μ g/ml and 30 units/ml respectively in sodium phosphate buffer. Incomplete or partial desialylation with neuraminidase was obtained after 30 min of incubation at 37 °C. O-Glycosidase from *Diplococcus pneumoniae* (Genzyme, Inter-Medico, Willowdale, Ontario, Canada) was used on neuraminidase-pretreated proteins, at a final concentration of 40 m-units/ml at

37 °C for 18 h. N-Deglycosylation was carried out on denatured proteins. T-MGH-U3 protein samples were boiled for 3 min in 1% SDS and then treated with 20 units/ml recombinant N-glycosidase F (Boehringer-Mannheim) for 18 h at 37 °C in a volume of buffer (35 mM sodium phosphate buffer, pH 8.0, containing 18 mM EDTA, 1% β -mercaptoethanol and 0.7% Nonidet P40) sufficiently large to have a sevenfold excess of Nonidet P40 to SDS. For digestion with heparinase, hyaluronidase and chondroitinase ABC, T-MGH-U3 protein samples were treated at a final concentration of 1 mg/ml at 37 °C for 18 h in enzyme-specific buffers. Heparinase from *Flavobacterium heparinum* (Sigma) was used at a final concentration of 2.5 units/ml in 0.1 M Tris/HCl, pH 7.2. Hyaluronidase from bovine testes (Sigma) was used at a final concentration of 20 units/ml in 0.1 M acetate buffer, pH 5.0. Chondroitinase ABC from *Proteus vulgaris* (Sigma) was used at a final concentration of 2.5 units/ml in 250 mM Tris/HCl, pH 8.0, containing 176 mM sodium acetate and 250 mM NaCl.

Electrophoresis and blotting

Proteins (50 μ g per track) were electrophoresed on 4% stacking and 7.5% separating SDS/polyacrylamide gels using Laemmli's buffer system [30] and transferred to Hybond-C nitrocellulose filters (Amersham, Oakville, Ontario, Canada) [31]. Analysis of the effect of chemical, physical and enzymic treatments and analysis of mAb reactivity on purified mucins were carried out by dot-blotting 10 μ g of treated and untreated proteins (positive controls) or 5 μ g of purified mucins on to Hybond-C nitrocellulose membranes.

Immunodetection

Immunodetection was carried out as previously described [21] with 4 μ g/ml purified antibody (mAbs M75, M300, M344 and 49H8), a 20% (v/v) dilution for hybridoma tissue culture supernatant (HMFG-2) or a 1:1000 dilution for ascites (mAb B72.3). The second antibody was 125 I-labelled goat anti-mouse polyclonal antibody [27] (Bio/Can Scientific, Mississauga, Ontario, Canada) for autoradiography or horseradish peroxidase-conjugated goat anti-mouse (Bio/Can Scientific) for enhanced chemiluminescence (ECL) detection.

RESULTS

Epitope coexpression and competition assays

Sandwich assays were carried out on either native or reduced and carboxymethylated T-MGH-U3 proteins using iodinated M344

Table 2 Solid-phase competition assay using M series mAbs and 125 I-labelled M344 mAb

The values are the means $\pm$ S.D. for triplicate determinations. Percentage control binding of 125 I-labelled M344 mAb is determined by comparing c.p.m. obtained in the presence and absence of competing mAb. c.p.m. obtained in the absence of unlabelled competing antibody was set at 100%. mAb 19A211, which is directed against an antigen unrelated to the antigen recognized by M series mAbs, was used as a negative control.

Competing mAb	Amount of competing mAb	Radioactivity (c.p.m.)	Percentage of control binding
M75	None	2047 $\pm$ 127	100
	20-fold excess	1340 $\pm$ 21	65
	100-fold excess	570 $\pm$ 24	28
M300	None	2011 $\pm$ 98	100
	20-fold excess	1129 $\pm$ 9	56
	100-fold excess	777 $\pm$ 40	39
M344	None	2268 $\pm$ 54	100
	20-fold excess	811 $\pm$ 25	36
	100-fold excess	476 $\pm$ 25	21
19A211	None	2299 $\pm$ 59	100
	20-fold excess	2183 $\pm$ 80	95
	100-fold excess	2280 $\pm$ 81	99

mAb as tracer antibody and the three M series mAbs as catcher antibodies. mAb 19A211, which recognizes an unrelated antigen also strongly expressed by T-MGH-U3 cells [23], was used as control. Strong signals were obtained when mAb M344 was used as catcher antibody indicating that the M344 epitope is a repeated rather than unique determinant (Table 1). Strong signals were also obtained when mAbs M300 and M75 were used as catcher antibodies. There was no difference in reactivity between native and reduced and carboxymethylated T-MGH-U3 proteins, confirming that the three epitopes are found on the same molecule. In a solid-phase competition assay, the three mAbs competed almost equally well with mAb M344, indicating that they react with a unique or closely situated epitopes (Table 2).

Physicochemical treatments

The M344, M300 and M75 epitopes were found to be resistant to heat denaturation (100 °C, 15 min). Their reactivity was greatly reduced by treatment with 0.1 M NaOH but it was resistant to 0.1 M HCl after 3 h but not after 24 h of treatment (results not shown). T-MGH-U3 antigen preparations were also treated with various concentrations of NaIO₄. mAb B72.3, against the sialyl-Tn epitope also expressed by T-MGH-U3 cells [21], was used as a control. The reactivity of mAbs M344, M300 and B72.3 was greatly reduced or abolished by 25 mM NaIO₄ whereas mAb M75 reactivity was increased after NaIO₄ treatment (Figure 1).

Protease treatments

T-MGH-U3 antigen preparations at different protein concentrations (1.0, 0.5 and 0.25 mg/ml) were treated with Pronase, papain, trypsin and α -chymotrypsin. The reactivity of the M series mAbs was sensitive to Pronase and papain but was resistant to α -chymotrypsin and trypsin after 3 h and 24 h treatments (results not shown).

Deglycosylation

Reactivity of the three mAbs was reduced by treatment with neuraminidase from *V. cholerae*, which removes $\alpha(2 \rightarrow 3)$,

Table 1 Double-determinant RIA using M series mAbs and 125 I-labelled M344 mAb

The values are the means $\pm$ S.D. for triplicate determinations. mAb 19A211, which is directed against an antigen unrelated to the antigen recognized by M series mAbs, was used as a negative control.

Native T-MGH-U3 proteins		Reduced and carboxymethylated T-MGH-U3 proteins	
Catcher mAb	(c.p.m.)	Catcher mAb	(c.p.m.)
M75	1216 $\pm$ 145	M75	1114 $\pm$ 107
M300	1272 $\pm$ 126	M300	1068 $\pm$ 68
M344	1257 $\pm$ 113	M344	1047 $\pm$ 103
19A211	0 $\pm$ 132	19A211	3 $\pm$ 50

Figure 5 Western-blot analysis of the cryptic expression of $\text{Gal}\beta(1 \rightarrow 3)\text{GalNAc}$ on T-MGH-U3 glycoproteins

mAbs M344 and 49H8 were tested on T-MGH-U3 proteins untreated (lanes 1) and partially desialylated (lanes 2) with neuraminidase. mAb 49H8 specifically recognizes $\text{Gal}\beta(1 \rightarrow 3)\text{GalNAc}$. The positions of the molecular-mass standards (43 to 200 kDa) are indicated. The arrow indicates the interface between the stacking and separating gels (ECL detection).

Similar results were obtained with enzymic O-deglycosylation, which completely abolished reactivity to mAb M75 whereas reactivity to HMFG-2 was increased (Figure 4B, lane 3). As reported in other studies, reactivity of mAb HMFG-2 was increased after desialylation of the proteins [32] whereas the electrophoretic mobility of the mucin decreased, probably because of changes in intrinsic molecular charges, as suggested by other investigators [33,34]. N-Glycosidase F treatment had no effect on the antigenicity of M344 epitope nor did it influence the electrophoretic mobility of the antigen when tested with mAb M344 by Western blot (results not shown).

Analysis of core glycans

The increased reactivity of mAb HMFG-2 after treatment with O-glycosidase indicates that the $\text{Gal}\beta(1 \rightarrow 3)\text{GalNAc}$ disaccharide (T-antigen) is found on T-MGH-U3 glycoproteins. The expression of this disaccharide on desialylated and untreated T-MGH-U3 proteins was analysed with mAb 49H8 in Western blot. Figure 5 shows that the T-antigen is a cryptic epitope since reactivity with mAb 49H8 is detectable only after neuraminidase treatment. The expression of T-antigen on the molecule recognized by the M series mAbs was tested in an heterologous sandwich assay using partially desialylated proteins obtained after a short-period of incubation with neuraminidase and tested with both mAb M75 and the PNA lectin which has affinity for $\text{Gal}\beta(1 \rightarrow 3)\text{GalNAc}$. PNA was unreactive with the untreated antigen but reacted strongly with the partially desialylated antigen captured by mAb M75 (Table 3). Under similar conditions a strong reactivity of PNA was observed with the neuraminidase-treated antigen captured by mAb HMFG-2. As a control, no PNA reactivity was observed with the antigen immunocaptured by mAb 19A211.

Table 3 Identification of cryptic $\text{Gal}\beta(1 \rightarrow 3)\text{GalNAc}$ on mucin antigens expressed by T-MGH-U3 cells as measured by heterologous sandwich assay using ^{125}I -labelled PNA

The values are the means $\pm$ S.D. of triplicate determinations. The data represent c.p.m. obtained after subtraction of the background as determined using no catcher mAb. Neu — represents the untreated T-MGH-U3 proteins and Neu + represents the neuraminidase-treated proteins (partially desialylated). mAb 19A211, which is directed against an antigen unrelated to the antigen recognized by M series mAbs, was used as a negative control.

Catcher mAb	c.p.m.	
	Neu —	Neu +
M75	97 $\pm$ 231	47 438 $\pm$ 1961
HMFG-2	29 $\pm$ 209	53 092 $\pm$ 3117
LDQ10	187 $\pm$ 235	5646 $\pm$ 1503
19A211	13 $\pm$ 228	70 $\pm$ 805

Figure 6 Western-blot analysis of the reactivity of mAbs M344 and M75 after thiol reduction of proteins from T-MGH-U3 cells and from a superficial bladder tumour specimen

Protein samples from a clinical superficial bladder tumour specimen (lanes 1) and from T-MGH-U3 cells (lanes 2) were boiled for 3 min after the addition of an equal volume of Laemmli's loading buffer [30] containing (+) or not containing (—) 5.0% β -mercaptoethanol and subjected to electrophoresis on a 7.5% SDS/polyacrylamide gel. The arrow on the right shows a non-specific signal (approx. 180 kDa) revealed by the secondary antibody alone under reducing conditions only. A similar signal was observed with mAb M344 but is not evident here because results presented are from a shorter exposure time. The arrowheads on the right indicate the molecular mass (54 and 65 kDa) of two molecular species detected with mAb M75 only. The arrow on the left indicates the interface between the stacking and separating gels. The positions of the molecular-mass standards (43 to 200 kDa) are indicated (^{125}I detection).

Effect of thiol reduction and molecular-mass variability of the antigen

The effect of thiol reduction on the electrophoretic mobility of the antigens was analysed on proteins from T-MGH-U3 and from a clinical specimen of papillary bladder tumour known to express both the high-molecular-mass component and the 62 kDa component. The reduced and unreduced antigens were detected with M75, M300, M344 and HMFG-2 mAbs. Figure 6 shows an increase in electrophoretic mobility of the high-molecular-mass components detected with M344 and M75 after reduction with β -mercaptoethanol whereas no change was observed with the mucin-like MUC1 as detected with mAb HMFG-2 (results not

shown). In addition, a slight difference in the electrophoretic mobility of the high-molecular-mass components can be observed between the two protein samples which may suggest a variability in the molecular mass of the antigen between individuals. Such a variability was also observed in a series of tumour samples from several individuals (results not shown). Under non-reducing conditions almost no signal was detected with the clinical bladder tumour sample with either M75 or M344 mAb (Figure 6, lanes 1), suggesting that the native antigen of this sample was too large to enter the stacking gel. The low-molecular-mass component, recognized by mAb M75 but not by mAb M344, was detected only under reducing conditions. In addition a faint 50 kDa species was also detected with mAb M75 but not with mAb M344 under non-reducing conditions. mAb M300 reactivity was similar to that of mAb M344 (results not shown).

Reactivity with purified mucins

As neuraminidase treatment suggests that the three mAbs react with sialylated epitopes, they were tested on BSM, OSM, PSM A⁺ and PSM A⁻ purified mucins expressing sialyl-Tn and some other sialylated oligosaccharides [35]. mAb B72.3 to sialyl-Tn was positive on the four mucins whereas the M series mAbs were negative (results not shown).

DISCUSSION

The results of the present study clearly confirm our original assumption that the determinants recognized by mAbs M344, M300 and M75 are expressed on a mucin. First the antigen was shown to be a protein and not a lipid by its sensitivity to treatments with Pronase, papain and, as previously reported [3], proteinase K. Moreover, the acid-resistance of the antigen and its lack of sensitivity to treatments by trypsin and α -chymotrypsin are characteristics commonly observed with other mucin antigens [10,36,37]. Variability in the electrophoretic mobility of the antigen observed between two distinct tumours in this study and between several clinical tumour samples is another characteristic of mucins. It is in part due to various glycosylation patterns, but also to genetic polymorphism leading to polypeptides with a variable number of tandem repeats [10]. The bladder cancer mucin antigen was also resistant to treatments with heparinase, chondroitinase ABC and hyaluronidase, ruling out the possibility that it is a proteoglycan, even though glycosaminoglycans are major components of the mucus produced by the bladder epithelium [38,39].

The biochemical nature of the epitopes recognized by the M series mAbs was also further defined in the present study. The lack of sensitivity to heat denaturation and the loss of reactivity after alkali treatment suggest carbohydrate epitopes. Furthermore, reactivity of all three mAbs was reduced by neuraminidase digestion, indicating that the epitopes may be sialylated. Such indications are, however, not always definitive, as for example in the case of the DF3 antigen which is sensitive to neuraminidase and yet is a peptide epitope from the tandem repeat of the MUC1 apoprotein [40]. On the other hand, M300 and M344 epitopes were sensitive to periodate oxidation which is consistent with the carbohydrate nature of these determinants. However, the reactivity of mAb M75 was not only maintained but was rather enhanced by this treatment, suggesting that mAb M75 reacts with a cryptic epitope that is unmasked by periodate oxidation. To determine whether the M75 epitope resides in the core protein, chemical deglycosylation with TFMSA and treatment with *O*-glycosidase were performed. No reactivity was observed with mAbs M344 and M300, nor with M75, whereas reactivity with

mAb HMFG-2 to the core protein of the mucin-like MUC1 was enhanced indicating that total or at least partial removal of glycans had been accomplished.

These results suggest that mAb M75 reacts with a partially cryptic-carbohydrate epitope that is unmasked by oxidation of the surrounding glycans. Its reactivity is also sensitive to neuraminidase, indicating that sialic acid is part of the determinant. More than 20 different forms of sialic acid have been identified so far [41]. The most common are *N*-acetylneuraminic acid (NeuAc) or *N*-glycolylneuraminic acid (NeuGc) which may or may not be substituted with acetyl, lactyl, sulphate, phosphate or methyl groups at one or several of the hydroxyl positions. Specific substitutions, such as *O*-acetyl substitution at C-8 or C-9 of sialic acid, or particular types of linkage, as for example α (2 $\rightarrow$ 3) linkage between the sialic acid and internal GalNAc, will confer on these sugars resistance to periodate oxidation [41]. Further analysis of the type of linkage and identification of the form of sialic acid involved in the M75 epitope will be necessary to explain its resistance to periodate oxidation. Based on periodate sensitivity, the M75 epitope appears distinct from the two other epitopes M344 and M300. Similarly, the rare discrepancies reported between tumour reactivity of mAb M344 and M300 suggest that these two epitopes are not identical [3], although they are clearly closely associated, since all three mAbs compete equally for the binding of iodinated M344 mAb.

Several cancer mucin antigens were found on closer examination to be sialylated blood group determinants. Results of the initial study on the M series mAbs ruled out their reactivity with blood group A, B, H, I, Le^a, Le^b, X and Y antigens [3]. More recently, no reactivity was found in inhibition assays with synthetic oligosaccharides S-T and S-Tn (M. Reddish, personal communication). In the present study, the M series mAbs were unreactive with three purified submaxillary mucins (OSM, BSM and PSM). However, in a recent immunohistochemical study, mAb M344 was shown to react with porcine submaxillary glands but not with human, bovine or ovine submaxillary tissues [42]. The discrepancy between this result and the lack of reactivity of mAbs M344, M300 and M75 with PSM expressing either blood group A⁺ or A⁻ in our analysis remains unexplained. The identification of the sialyl carbohydrate epitopes recognized by the M series mAbs will require further characterization of the glycan chains.

Whereas the M series mAbs do not react with sialylated blood group determinants, the bladder cancer mucin immunocaptured by mAb M75 expressed the sialyl-T antigen, which is also expressed on MUC1 in the same tumour cells. Sialyl-Tn was also shown in a separate study to be expressed on both MUC1 and the bladder cancer mucin [21]. The expression of at least two different types of *O*-glycans on the bladder cancer mucin and the lack of sensitivity of the M344 epitope to *N*-glycosidase F treatment indicate that the antigen is *O*-glycosylated, providing additional indications that it is a mucin. Ongoing experiments aimed at the purification of the antigen have also shown that in caesium chloride gradient it has, like most mucins, a density of about 1.4 g/ml (S. Champetier, personal communication). Although sharing similar glycan chains with the mucin-like MUC1, the bladder cancer mucin is a typical secreted mucin. The change in the electrophoretic mobility of the antigen after reduction of disulphide bonds indicates an oligomeric nature. In contrast, the mucin-like MUC1 is membrane bound and is monomeric as it lacks domains rich in cysteine residues necessary for the formation of the disulphide links [12].

The detection of a 62 kDa component by mAb M75 raises interesting questions. It was detected in clinical tumour samples under reducing conditions only, suggesting that in its native form

this glycoprotein may be closely associated with the high-molecular-mass component detected by the M series mAbs. Similar low-molecular-mass components associated with mucins and detected only under reducing conditions have previously been observed during the purification of porcine gastric [43], canine tracheobronchial [44] as well as human intestinal [45] and tracheobronchial [46] mucins. These components were described as putative 'link' glycoproteins by some investigators on the assumption that they were involved in disulphide-linked oligomerization of mucins [45]. Although still controversial, the existence of link glycoproteins has been further supported by the demonstration that a 118 kDa link glycoprotein from rat intestinal mucin is the proteolytic fragment of a rat MUC2 mucin homologue [47]. Recently, dimerization of MUC2 [48] was shown to be analogous to that of the von Willebrand factor to which the N- and C-terminal regions of MUC2 show some similarity [49,50]. von Willebrand factor has been shown to undergo proteolytic cleavage during its biosynthesis to yield a pre-propeptide, which in turn seems to play an important role in the oligomerization process [51]. Whether the 62 kDa molecular species recognized by mAb M75 is a putative link glycoprotein will require further molecular characterization.

In summary, the M series mAbs identify sialylated carbohydrate epitopes which remain undefined. These determinants are expressed on an antigen that we demonstrated to be a mucin presenting structural features of typical mucins with a glycosylation pattern similar to that of the mucin-like MUC1. We thus named this antigen MAUB for mucin antigen of the urinary bladder. Data published elsewhere have shown that MAUB is distinct from MUC1 and MUC2 and that the MGH-U3 tumour cell system does not express MUC3, MUC4, MUC5AC, MUC5B and MUC6 mRNA [21].

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