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MAUB Is a New Mucin Antigen Associated with Bladder Cancer*

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The M344 tumor-associated antigen, expressed in 70% of superficial bladder tumors, is a sialylated carbohydrate present on a high molecular mass thiol-reducible secreted mucin, which we named MAUB for mucin antigen of the urinary bladder. Herein we studied the relationship between MAUB and other known mucins in the MGH-U3 bladder cancer line where MAUB expression is modulated by culture conditions. Northern blots, immunoradiometric assays, and Western blots showed that only MUC1 and MUC2 are expressed in this MAUB-positive cell line. MUC1 differs from MAUB by its molecular mass and its non-oligomeric nature, while MUC2 has similar molecular mass and response to culture conditions. However, in double determinant immunoradiometric assays, MAUB and MUC2 did not cross-react. Moreover, confocal microscopy showed different subcellular localization of the two antigens. Treatment of MGH-U3 cells with MUC2 antisense oligodeoxynucleotides resulted in decreased expression of MUC2 and increased expression of MAUB, ruling out the possibility that monoclonal antibody M344 recognizes a different glycosylated form of MUC2. In addition, we identified a tumor specimen expressing MAUB but no MUC2 antigen or mRNA. Together, these results suggest that there is expression of at least three mucins in MGH-U3 cells and that MAUB is a cancer-associated mucin distinct from those identified so far.

Mucins are the major macromolecules of the mucus produced by epithelial or glandular cells. They are highly heterogeneous membrane-bound or secreted oligomeric or non-oligomeric molecules characterized by high molecular masses ranging from 200 to many thousands of kDa (1–4). These proteins are heavily *O*-glycosylated through serine and/or threonine residues, which account for 30–40% of the total amino acid composition of their protein backbone. The diversity of the carbohydrate chains has been shown to contribute largely to the heterogeneity observed in mucins. However, molecular cloning of cDNAs encoding mucins has revealed another level of heterogeneity and a higher degree of complexity. Up to now at least eight human mucin genes have been identified, namely MUC1 (5, 6), MUC2 (7, 8), MUC3 (9), MUC4 (10), MUC5B-MUC5AC (11), MUC6 (12), and MUC7 (13). They are characterized by variable numbers of tandemly repeated sequences, the exact number of repetitions differing from one individual to another (14). The growing number of identified genes, the genetic polymorphism due to the variable number of tandem repeats, along with the unpredictable number of glycoforms of these proteins explain their marked heterogeneity and polymorphism.

Mucins have long been known to play an important role in the physiopathology of a number of diseases (1, 3). However, the recent finding of aberrant expression of mucin gene products in cancers has stimulated a rapidly growing interest in them. Because of their unique features, cancer mucin antigens are ideally suited as tumor markers for cancer diagnosis and prognosis, and also as immunostimulants potentially useful in the design of cancer vaccines. Numerous tumor-associated antigens defined by mAbs[1] were found to be expressed on mucins (15–24) and many of them are already clinically useful (25, 26). Although mucins are natural components of normal epithelial tissues, their abnormal expression in cancers may be due either to aberrant regulation of mucin gene expression or aberrant glycosylation of the gene products. The heterogeneity of mucin gene expression has been studied in several cancer types (27–31). Mucin genes may be up- or down-regulated in cancers originating from tissues where they are constitutively expressed, or they may be ectopically expressed in cancers derived from tissues that do not normally express them. Aberrant glycosylation, on the other hand, is often responsible for the appearance of cancer determinants identified by mAbs (32–37). The abnormal glycosylation process in cancer cells leads in most instances to a shortening of the glycan chains, exposing new carbohydrate epitopes and enhancing the accessibility to some protein epitopes. To date, most mAbs reactive with tumor-associated mucins identify protein epitopes of MUC1 product or carbohydrate epitopes found on multiple mucins.

We have identified a tumor-associated antigen of human superficial bladder tumors using a series of mAbs of which mAb M344 was the prototype used in several clinical studies (38–40). The antigen is expressed in 70% of papillary bladder tumors, the most common form of bladder cancer (41, 42), but not in normal bladder urothelial cells or other normal adult or fetal tissues (38). The M344 antigen has also been identified on a small subset of adenocarcinomas of various origins (43). The measure of this antigen provides a basis for promising diagnostic and prognostic tests for the management of bladder cancer (40, 44, 45). Biochemical studies have shown that mAb M344 reacts with a sialylated carbohydrate epitope expressed on a very high molecular mass protein.[2] Several other characteristics of the antigen such as the molecular mass variability between individuals, the decrease of the apparent molecular

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[1] The abbreviations used are: mAb, monoclonal antibody; MAUB, mucin antigen of the urinary bladder; M-MGH-U3, MGH-U3 cells cultured as monolayer; T-MGH-U3, MGH-U3 cells grown as nude mouse tumors; RIA, radioimmunoassay; ELISA, enzyme-linked immunosorbent assay; GAM, goat anti-mouse; TBS, Tris-buffered saline; PBS, phosphate-buffered saline; BSA, bovine serum albumin; ECL, enhanced chemiluminescence; oligo, oligonucleotide.

[2] A. Bergeron, H. LaRue, and Y. Fradet, manuscript in preparation.

mass upon thiol group reduction, the association of the antigen with cytoplasmic vacuoles and its secretion by tumor cells all indicate that the M344 antigen belongs to the mucin family.[3,4] We named this antigen MAUB for mucin antigen of the urinary bladder. It is not known at this time whether MAUB is a new mucin or a product of a known mucin gene.

Little is known on the expression of mucin genes in normal bladder and bladder cancer. However, reports based on immunohistology studies with mAbs have suggested the expression of at least the MUC1 and MUC2 mucins. MUC1 epitopes were found expressed in the superficial layer of normal urothelium and at higher frequency in the most aggressive forms of bladder cancer (46–49). On the other hand, MUC2 protein epitopes were not expressed in normal bladder but were expressed in approximately 40% of bladder cancers of all types in the only study reported (50).

The objective of the present study was to establish the relationship between MAUB and the other known MUC gene products. We took advantage of the characteristics of MAUB expression in the human bladder cancer line MGH-U3 established from a low grade papillary bladder tumor. In this cellular system, MAUB is expressed at very high levels when MGH-U3 cells are grown as nude mice xenografts but is not expressed on the cells grown as monolayer *in vitro*. We report here the coexpression of at least three mucins in this bladder cancer line and that MAUB is distinct from those mucins already identified.

EXPERIMENTAL PROCEDURES

Cell Culture Conditions and Tissues

The human bladder cancer MGH-U3 cells were cultured as monolayer (M-MGH-U3) in minimum essential medium (Life Technologies, Inc.) supplemented with penicillin/streptomycin and 7.5% fetal calf serum. Nude mice MGH-U3 tumors (T-MGH-U3) were obtained by subcutaneous injection of 10^7 M-MGH-U3 cells in four sites to 6-week-old female nude mice (Charles River Canada, St-Constant, Québec). Three weeks later, mice were sacrificed and tumors were harvested under sterile conditions. Tumor cells were dispersed on a metal grid and thus prepared as a monocellular suspension. Capan-1, LS180, and MCF-7 cell lines from human pancreatic, colonic, and breast cancers, respectively, were used as control cell lines for the expression of some mucin genes. These cells were cultured in monolayer in conditions similar to those of MGH-U3 cells. LS180 nude mouse xenografts were obtained as were MGH-U3 xenografts. Samples of superficial papillary bladder tumors along with normal stomach tissue, used as a positive control for the expression of some mucin genes, were obtained from the Pathology Department of the Hôtel-Dieu de Québec. Cells from the bladder tumor samples were obtained after processing the tumors as for nude mouse tumors.

Protein Extractions

Proteins of the different cell preparations were solubilized with 0.25% *N*-dodecyl β-D-maltoside detergent (Calbiochem) in 50 mM Tris-HCl, 150 mM NaCl, pH 8.0, added with 2 mM phenylmethylsulfonyl fluoride (Sigma) in the proportion of 1 ml/10^7 cells. Extractions were carried out at 4 °C for 1 h, with gentle mixing. Solubilized proteins were collected after a 45-min centrifugation at 100,000 × *g*. Protein concentration was determined by the Bradford assay (51).

Antibodies

mAb M344 was obtained by simultaneous immunization with superficial papillary bladder tumor cells and mouse polyclonal serum against normal urothelium (38). mAb 19A211 defines another superficial papillary bladder tumor-associated sialylated carbohydrate antigen expressed in T-MGH-U3 but not in M-MGH-U3 (52). These two IgG_1 mAbs were purified from ascites by caprylic acid and ammonium sulfate precipitations (53) or by fast preparative liquid chromatography using a Mono-Q column. The following mAbs directed against carcinoma-associated mucins were used in this study. HMFG-2 (54) and DF3 (23) mAbs, which are directed against the core protein of the polymorphic epithelial mucin MUC1 were provided, respectively, by Dr. J. Taylor-Papadimitriou (Imperial Cancer Research Fund, London, United Kingdom) and by Dr. D. W. Kufe (Dana-Farber Cancer Institute, Boston, MA). DUPAN-2 mAb, which is directed against a carbohydrate epitope found on the MUC1 pancreatic product (55), and B72.3 mAb, which recognizes the sialyl-Tn antigen (15, 56), were, respectively, provided by Dr. R. S. Metzgar (Duke University Medical Center, Durham, NC) and by Dr. J. Schlom (National Institutes of Health, Bethesda, MD). AR-3 and BD-5 mAbs (21, 22) reactive with gastric and pancreatic mucins were provided by Dr. M. Prat (Universita di Torino, Italy). MOV-2 mAb, which is directed against the $Lewis^a$ hapten (57), was provided by Dr. M. I. Colnaghi (Institute Nazionale per lo Studio e la Cura dei Tumori, Milan, Italy). LDQ10 mAb, which recognizes an epitope found in the core protein of MUC2 (58), and 49H8 mAb, which is directed against the Thomsen-Friedenreich (T or TF) hapten (24), were, respectively, provided by Dr. F. X. Real (Institut Municipal d'Investigatió Mèdica, Barcelona) and by Dr. B. M. Longenecker (BioMira, University of Alberta, Edmonton). PD41 mAb, which was provided by Dr. G. L. Wright (Eastern Virginia Medical School, Norfolk), recognizes a mucin antigen preferentially expressed on prostate carcinomas (59). In the different assays, purified antibodies (M344, 49H8, and PD41) were used at a concentration of 4 μg/ml, while ascites (B72.3, MOV-2, and LDQ10) were used at a dilution of 1:1000. Hybridoma tissue culture supernatants (HMFG-2, DF3, DUPAN-2, AR-3, and BD-5) were used undiluted for immunoassays but diluted to 20% (v/v) for Western blot analyses.

For some immunoassays, M344 mAb and a goat anti-mouse (GAM) polyclonal antibody (Bio/Can Scientific, Missaussauga, Ontario, Canada) were labeled with ^{125}I according to the IODOGEN method (60). For double labeling immunofluorescence assay, mAb M344 was labeled with biotin as follows. Briefly, 200 μl of biotinamidocaproate *N*-hydroxysuccinimide ester (Sigma) dissolved in *N*-dimethylformamide at a concentration of 2 mg/ml was added dropwise to 10 ml of a 1 mg/ml solution of purified M344 mAb in 0.2 M bicarbonate buffer, pH 8.8, containing 0.15 M NaCl. After 15 min of agitation at room temperature, the solution was dialyzed against 0.1 M sodium phosphate buffer, pH 7.4.

Western Blot Analyses

50 μg of solubilized proteins were resolved on 7.5% SDS-polyacrylamide gels according to the Laemmli's buffer system (61) and transferred onto Hybond C nitrocellulose filters (Amersham, Oakville, Ontario, Canada) in a Hoeffer apparatus at 500 mA overnight (62). The filters were blocked with 5% powdered skim milk in TBS (50 mM Tris-HCl, pH 8.0, 150 mM NaCl) and incubated for 3 h at 37 °C with first antibody diluted in 1% powdered skim milk in TBS. The filters were washed with several changes of TBS and then incubated with the second antibody. For radioactive detection, ^{125}I-labeled GAM polyclonal antibody was used at a concentration of 5×10^5 cpm/ml in 1% powdered skim milk in TBS for 1 h at room temperature. The nitrocellulose filters were finally washed in TBS and then autoradiographed. For the enhanced chemiluminescence (ECL) detection, horseradish peroxidase-conjugated GAM polyclonal antibody (Bio/Can Scientific) was used at the manufacturer's recommended dilution in 1% powdered skim milk in TBS for 1 h at 37 °C. Filters were washed with TBS, and then detection of bound antibodies was performed using ECL Western blotting detection reagents from Amersham.

Immunoassays

For indirect radioimmunoassays (RIA), each well of Falcon polyvinyl chloride microwell plates (Fisher Scientific, Montréal, Québec) was coated with 5 μg of solubilized proteins by complete drying overnight at 37 °C. Plates were washed with TBS and then blocked with 5% bovine serum albumin (BSA) in TBS for 1 h at 37 °C. Incubations were performed for 3 h at 37 °C with antibodies diluted in 1% BSA-TBS. Plates were washed several times with TBS and then incubated for 1 h at room temperature with ^{125}I-labeled GAM polyclonal antibody at a concentration of 2×10^5 cpm/ml in 1% BSA-TBS (50 μl/well). Plates were washed again several times with TBS, and then each well was counted.

For enzyme-linked immunosorbent assay (ELISA), each well of MaxiSorb immunoplates (Nunc, Life Technologies, Inc.) was coated with 5 μg of solubilized proteins, blocked, and then incubated with first antibody as described for RIA. After several washes in TBS plates were incubated at room temperature for 1 h with alkaline phosphatase-conjugated GAM (Bio/Can Scientific) diluted at the manufacturer's

[3] Y. Fradet, R. Pankov, A. Bergeron, and C. Parent-Vaugeois, manuscript in preparation.

[4] H. LaRue, C. Parent-Vaugeois, A. Bergeron, S. Champetier, and Y. Fradet, manuscript in preparation.

recommended dilution in 1% BSA-TBS. Plates were washed and then incubated for 30 min in presence of p-nitrophenyl phosphate (Sigma) at a concentration of 0.5 mg/ml in 1 M diethanolamine, 0.5 mM $MgCl_2$, pH 9.8. After stopping the reaction by adding an equal volume of 0.1 N NaOH, the plates were read at 405 nm on an automatic plate reader.

For double determinant immunoradiometric assays, two different techniques were used. The first was a sandwich assay in which each well of polyvinyl chloride microwell plates was coated with 1 μg of GAM polyclonal antibody as described above. After blocking the unreacted binding sites with 5% BSA in TBS, calculated amounts of antibody solutions were added in order to capture an equivalent amount of antibodies (≈50 ng) in each well. This incubation was performed at 4 °C overnight. Plates were washed extensively in TBS, and then 5 μg of the appropriate antigen was added to each well. The capture of the antigen was allowed to proceed at 37 °C for 3 h. Plates were washed several times in TBS, and then available GAM paratopes were blocked with a solution of 0.01% purified normal mouse immunoglobulins (Bio/Can Scientific) in TBS for 5 h at 37 °C in a humid chamber. After this blocking step, 2×10^5 cpm of ^{125}I-labeled M344 mAb was added to each well and the plates were incubated for 1 h at room temperature. After extensive washing with TBS, each well was counted. The second technique was a slightly modified version of that described by Würzner *et al.* (63). It is similar to the first except that the captured antigens were detected using ^{125}I-labeled immune complexes instead of using radiolabeled mAbs. Immune complexes were obtained by incubating optimal amounts of mAbs in microtubes with ^{125}I-labeled GAM polyclonal antibody (2×10^5 cpm/50 μl) for 30 min at 37 °C. Normal mouse immunoglobulins were then added to a final concentration of 0.002% and incubated for an additional 30 min. The immune complexes were added to captured antigen in wells and the plates incubated for 1 h at 37 °C. After several washes in TBS, each well was counted.

Confocal Fluorescence Microscopy

Indirect Immunofluorescence—T-MGH-U3 cells grown on fibronectin-coated cover glasses were fixed in acetone:methanol (1:1) and washed twice with PBS (130 mM NaCl, 9.42 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4, pH 7.4). After these washes, cells were incubated with first antibody (hybridoma tissue culture supernatant (M344) or ascites fluid diluted 1:1000 in Iscove's modified Dulbecco's medium supplemented with 7.5% of goat serum (LDQ10)) for 1 h in a humid chamber. After four washes in PBS, 0.05% Tween 20, cells were overlaid with fluorescein-conjugated GAM polyclonal antibody (Bio/Can Scientific) and incubated for 30 min in the dark. Cells were washed four times with PBS, 0.05% Tween 20 and then immediately mounted with SlowFade (Molecular Probes, Eugene, OR) for examination or treated for double labeling.

Double Labeling—GAM polyclonal antibody residual binding sites were blocked by incubating the indirectly fluorescein-labeled cells with a solution of 0.01% purified normal mouse immunoglobulins for 90 min. Cells were washed four times with PBS, 0.05% Tween 20 and then incubated for 1 h with biotinylated M344 mAb. After four washes with PBS, 0.05% Tween 20, cells were incubated with Texas Red-labeled streptavidin (Calbiochem) for 30 min. Cells were finally washed four times with PBS, 0.05% Tween 20 and mounted on slides as described above. All steps were carried out at room temperature.

Specimens were analyzed with a Bio-Rad MRC-600 confocal imaging system mounted on a Nikon Diaphot-TDM. A 60× objective lens with a 1.5 numerical aperture was used. Confocal settings were as follows: 0.3-milliwatt laser power, 1.5 zoom, 1 s/scan kalman filter, and six frames/image. The photomultiplier gain was set at maximum, and the confocal aperture was adjusted to obtain maximum resolution.

Oligodeoxynucleotides (Oligos) and cDNA Probe

Oligos corresponding to partial tandemly repeated sequences of all mucin cDNAs (except MUC2) were synthesized by Bio/Can Scientific. The antisense oligo sequences were 5′-CGAGGTGACACCGTGGGCTGG-3′ for MUC1 (6), 5′-AGAAGTGAAGCTGGGAGTACTGTG-3′ for MUC3 (9), 5′-GGTGACAGGAAGAGGGG-3′ for MUC4 (10), 5′-TGTGGTCAGCTCTGTGAGGATCCA-3′ for MUC5B (64), 5′-AGGGGCAGAAGTTGTGCTCGTTGT-3′ for MUC5AC (64), and 5′-GGTTGGATAGGTAGTGGTGGT-3′ for MUC6 (12). The sense oligo sequence for MUC1 was 5′-CCAGCCCACGGTGTCACCTCG-3′. Purity was confirmed by polyacrylamide gel electrophoresis.

Oligos were end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase (Life Technologies, Inc.) according to the manufacturer's protocol. A 0.8-kilobase pair partial MUC2 cDNA insert from plasmid SMUC41, kindly provided by Young S. Kim (University of California, San Francisco), was labeled by random priming using [α-^{32}P]dCTP and Klenow enzyme (Life Technologies, Inc.) (65). Specific activities of labeled probes ranged from 10^8 to 10^9 cpm/μg.

Northern Blot Analyses

RNA analyses were based on standard protocols for hybridization with either labeled oligos (66) or cDNAs (67). Brifely, total RNA was isolated as described by Chomczynski and Sacchi (68). 10 μg of total RNA were size-fractionated by electrophoresis on a formaldehyde, 1% agarose gel as described elsewhere (67). The quality and relative amounts of RNA were assessed by ethidium bromide staining. RNA was transferred via capillary blotting to Hybond-C-extra nitrocellulose membranes (Amersham), which were thereafter baked for 1–2 h at 80 °C under vacuum, prior to prehybridization.

For analyses with oligo probes, membranes were prehybridized at either 42 °C (MUC1, MUC3, MUC5B, MUC5C, and MUC6) or 37 °C (MUC4) for 2–4 h in a solution containing 6 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 5 × Denhardt's reagent (1 × Denhardt's reagent is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% BSA), 0.05% sodium pyrophosphate, 100 μg/ml denaturated salmon sperm DNA, and 100 μg/ml yeast tRNA. The probe was then added ($1–4 \times 10^6$ cpm/ml), and hybridization was carried out overnight under the same conditions. Membranes were washed at room temperature for 20 min in 3 × SSC, 0.05% sodium pyrophosphate, then washed twice for 2 min in the same solution, at 50 °C for MUC4, at 55 °C for MUC3, MUC5B, MUC5C, and MUC6, or at 60 °C for MUC1, before autoradiography.

For the analysis of MUC2, the membrane was prehybridized at 42 °C for 2 h in a solution consisting of 50% formamide, 5 × SSC, 5 × Denhardt's reagent, 0.1% SDS, and 100 μg/ml denatured salmon sperm DNA. The cDNA probe was added ($1–4 \times 10^6$ cpm/ml), and incubation was continued overnight. The membrane was washed at room temperature for 20 min in a 1 × SSC, 0.1% SDS solution, and then three times at 68 °C for 20 min in the same solution and subjected to autoradiography.

Inhibition of Protein Expression with Oligos

T-MGH-U3 cells freshly harvested from nude mice under sterile conditions were cultured in 24-well tissue culture plates (Nunc, Life Technologies, Inc.) until they reached 50–75% confluence. Subconfluent cells were cultured for 72 h in presence of 25 or 50 μg/ml MUC1 sense, MUC1 antisense or MUC2 antisense oligos diluted in minimal essential medium supplemented with 7.5% of heat-treated fetal calf serum (65 °C for 30 min) and antibiotics. Cells were trypsinized and washed in PBS. Antigen expression was analyzed by ELISA on protein extracts.

RESULTS

Expression of Mucin Gene mRNA in MGH-U3 Bladder Cancer Cells—The MGH-U3 superficial bladder cancer cell line strongly expresses MAUB when grown as a tumor (T-MGH-U3) in nude mice, but not when cultured as monolayer (M-MGH-U3) *in vitro*.[4] To determine which mucins are expressed in the MGH-U3 cells, we first analyzed by Northern blot the levels of MUC1, MUC2, MUC3, MUC4, MUC5AC, MUC5B, and MUC6 mRNAs in M-MGH-U3 and T-MGH-U3 cells (Fig. 1). Total RNA extracted from these cells was probed with oligos corresponding to antisense sequences of the tandem repeats of the different mucins (MUC1, MUC3, MUC4, MUC5AC, MUC5B, and MUC6) or tested with a cDNA probe corresponding to a fragment containing several tandem repeats (MUC2). MUC1 mRNA was present in both M-MGH-U3 and T-MGH-U3 cells. The MUC1 probe revealed two bands of about 4.7 and 6.4 kilobases in both MGH-U3 cells and the control MCF7 cells. MUC2 mRNA was also present at high level in the T-MGH-U3 but not in the M-MGH-U3 cells. The MUC2 probe revealed a polydisperse signal similar to that previously reported (7) and similar to that observed with RNA from nude mouse xenografts of LS180 cells. MUC3, MUC4, MUC5AC, MUC5B, and MUC6 mRNAs were not detected in M-MGH-U3 or in T-MGH-U3 cells, while positive controls showed the characteristic polydisperse signals usually observed for these mucin mRNAs (31). Thus, of the seven mucin genes tested, only MUC1 and MUC2 mRNAs were detectable in the MGH-U3 bladder cancer cells.

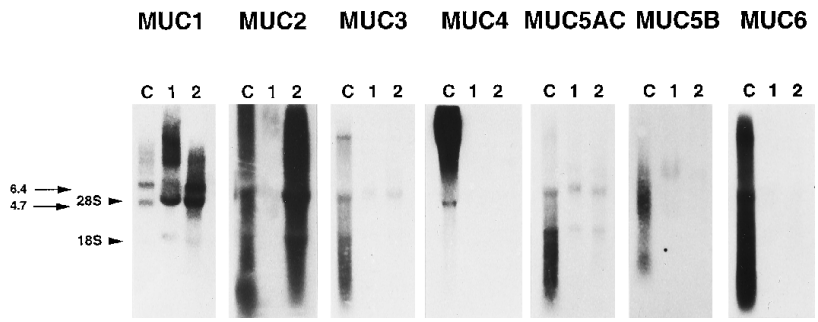

FIG. 1. **Northern blot analysis of the levels of seven mucin mRNAs in the MGH-U3 cellular system.** Total RNA (10 μg/lane) from M-MGH-U3 cells (*lane 1*) and T-MGH-U3 cells (*lane 2*) have been tested for mucin gene expression with MUC1, MUC3, MUC4, MUC5AC, MUC5B, and MUC6 oligo probes or with a MUC2 cDNA probe. Controls (*lanes C*) were total RNA from MCF-7 cells (MUC1), CAPAN-1 cells (MUC4), nude mouse xenografts of LS180 cells (MUC2), and from normal stomach mucosa (MUC3, MUC5AC, MUC5B, and MUC6).

Expression of Tumor-associated Mucin Antigens—The expression of carcinoma-associated epitopes commonly found on mucin or mucin-like glycoproteins was tested by indirect RIA on both M-MGH-U3 and T-MGH-U3 protein extracts using mAbs directed against carbohydrate or protein epitopes of MUC1 and MUC2 mucins, as well as other mucin antigens (Table I). MAUB was strongly expressed on T-MGH-U3 but not on M-MGH-U3. The two mAbs to protein epitopes of MUC1, DF3 and HMFG-2, both reacted with T-MGH-U3, but only DF3 was also reactive with M-MGH-U3. Consistent with the data obtained by Northern blot analysis, the LDQ10 mAb to a protein epitope of the MUC2 gene product reacted strongly with T-MGH-U3, but remained negative with M-MGH-U3 protein extracts. Sialyl-Tn was the only of the five carbohydrate determinants studied that was expressed by MGH-U3 cells. As for MAUB, this antigen was detected on T-MGH-U3 but not in M-MGH-U3 cells grown *in vitro*. mAbs to mucin antigens of prostate cancer (PD41) and gastric cancer (BD5) were unreactive. mAb 19A211 identifies a tumor-associated antigen of superficial bladder tumors distinct from MAUB and also expressed in T-MGH-U3 but not in M-MGH-U3 cells (52). The 19A211 epitope is not expressed on mucins and thus mAb 19A211 was used as a control in the various immunoassays.

Western Blot Analysis—mAbs reactive with MGH-U3 protein extracts in RIA were tested on M-MGH-U3 and T-MGH-U3 protein extracts in Western blot for band pattern comparison (Fig. 2). The two mAbs to MUC1 apoprotein, DF3 and HMFG-2, revealed two proteins of high molecular mass in T-MGH-U3, which were also observed in M-MGH-U3 with mAb DF3. mAb LDQ10 to MUC2 apoprotein and mAb M344 to MAUB both revealed molecular species of much higher molecular mass not migrating further than the interface between the running and stacking gels. Reactivity with M344 and LDQ10 mAbs was limited to T-MGH-U3, and even after long exposure no reactivity was observed with M-MGH-U3, in contrast to HMFG-2, which then showed a faint reactivity (data not shown). mAb B72.3 reacted with T-MGH-U3 but not with M-MGH-U3 protein extracts and revealed three molecular species: one co-migrating with the protein revealed by M344 and LDQ10 mAbs, and two other proteins co-migrating with those revealed by MUC1 mAbs. These results suggest that in MGH-U3 bladder cancer cells sialyl-Tn is expressed on MUC1 and on MUC2 and/or MAUB. Moreover, mAb M344 does not appear to react with MUC1 mucin, but its pattern of reactivity is similar to that of mAb LDQ10 to MUC2 mucin.

Double Determinant Analysis—The relationship between MAUB and MUC1 and MUC2 mucins was further examined by double determinant immunoradiometric assays. In a first series of experiments, antigens from T-MGH-U3 protein extracts were immunocaptured by mAbs M344, B72.3, HMFG-2, LDQ10, and 19A211 and tested in direct immunoassay with radiolabeled M344 mAb. Similar tests performed on protein extracts from M-MGH-U3 cells were used as negative control. As shown in Table II, mAb M344 reacted strongly with antigen

TABLE I
Expression of some carcinoma-associated mucin antigens in MGH-U3 cells as determined by indirect RIA

mAbs	Epitope	counts/min	
		M-MGH-U3	T-MGH-U3
MAUB (M344)	ND[a]	0	4263
MUC1 apoprotein			
HMFG-2	DTR (72)	32	1238
DF3	TRPAPGS (84)	987	3821
MUC2 apoprotein (LDQ10)	PTGT (85)	0	2088
Carbohydrate determinants			
B72.3	Sialyl-Tn (56)	0	3609
MOV-2	Lewis[a] (57)	0	0
DU-PAN-2	LsTa (86)	0	0
49H8	T (24)	0	0
AR3	ND	0	0
Other mucin antigens			
PD41	ND	0	0
BD5	ND	0	0
Control mAb (19A211)	ND	0	3917

[a] ND, unknown.

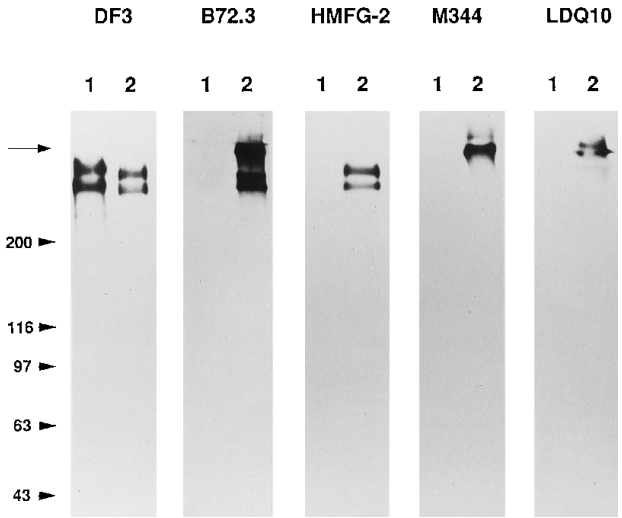

FIG. 2. **Western blot analysis of the expression of some carcinoma-associated mucins in the MGH-U3 cellular system.** M-MGH-U3 (*lane 1*) and T-MGH-U3 proteins (*lane 2*) (50 μg/lane) were analyzed with mAbs DF3, HMFG-2, B72.3, M344, and LDQ10 for band pattern comparison. The *arrow* represents the interface between the stacking and running gels (^{125}I detection).

captured by mAb M344 and 11-fold less with antigen captured by mAb B72.3. No reactivity (above that observed with antigen captured by the negative control mAb 19A211) was observed with antigens captured by mAbs to MUC1 and MUC2 mucins. In the second set of experiments, antigens immunocaptured by the same five mAbs were tested in indirect immunoassay with each mAb used for capture (Table III). The results were similar to those in Table II when mAb M344 was tested. mAb B72.3 reacted with MUC1 mucin captured by mAb HMFG-2 and at a lower level with antigen captured by mAb M344, but not with MUC2 mucin captured by mAb LDQ10. On the other hand, mAb LDQ10 showed no reactivity with antigen captured by mAbs M344, B72.3, or HMFG-2. These results showing a lack of cross-reactivity between mAb M344 and mAbs to MUC1 and MUC2 mucins suggest that MAUB is a distinct mucin.

Confocal Immunofluoresence Microscopy—In order to better define the pattern of expression of MAUB and MUC2 mucin in T-MGH-U3 cells, a double labeling immunofluorescence assay was performed using LDQ10 mAb detected by a fluorescein-labeled GAM polyclonal antibody and biotinylated M344 mAb detected with Texas Red-labeled streptavidin reagent. The reactivity patterns of positive cells was analyzed with the high resolution power of confocal microscopy. About 10% of T-MGH-U3 cells reacted strongly with one or both mAbs. Most commonly, all positive cells expressed both antigens but the relative amount of each antigen in the positive cells varied greatly from one cell to another (Fig. 3). The reactivity was always observed in the cytoplasm and was either diffuse or patchy. No reactivity was found with unfixed cells. When the two fluorescent signals were simultaneously visualized, heterogeneous reactivity patterns were observed. Colocalization of both antigens was not observed in any cell group analyzed. These results obtained by double-labeling immunofluorescence assay are in agreement with the results previously obtained by double determinant analyses and suggest that the M344 and LDQ10 epitopes are not coexpressed on the same molecule.

Inhibition of Mucin Gene Expression with Antisense Oligos—While MAUB and MUC1 mucins can be easily distinguished, the above results do not conclusively demonstrate that MAUB and MUC2 mucins are two distinct molecules. Indeed, since mAb LDQ10 recognizes a protein epitope and mAb M344 a carbohydrate epitope, it is possible that both mAbs recognize different glycosylated forms of a unique mucin on which the accessibility of the protein epitopes may be modulated by the level of glycosylation. To test this possibility, an experiment was designed to inhibit the expression of MUC2 gene and assess its effect on MAUB expression. Cells from freshly excised MGH-U3 xenografts were cultured on plastic and treated with 25 and 50 μg of MUC2 antisense oligo for 72 h. Control cell populations were treated with the same amount of MUC1 antisense oligo and with the same amount of an unrelated oligo, the MUC1 sense oligo. Neither the MUC1 sense nor the MUC1 antisense oligos had any significant effect on the expression of either M344 or LDQ10 antigens (Fig. 4). However, when T-MGH-U3 cells were treated with increasing amounts of the MUC2 antisense oligo, a significant dose-related decrease ($p = 0.05$) in the reactivity of mAb LDQ10 as well as a significant dose-related increase ($p = 0.03$) of the expression of M344 antigen was observed. Statistics were obtained from analysis of variance between the three doses using the Kruskal-Wallis test. These results suggest that the M344 carbohydrate epitope is found on a mucin different from the MUC2 mucin.

Tumor Sample Analysis—Several superficial papillary bladder tumors were analyzed by Western blotting to determine the expression of the two mucins in clinical samples. One of these tumor specimens reacted with mAb M344 but not with mAb LDQ10 (Fig. 5). Since protein epitopes are frequently masked on certain mucin glycoforms, the expression of the MUC2 mucin was also assessed at the transcriptional level by Northern blot analysis. The characteristic polydisperse signal of MUC2 was only detected with RNA isolated from T-MGH-U3 cells and not with RNA isolated from the tumor specimen, indicating that no MUC2 mucin is expressed in this tumor sample. This result also indicates that MAUB is different from the MUC2 mucin.

TABLE II
M344 epitope expression on carcinoma-associated mucin antigens expressed in T-MGH-U3 cells as determined by double determinant analysis with ^{125}I-labeled M344 mAb

Catcher mAb[a]	Bound counts/min
M344	9272[b]
B72.3	811
HMFG-2	40
LDQ10	28
19A211[c]	148

[a] Catcher mAb is the coated antibody (via GAM) and tracer mAb is ^{125}I-labeled M344 mAb.
[b] The data represent the number of counts/min after subtraction of the background obtained using M-MGH-U3 as the source of antigen.
[c] MAb 19A211 is not directed against a carcinoma-associated mucin but is used here as a negative control.

DISCUSSION

Superficial papillary tumors are the most common form of bladder cancer, representing more than 70% of cases at initial diagnosis. These tumors can be effectively treated by endoscopic excision, but more than 60% of patients will experience multiple tumor recurrences and thus require careful monitoring (41, 42). Intravesical immunotherapy with bacillus Calmette-Guerin is currently one of the most effective methods to prevent bladder tumor recurrence and bladder cancer is certainly the best example of success of nonspecific immunotherapy in the treatment of cancer (69, 70). MAUB defined by mAb M344 has several characteristics of a promising tumor marker for the management of bladder cancer. The expression of MAUB in 70% of superficial tumors and its complete lack of expression in normal cells provided a basis for the design of an effective non-invasive diagnostic test on exfoliated cells of urine (44). Other studies also indicated that primary superficial blad-

TABLE III
Epitope coexpression on carcinoma-associated mucin antigens expressed in T-MGH-U3 cells as determined by double determinant analysis

Tested mAb[a]	Catcher mAb[a]				
	M344	HMFG-2	B72.3	LDQ10	19A211[c]
M344	**2146**[b]	101	233	101	142
HMFG-2	20	**498**	154	4	45
B72.3	160	312	**1168**	77	70
LDQ10	0	0	0	**2359**	100
19A211	7	19	7	0	**396**

[a] Catcher mAb is the coated antibody (via GAM) and the tested mAb is the antibody complexed with ^{125}I-labeled GAM.
[b] The data represent the number of counts/min after subtraction of the background obtained using M-MGH-U3 as the source of antigen.
[c] mAb 19A211 is not directed against a carcinoma-associated mucin but is used here as a negative control.

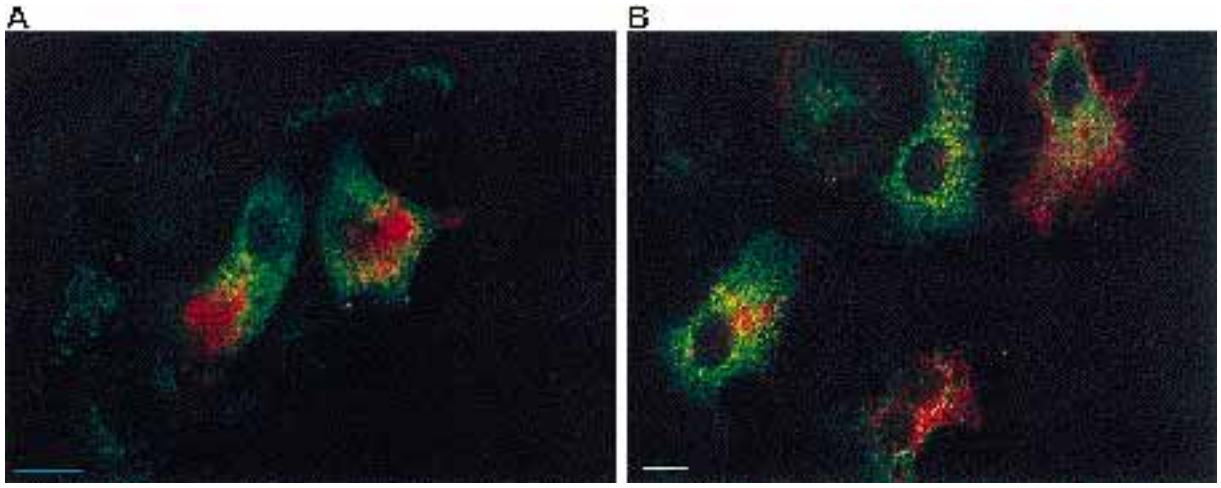

FIG. 3. **Confocal immunofluorescence micrographs of T-MGH-U3 cells double-labeled with M344 and LDQ10 mAbs.** *A*, micrograph showing cells where LDQ10 reactivity (*green* fluorescence) is diffusely distributed in the cytoplasm and where M344 reactivity (*red* fluorescence) is found in vacuolar structures. *B*, micrograph showing the diversity of the expression patterns observed. *Scale bars* represent 10 μm.

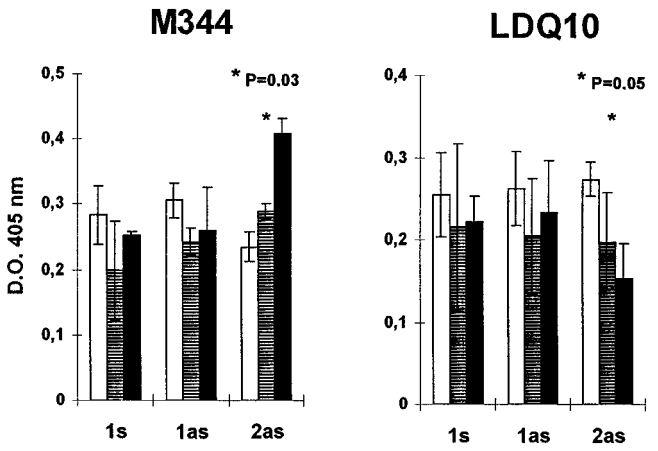

FIG. 4. **Inhibition of the MUC2 gene expression with MUC2 antisense oligos.** T-MGH-U3 cells were treated with 25 and 50 μg/ml MUC2 antisense oligos (*2as*). Control cells were treated with identical amounts of MUC1 sense (*1s*) and MUC1 antisense oligos (*1as*). After 72 h of treatment, proteins from the treated cells were extracted and analyzed by ELISA with M344 and LDQ10 mAbs. The *asterisk* (*) indicates experiments showing statistical difference by Kruskal-Wallis test. □, 0 μg/ml; ▤, 25 μg/ml; ■, 50 μg/ml.

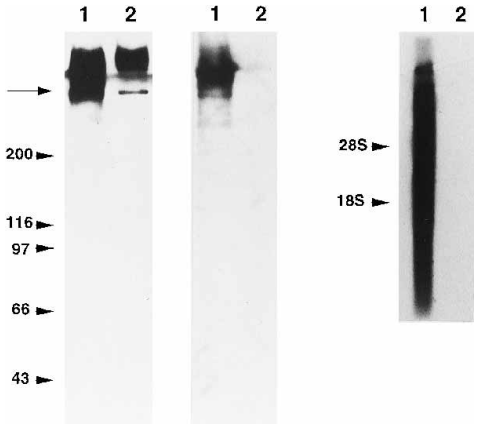

FIG. 5. **Analysis of the expression of MAUB and MUC2 mucins in a human superficial bladder tumor specimen.** *A*, proteins from T-MGH-U3 cells (*lane 1*) and from a superficial bladder tumor sample (*lane 2*) were analyzed by Western blot with M344 and LDQ10 mAbs. The *arrow* represents the interface between the running and the stacking gels (ECL detection). *B*, Northern blot analysis of the expression of the MUC2 gene in T-MGH-U3 cells (*lane 1*) and in the same human superficial bladder tumor sample (*lane 2*) expressing MAUB but not MUC2 mucin as assessed by Western blot analysis.

der tumors expressing MAUB had a significantly higher rate of tumor recurrence (71). This observation may find an explanation in the fact that MAUB is expressed at high frequency in the normal appearing urothelium of patients with a MAUB-positive tumor, thus suggesting that MAUB expression is occurring early in the process of bladder tumorigenesis (45). The identification of MAUB as a new mucin antigen associated with bladder cancer may have important implications for the treatment of superficial bladder tumors, since cancer mucins appear to have immunomodulatory properties and thus are good candidates for the design of specific cancer vaccines (72, 73).

The study of MAUB and other mucin antigens on the MGH-U3 cell line was very informative. The MGH-U3 cell line is derived from a grade I non-invasive papillary bladder tumor (74). It is tumorigenic in nude mice, and the xenografts obtained reproduce the histopathologic appearance of the original tumor. There are, however, important differences between MGH-U3 cells grown as nude mouse tumors or as monolayer *in vitro*. Ultrastructural studies showed that T-MGH-U3 cells contain electron-lucid cytoplasmic vacuoles typical of mucin secretion, which are not found in M-MGH-U3 cells cultured *in vitro*. The expression of MAUB followed closely the pattern of appearance of these vacuoles, and immunogold electron microscopy studies clearly demonstrated reactivity of mAb M344 with these vacuoles.[3] In the present study, the expression of MUC2 mucin detected by mAb LDQ10 was only observed in T-MGH-U3 cells and was located to cytoplasmic granules as assessed by confocal immunofluorescence microscopy. MUC2 mRNA was also present in T-MGH-U3 but not in M-MGH-U3 cells. A similar finding was reported with the pancreatic cancer cell line SW1990, which did not express MUC2 mRNA while the cells from tumor xenografts showed intense expression (75, 76). Of the seven mucin genes tested, MUC1 was the only other gene expressed in MGH-U3 cells. The presence of MUC1 mRNA in both M-MGH-U3 and T-MGH-U3 cells, the differences in band patterns observed in Western blots between MUC1 and MAUB, and the results of immunocaptures convincingly ruled out the possibility that MAUB is related to MUC1. To determine whether MAUB is a new mucin or is a glycoform of MUC2 mucin required more detailed analysis.

Several carcinoma-associated antigens were found to result from early sialylation of shorter carbohydrate chains in cancers

compared to normal cells (32, 36, 77). The results of the present study indicate that such changes in glycosyltransferase activity may also be influenced by the three-dimensional conformation of the cancer cells as shown previously for mAb B72.3 reactivity (78). In addition to revealing MUC1 bands in T-MGH-U3, mAb B72.3 also showed in Western blots a band comigrating with the high molecular mass one revealed by mAbs M344 and LDQ10. Immunocapture experiments using T-MGH-U3 protein extracts showed that B72.3 reactivity was limited to the antigen captured by mAb M344 and not by mAb LDQ10 to MUC2 mucin. Even though the M344 epitope is a sialylated carbohydrate, the absence of reactivity of mAb M344 with the MUC1 bands and also the lack of reactivity with bovine submaxillary mucin,[2] which is rich in sialyl-Tn antigen, rule out that mAb M344 reacts with the epitope recognized by mAb B72.3. The immunocapture experiments also showed no reactivity of mAb M344 with MUC2 mucin captured by mAb LDQ10, and conversely no LDQ10 reactivity with the antigen captured by mAb M344, suggesting that MAUB and MUC2 are two distinct molecules.

That MAUB and MUC2 mucins are distinct is also suggested by their very different patterns of expression in normal and cancerous human tissues. Several mAbs reactive with the core protein of MUC2 gene product have been described (58, 79–81). In at least two studies of human tissues, expression of MUC2 mucin measured by immunohistochemistry was found to correlate with expression of MUC2 mRNA as measured by *in situ* hybridization and by semiquantitative analysis of gene expression by reverse transcription followed by polymerase chain reaction (82, 83). All studies showed restricted expression of MUC2 gene product to normal epithelium of stomach and small and large bowel. The LDQ10 mAb that was used in the present study reacts with deglycosylated colon cancer mucin and with a synthetic peptide encompassing the MUC2 tandem repeat sequence. LDQ10 showed strong reactivity with goblet cells in the gastrointestinal tract and with a majority of colorectal, stomach, pancreatic, and breast cancers (58). By contrast, in two different studies, mAb M344 was unreactive with any normal adult or fetal tissue tested and was reactive with only few colon and breast carcinomas (38, 43). MAUB is not expressed in normal urothelium but is expressed in 70% of superficial (stages pTa and pT1) bladder tumors and in less than 15% of muscle invasive cancers (stage T2+) (38). The expression of MUC2 in urothelial cancers was only studied in depth by one group using mAb 4F1 also reactive with MUC2 core protein. MUC2 was not expressed in normal urothelium, but in contrast with MAUB it was expressed in 40% of muscle-invasive cancers and in only 40% of superficial pTa and pT1 bladder tumors (50). Since immunodetection of MUC2 mucin was found to correlate well with MUC2 mRNA expression, the lack of expression of MUC2 in tumors positive with mAb M344 strongly suggests that MAUB is distinct from MUC2. This conclusion is further supported by the lack of colocalization of MUC2 and MAUB mucins in confocal microscopy studies of T-MGH-U3 cells co-expressing both antigens.

Complex influences may come into play in the detection of various mucin epitopes by mAbs. It was thus important to obtain further evidence at the molecular level to substantiate the hypothesis raised by immunohistochemistry studies suggesting that MAUB and MUC2 are distinct mucins. One indication is provided by the results of the inhibition of MUC1 and MUC2 gene expression using antisense oligos. While the antisense MUC1 oligos had no effect on MUC2 nor MAUB expression, the antisense MUC2 oligos resulted in specific inhibition of MUC2 mucin expression and increased expression of MAUB. Further evidence came from the identification of a superficial bladder tumor, which strongly expressed MAUB and had no expression of MUC2 peptide and mRNA. Thus MAUB is a mucin distinct from those identified so far, although it shares several common features with MUC2 mucin. Indeed, both are typical secreted mucins and their expression in cancer cell lines appear to be influenced by the spatial configuration of tumor cells. The complete characterization of MAUB and its accurate tissue distribution will await the cloning of cDNA encoding its core protein. It is, however, possible to already conclude that cells from MGH-U3 tumor xenografts express three distinct mucins. Coexpression of distinct mucins is a normal feature of mucus secreting epithelium such as stomach and colon. A recent study reported that increased heterogeneity of mucin gene expression in gastric adenocarcinomas was associated with advanced cancer stage (31). The finding of multiple mucin expression in a well differentiated bladder tumor originating from a typically non-mucous-secreting epithelium suggests that ectopic expression of mucin gene products may be an early feature of urothelial tumorigenesis.

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