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Production and Characterization of Mouse Monoclonal Antibodies to Human Bladder Tumor-associated Antigens[1]

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ABSTRACT

Monoclonal antibodies (McAbs) to human bladder carcinoma were generated by fusion of NS-1 mouse myeloma cells with spleen cells from BALB/c mice immunized with either cultured human bladder cancer cells or cells obtained from a fresh surgically removed bladder tumor. Four hybridomas which reacted strongly with bladder tumor cells and not to normal skin fibroblasts or urothelial cells were identified and cloned by limiting dilution to obtain monoclonality. One McAb, 3G2-C6, raised with cultured tumor bladder cells MGH-U1 (EJ) as the immunogen reacted more strongly to the bladder tumor lines tested than any of the other McAbs resulting from various fusion experiments. Hybridoma 3G2-C6 was found to secrete murine immunoglobulin G1 and to produce high titer ascites fluid when grown in BALB/c mice. Results from quantitative enzyme-linked immunosorbent assays on a panel of more than 35 cell lines demonstrated that McAb 3G2-C6 reacted with several bladder tumor cell lines 50 to 90 times more than with normal transitional urothelium. Two kidney and two testicular tumor lines also bound 10 times more 3G2-C6 than with normal cells. The 3G2-C6 antigen was only marginally detected on a number of other cancer and noncancerous cells tested such as breast and lung tumor cells, melanoma, fetal cells, and peripheral blood lymphocytes. To identify the antigen ^{125}I-labeled membrane components from MGH-U1 cells were extracted with detergent, immunoprecipitated with Protein-A bound 3G2-C6, and analyzed by sodium dodecyl sulfate-gel electrophoresis. This revealed that McAb 3G2-C6 binds to a M_r 90,000 cell surface component. Indirect immunofluorescence microscopy with fluorescein isothiocyanate-anti-mouse immunoglobulin G also identified the antigen on the surface of cultured and fresh tumor cells and detected the antigen on 16 of 17 Grade 3 bladder tumor specimens as well as on some kidney and testicular tumor cells. This study confirms the potential of the hybridoma technique for producing McAbs capable of identifying tumor associated antigens which may be useful in the diagnosis and treatment of bladder cancer.

INTRODUCTION

The generation of McAbs[4] via the technique of somatic cell hybridization (22) has rapidly become a standard procedure for the identification of tumor-associated antigens. McAbs of relevant specificities can be valuable reagents not only for immunodiagnosis and immunotherapy but also in the study of tumor cells in general. Several examples of the current repertoire include McAbs which identify antigens expressed in leukemia (1, 42, 46), melanoma (6, 10), glioma (3, 44), and carcinomas including breast (7, 43), bladder (13, 16, 29, 30), colon (18, 50), lung (8), and prostate (14, 48, 55).

The hybridoma technique is particularly suited to the area of bladder cancer because of the difficulties encountered in the early detection of the disease and the suspected antigenic differences existing between normal and malignant cells. TCC of the urinary tract is a highly variable, heterogeneous, and complex disease. The cancer may be solid, papillary, sessile, or *in situ* and possess different propensities in recurrence, progression and invasion, and response to treatment (36, 47). Currently the detection of tumors is based primarily on cystoscopical appearance and conventional histological and cytological methods where cellular and tissue morphologies are the main criteria. These methods often prove to be inadequate for the detection of carcinoma *in situ* and low grade tumors (54). Tumor markers if available would be valuable assets in detecting and defining various types of bladder tumors, particularly in the early stages before invasion through the mucosa has occurred since some patients do not develop symptoms until the disease has progressed through the muscle layer.

In TCC of the bladder several investigators have reported the existence of antigens associated with bladder tumors that are not found in normal urothelium. A protein of M_r 72,000 has been identified with TCC *in vitro* (4) and the presence of the T-antigen in bladder carcinoma has been correlated with poor prognosis (51). In addition loss of ABO blood group antigen expression has been linked with invasiveness of bladder cancer (49) and a new epitope common to human erythrocyte membranes and bladder epithelium which is affected by neoplastic transformation has also been identified (39). Therefore specific McAbs which identify TCC tumor associated antigens have potentially significant clinical applications. This report describes the production of four murine McAbs against human bladder carcinoma and characterization of one McAb that appears to react with a membrane surface component expressed on bladder tumor cells.

MATERIALS AND METHODS

Cells and Cell Lines. Thirty-eight different human cell lines or cultures (listed in Table 1) representing various normal and neoplastic tissues were used to characterize the antibodies in this study. Malignant cell lines MGH-U3 and -U4, PN, AB, SV, RW, and DR were established in our laboratory from surgical tumor specimens. AB originated from a Grade 3 renal pelvis TCC, SV and PN from high grade renal cell carcinomas, and RW and DR from seminomatous and embryonal testicular carcinomas, respectively. The cellular morphology, growth characteristics, enzyme, and chromosome markers and tumorigenicity of the MGH series of bladder tumor cell lines has been described recently (27). These lines and bladder tumor lines T-24 (5), MGH-U1 (EJ) and -U2 (HM), subclones of T-24 (32), and RT-4 (41) were maintained in McCoy's Medium 5A supplemented with 5% heat-inactivated fetal bovine serum.

[1] A portion of this investigation was reported at the 75th Annual Meeting of the American Association for Cancer Research (58).

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[4] The abbreviations used are: McAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; SDS, sodium dodecyl sulfate; TCC, transitional cell carcinoma; DPBS, Dulbecco's phosphate-buffered saline; BSA, bovine serum albumin.

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Other cell lines utilized in this study were grown in nutrient media supplemented with serum as recommended by their suppliers. The colon carcinoma cell lines COLO 320 (37) and COLO 205 (45) and ovarian tumor cell line COLO 330 (57) were obtained from Dr. George Moore (Department of Surgery, Denver General Hospital, CO). Cell lines A673 (15), A431 (15), Hs804, Hs767, WI38 (17), and Hs769 (33) were obtained from the Naval Biosciences Laboratory (Naval Supply Center, Oakland, CA). Cell lines G-361 (34), ZR-75-1 (11), ZR-75-30 (11), RPMI 8226 (31), PC-3 (19), A549 (15), HT-1376 (38), and HT-1197 (38) were purchased from the American Type Culture Collection, Rockville, MD. Short-term primary cultures, FL-12 and FL-16 (Grade 3 TCC), FL-17, (seminoma), FL-20 (Grade 3 RCC), and BYF (penile fibroblasts) were grown from surgical specimens in our laboratory. The mouse myeloma cell P3-NSI/I-Ag4-1 (21) was obtained from the cell distribution center of the Salk Institute (San Diego, CA).

Normal bladder urothelial cell cultures were initiated by a microdissection technique exactly as described by Reznikoff *et al.* (40). Briefly the transitional epithelial cell layer was dissected from the muscle and connective tissue layers from fresh pieces of histologically normal human ureter or bladder mucosa. Explant cultures were grown on thick (2 ml/60 mm dish) rat tail collagen substrates in Ham's F12 medium supplemented with 5 μg/ml each of insulin, hydrocortisone, and transferrin to enhance epithelial cell growth (40). Successful primary transitional cell explant cultures grew as continuous sheets of tightly adherent cells and demonstrated the typical polygonal shape with prominent nucleoli as observed with phase contrast microscopy. Cultures which developed fibroblast growth or cells with questionable epithelial cell morphology were discarded. DNA ploidy of these cultures was monitored by staining with propidium iodide and analyzed by flow cytometry to establish that only diploid cells were present.

Mononuclear cells from heparinized peripheral blood were recovered from healthy adult donors by Ficoll-Hypaque density gradient centrifugation.

Immunization. In several separate experiments female BALB/c mice (8–12 weeks old; Charles River Breeding Laboratories, Wilmington, MA) were immunized with either cultured bladder tumor cells (MGH-U1 or -U2) or cells obtained from a fresh surgically removed Grade 3 TCC of the bladder. Confluent monolayers of cultured cells were harvested with the aid of a rubber policeman and washed three times with DPBS, pH 7.2. The dispersion of tumor into cell suspension was performed according to the method of Bashor (2). The fresh tumor was minced and digested in a trypsinizing flask with a solution of trypsin, 0.1 mg/ml, and collagenase, 0.2 mg/ml, (Type II; Sigma) in Earle's balanced salt solution. Released cells were either used immediately or preserved in liquid nitrogen. For immunization 1×10^7 cells in 0.2 ml DPBS were injected i.p. into each mouse followed by a second injection of 1×10^7 cells 1 month later. Spleens were removed 3 days later for fusion.

Cell Fusion. Spleen cells from immunized mice were fused with P3-NSI/1-Ag4-1 (NS-1) mouse myeloma cells according to a modified procedure of Kohler and Milstein (22). Fusion was accomplished by incubating 10^8 splenocytes with 10^8 NS-1 cells in 1 ml of 50% (w/v) polyethylene glycol 1540 for 1 min at 37°C. Fused cells were resuspended in hybridoma medium composed of RPMI 1640 medium containing 15% fetal bovine serum, penicillin (50 units/ml), streptomycin (50 μg/ml), 1 mM sodium pyruvate, and 2 mM L-glutamine, and distributed to 96-well culture dishes at a concentration of 1×10^6 cells/well in 0.1 ml hybridoma medium at 37°C with 7.5% CO_2 in a humid atmosphere. After 24 h 0.1 ml of hybridoma medium supplemented with 0.1 mM hypoxanthine, aminopterin (350 ng/ml), and 16 μM thymidine was added to each well to select for hybridomas. Every 3 days one-half of the medium in each well was replaced with fresh hypoxanthine, aminopterin, and thymidine medium until the hybridoma colonies were large enough to screen for antibody production (about 3 weeks).

Screening of Hybridoma Supernatants and Cloning. Culture supernatants were screened for antibody activity to normal and malignant cells with a solid phase ELISA (20). Target cells (1×10^5) were first allowed to attach to wells and then fixed for 15 min at room temperature with 0.5% glutaraldehyde in DPBS. Normal urothelial cells were removed from their collagen substrates with 0.1% EDTA (30), centrifuged into wells to promote attachment, and incubated overnight in culture medium before fixing. After fixation, wells were washed, incubated with 100 mM glycine in BSA solution (0.1% bovine serum albumin in RPMI 1640 with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4) and washed again. Fixed cells were either used immediately or stored frozen in BSA solution for future assays.

To assay antibody binding activity 50 μl of culture supernatant from each well in a hybridoma plate were transferred to a plate of fixed target cells and incubated for 1 h at 37°C. The amount of antibody bound was determined colorimetrically with the HyBRL screening kit (Bethesda Research Laboratory, Gaithersburg, MD) which used biotinylated goat anti-mouse immunoglobulin, Streptavidin-biotinylated horseradish peroxidase, and *o*-phenylenediamine as the substrate. Clones which reacted with tumor cells and not with normal cells were expanded in hypoxanthine and thymine medium. To obtain monoclonality healthy cultures exhibiting antibody activity were cloned by limiting dilution at concentrations of 5, 1, and 0.5 cells/well in 96-well dishes with BALB/c thymocytes as feeder cells.

Antibody Production and Purification. As a source of monoclonal antibodies, hybridomas were overgrown (about 4 to 6 days) and the supernatant was collected and stored frozen in appropriate aliquots until use. Ascites fluid was also produced by injecting 2×10^6 healthy hybrid cells i.p. into pristane-primed BALB/c mice. IgG1 monoclonal antibodies were purified from culture supernates by Protein A-Sepharose column chromatography (12) at pH 8.6 and eluted with 0.05 M citrate buffered saline, pH 5.5.

Characterization of Monoclonal Antibodies. The heavy and light chain composition of each monoclonal antibody of interest was determined with glutaraldehyde-fixed tumor cells in an ELISA assay with a monoclonal antibody isotype identification kit (Chemicon International Inc., Los Angeles, CA). McAb binding was detected by rabbit antiserum to murine light and heavy chains, followed by a peroxidase labeled affinity purified goat anti-rabbit IgG.

The extent of McAb binding to various normal and malignant cells (listed in Table 1) was determined with an expanded version of the ELISA used for screening described above, with 0.25 ml of supernatant and 5×10^5 target cells, and the production of substrate was measured spectrophotometrically at 450 nm. Cells in suspension such as murine and human myeloma cells and peripheral blood lymphocytes were assayed with a Microfold immunofiltration system (V & P Scientific Inc., San Diego, CA) which retained cells on glass fiber filters. Binding ratios were calculated between the average absorbance values of normal urothelium and each cell type tested as an indication of the relative amount of antibody binding.

Molecular Weight Determination of McAb Reacting Antigen. The procedures for iodination of membrane proteins and immunoprecipitation of the reacting antigen have been described previously (26). Briefly 5×10^7 cells in 0.5 ml DPBS were mixed with 1 mCi $Na^{125}I$ (New England Nuclear, Boston, MA) in 0.1 ml of 1.0 M phosphate buffer, pH 7.2, 200 μg (48 units) of lactoperoxidase, and 25 μl of 0.03% hydrogen peroxide at room temperature for 10 min. Membrane proteins were extracted from the labeled cells with cold 1.0% NP40 containing 1.0 mM phenylmethane sulfonyl fluoride. For immunoprecipitation, formalin fixed Cowan strain I *Staphylococcus aureus* cells (Pansorbin; Calbiochem, La Jolla, CA) were washed three times in washing buffer, phosphate-buffered saline, pH 8.6, 0.1% BSA, 0.02% NaN_3, 0.5% NP40, and 0.1% SDS and brought to a 10% suspension in whole anti-mouse IgG (Sigma). The precoated bacteria were mixed with the monoclonal antibody (spent hybridoma culture medium) and after washing the preparation was used to immunoprecipitate the ^{125}I-labeled membrane antigen with overnight incubation at 4°C. After extensive washing bound antigen was released by incubation for 5 min at 100°C in 100 μl of sample buffer for SDS-gel electrophoresis (25). The bacteria were spun down and 10 to 40 μl of

the supernatant was analyzed by electrophoresis on a 10% SDS-polyacrylamide slab gel (25) with molecular weight markers. Gels were stained, destained, dried, and autoradiographed with an intensifying screen (52). By superimposing the autoradiograph on the dried stained gel the molecular weight of the labeled protein was determined by comparison with the unlabeled markers in the same lanes using a semilog plot.

Immunofluorescence. Specimens for this study included tumors and nontumor tissues and urothelial cells obtained by saline barbotaging the bladders of patients with and without bladder tumor. One half of each specimen was used for pathological diagnosis and the other half for McAb staining. Single cell suspensions were prepared from fresh tumor specimens as described above for immunization with the omission of trypsin or in the case of cultured cells removed from the substratum with 0.1% EDTA in Ca^{2+} and Mg^{2+} free DPBS. Cell pellets were suspended in an equal volume of McAb solution, either from spent culture medium or a 1:50 dilution of mouse ascites fluid, incubated at 4°C for 30 min, and washed three times with cold DPBS. NS-1 myeloma spent medium or NS-1 ascites fluid were included as controls. The cell pellets were then resuspended in an equal volume of a 1:20 dilution of anti-mouse IgG fluorescein isothiocyanate conjugate (Sigma), incubated for 30 min at 4°C, washed three times, and resuspended in a small volume of 0.05% BSA in cold DPBS. Fluorescent stained cells were observed as a wet mount with a Zeiss fluorescent microscope.

Culture cells and cells from bladder barbotages also were fixed on slides with Aerocell, a water soluble fixative (Aeroceuticals Health Care Products, Southport, CT) and stained in the same manner except with 3 one-min washes between subsequent incubation steps.

RESULTS

Generation and Screening of McAbs. Within 2 weeks after fusion of the immunized mouse spleen cells with NS-1 myeloma cells colonies of hybrid cells were observed in nearly every well. In every case approximately 50% of these cultures reacted with both the normal and malignant human target cells tested in the initial screening process. From six separate fusions a total of twenty clones were identified that produced antibodies distinguishing between bladder tumor cells and normal urothelial or skin fibroblast cells. Four hybridomas, 3G2-C6 and C3 (derived from mice immunized with MGH-U1), 2H3 (from MGH-U2), and CD5F4 (from a surgical bladder tumor specimen) were confirmed by repeated ELISA testing to be continuously producing antibody and were selected for expansion and recloning. Each clone had been stable in culture for over 30 passages. Supernatants were tested to identify the immunoglobulin subclass secreted. Interestingly antibodies 3G2-C6, C3, and 2H3, resulting from mice immunized with cultured cells were all found to be of the IgGl,k isotype whereas CD5F4, derived from mice immunized with fresh tumor cells by the same protocol, produced an IgM antibody.

Characterization. Each McAb was characterized by binding to a panel of normal and malignant cells listed in Table 1. Overall, antibodies 3G2-C6, 2H3, and C3 exhibited higher binding ratios with bladder tumor cell lines MGH-U1, MGH-U2, T-24, and HT-1376 than with other bladder lines. These antibodies also displayed reactivity with other malignant cells including some kidney and testis lines, melanoma (G-361), colon (COLO 205), and breast (ZR-75-30) lines, but with generally lower binding ratios than those observed with the bladder lines. CD5F4, the IgM antibody, showed a wider range of reactivity than any of the other antibodies binding to all of the kidney and testicular tumor lines and in lower but detectable amounts to almost every one of the remaining cultured tumor lines. Reactivity of the McAbs with NS-1, over 30 different cultures of normal urothelium or bladder mucosa, human peripheral blood lymphocytes, normal type A, B, and O erythrocytes (Table 3), and human skin fibroblasts were very low and barely detectable by the ELISA. 3G2-C6 was selected for further study because it exhibited the greatest binding ratio between bladder tumor cells and normal cells and the highest specificity for the bladder lines as compared with the other tumor cell lines.

The titers of the 3G2-C6 ascites fluid and that of culture supernatant were compared. Among the bladder cell lines T-24, MGH-U1 and -U2, 3G2-C6 ascites McAb exhibited about a 100-fold greater titer than 3G2-C6 from culture supernatant in that a 1:500 dilution of ascites showed the same binding pattern and degree of activity as a 1:5 dilution of supernatant McAb. MGH-U1, a subline of T-24 demonstrated a higher overall pattern of binding than T-24. Even at a high concentration of 3G2-C6 (1:10 dilution of the ascites) binding to the bladder tumor lines MGH-U3 and -U4 derived from low grade TCC was very minimal, implying that the 3G2-C6 antigen is expressed at a very low level on these cells.

Indirect Immunofluorescence Staining. Indirect immunofluorescence was performed by staining either viable cell suspensions or fixed cell smears with McAb 3G2C6 and fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG. Treatment of MGH-U1 cells with trypsin resulted in some loss of staining and disruption of tissues with a collagenase cocktail containing no trypsin resulted in poor cell preparations. For these reasons cultured cells were removed from their substate with EDTA and cells were obtained from tissue specimens by mechanical agitation. Fig. 1 shows that the presence of 3G2-C6 can be visualized either as a perimeter of fluorescence or occur in membrane "patches" on MGH-U1 cells. Bladder tumor cells MGH-U3 and -U4 which did not bind significant levels of 3G2-C6 in ELISA assays did not exhibit any immunofluorescence above background levels. Positive culture tumor cells usually exhibited only membrane fluorescence with very little or no cytoplasmic staining. Cells from clinical specimens, however, often exhibited a range of fluorescence intensities within the same sample, with cells staining weakly to very strongly on the membrane and throughout the cytoplasm and others primarily on the surface. Due to these variations it was difficult to determine a specific intensity for each specimen. Therefore cells with all staining intensities were enumerated. Freshly prepared cell suspensions stained with much more clarity than did preparations fixed on slides. In our hands efforts to stain frozen sections by immunofluorescence or immunoperoxidase were inconsistent (data not shown), often resulting in preparations with high background staining. Attempts were also made to stain paraffin-embedded MGH-U1 cells with an immunoperoxidase technique (results not shown), but the antigen could not be detected indicating that it was probably not stable throughout the embedding or deparaffination process.

Listed in Table 2 are the results of 3G2-C6 fluorescence staining of malignant and nonmalignant bladder specimens. These included bladder tissues obtained by biopsy, partial excision and cystectomy, and urothelial cells by barbotage. Throughout the study MGH-U1 and MGH-U4 cells were used as positive and negative controls, respectively. Bladder specimens from patients with Grade 3 TCC showed positive staining with 3G2-C6 in 16 of 17 cases. In all cases both positively and negatively stained cells were observed. 3G2-C6 stained only 2 of 13 and 1

Table 1

Binding of monoclonal antibodies to human malignant and nonmalignant cells in culture

Undiluted supernatants from hybridomas 3G2-C6, 2H3, CD5F4, and C3 were tested in triplicate with a streptavidin solid-phase ELISA as described in "Materials and Methods." Each number represents the average absorbance of the substrate end product at 450 nm. The value in parentheses is the binding ratio of the absorbance obtained with the target test cell to the absorbance of normal transitional cells. Absorbance values for normal transitional cells are the average of at least four different primary cultures. Controls without the primary antibody (McAb) exhibited an average absorbance of 0.02.

Target cells	ELISA binding of monoclonal antibodies			
	3G2-C6	2H3	CD5F4	C3
Bladder tumors, TCC				
MGH-U1	3.54 (86.0)	2.45 (42.0)	0.35 (6.4)	2.08 (39.0)
MGH-U2	3.85 (94.0)	2.50 (43.0)	1.17 (21.0)	1.73 (33.0)
MGH-U3	0.09 (2.1)	0.64 (11.0)	0.61 (11.0)	1.29 (24.0)
MGH-U4	0.01 (0.3)	0.88 (15.0)	0.26 (4.7)	0.86 (16.2)
T-24	2.32 (56.0)	3.24 (56.0)	0.43 (7.8)	2.34 (44.0)
RT-4	0.15 (3.7)	0.56 (9.7)	0.58 (10.5)	0.28 (15.3)
FL-12	0.04 (0.8)	0.78 (13.4)	1.65 (30.0)	0.40 (7.5)
FL-16	0.95 (23.0)	0.46 (7.9)	0.96 (17.5)	0.68 (12.8)
HT-1197	0.42 (10.2)	2.20 (37.9)	1.82 (33.1)	1.16 (21.9)
HT-1376	1.08 (26.3)	3.24 (56.0)	1.79 (32.5)	3.48 (65.7)
Kidney tumors				
TCC				
AB	0.35 (8.4)	0.88 (15.1)	0.80 (14.5)	1.79 (33.8)
Renal cell carcinoma				
PN	0.98 (24.0)	0.28 (4.9)	1.42 (26.0)	0.50 (9.4)
SV	0.09 (2.1)	0.85 (14.7)	0.36 (6.5)	0.84 (15.8)
FL-20	0.54 (13.0)	0.08 (1.4)	0.40 (7.3)	0.92 (17.4)
Testicular tumors				
RW	0.50 (12.0)	0.20 (3.5)	2.75 (50.0)	1.61 (30.0)
DR	0.30 (7.2)	0.17 (2.9)	0.86 (15.6)	1.22 (23.0)
FL-17	3.74 (91.0)	0.68 (11.7)	0.52 (9.5)	0.48 (9.0)
Colon carcinoma				
COLO 320	0.03 (0.6)	0.04 (0.6)	0.38 (6.9)	0.05 (0.9)
COLO 205	0.12 (2.9)	0.55 (9.5)	0.16 (2.9)	0.68 (12.8)
Rhabdosarcoma				
A673	0.16 (3.8)	0.10 (1.7)	0.50 (9.4)	0.05 (0.9)
Ovarian tumor				
COLO 330	0.03 (0.7)	0.09 (1.5)	0.48 (8.7)	0.36 (6.8)
Carcinoma of vulva				
A431	0.08 (1.8)	0.10 (1.7)	0.31 (5.6)	0.23 (4.4)
Melanoma				
JoCat	0.03 (0.7)	0.12 (2.1)	0.55 (10.0)	0.29 (5.5)
G-361	0.34 (8.3)	0.43 (7.4)	0.42 (7.6)	0.23 (4.4)
Prostate carcinoma				
Hs804/Au071	0.12 (2.9)	0.05 (0.9)	0.36 (6.5)	0.12 (2.3)
PC3	0.15 (3.6)	0.15 (2.6)	0.18 (3.3)	0.12 (2.3)
Hs767/At977	0.09 (2.2)	0.20 (3.4)	0.26 (4.7)	0.34 (6.4)
Lung carcinoma				
A549	0.08 (1.9)	0.20 (3.4)	0.72 (13.0)	0.09 (1.7)
Breast carcinoma				
ZR-75-1	0.11 (2.6)	0.25 (4.3)	0.01 (0.2)	0.48 (9.0)
ZR-75-30	0.52 (12.7)	0.19 (3.3)	0.33 (6.0)	0.65 (12.3)
Myeloma				
RPMI 8226	0.05 (1.2)	0.05 (0.9)	0.04 (0.7)	0.04 (0.8)
Mouse myeloma				
NS-1	0.01 (0.1)	0.02 (0.3)	0.05 (0.9)	0.04 (0.8)
Normal fetal lung				
WI 38	0.52 (1.3)	0.04 (0.7)	0.09 (1.6)	0.04 (0.8)
Skin fibroblast				
HSF	0.03 (0.6)	0.04 (0.7)	0.11 (2.0)	0.10 (1.8)
Normal bladder				
Hs769/BD156	0.01 (0.2)	0.01 (0.2)	0.11 (2.0)	0.09 (1.6)
Corpora fibroblast				
BYF	0.09 (2.2)	0.27 (4.6)	0.09 (1.5)	0.01 (0.2)
Human peripheral blood lymphocytes	0.05 (1.3)	0.08 (1.3)	0.05 (0.9)	0.06 (1.1)
Normal urothelium	0.041 (1.0) ± 0.025[a]	0.058 (1.0) ± 0.038	0.055 (1.0) ± 0.024	0.053 (1.0) ± 0.012

[a] Mean ± SE, absorbance values of transitional cells used to determine the binding ratio of 4 different cultures.

Table 2

Reaction of McAb 3G2-C6 with malignant and nonmalignant bladder specimens by indirect immunofluorescence

Indirect immunofluorescence assay was carried out on cell suspensions or fixed smears of tumor specimens. 3G2-C6 ascites fluid diluted 1:50 and fluorescein-labeled goat anti-mouse IgG were used. Specimens were graded as highly positive if greater than 50%, moderately positive if between 20 and 50%, weakly positive if between 5 and 20%, and negative if less than 5% of cells showed fluorescence staining over controls and background.

Specimen	Pathological diagnosis			
	Type	Grade[a]	Stage[b]	Immunofluorescence
Biopsy	TCC	3	T3a	Highly positive
Biopsy	TCC	3	T3a	Highly positive
Biopsy	TCC	3	Tis	Highly positive
Cystectomy	TCC	3	T3b	Highly positive
Cystectomy	TCC	3	T4b	Highly positive
Cystectomy	TCC	3	T3b	Highly positive
Cystectomy	TCC	3	T3b	Highly positive
Cystectomy	TCC	3	T2	Highly positive
Cystectomy	TCC	3	Tis	Highly positive
Cystectomy	TCC	3	T4a	Highly positive
Cystectomy	TCC	3	T3b	Highly positive
Cystectomy	TCC	3	T3a	Highly positive
Excision	TCC	3	Tis	Highly positive
Biopsy	Severe atypia	3	Tis	Highly positive
Barbotage	TCC	3	T3a	Negative
Barbotage	TCC	3	T3b	Highly positive
Barbotage	TCC	3	T3b	Highly positive
Barbotage	Squamous cell carcinoma	2	T3b	Highly positive
Excision	Papillary TCC	2	Ta	Moderately positive
Biopsy	Papillary TCC	2	Ta	Highly positive
Biopsy	Papillary TCC	2	T1	Negative
Biopsy	Papillary TCC	2	Ta	Negative
Biopsy	Papillary TCC	2	T3a	Negative
Biopsy	Papillary TCC	2	Ta	Negative
Biopsy	Papillary TCC	2	Ta	Negative
Biopsy	Papillary TCC	2	Ta	Negative
Biopsy	Papillary TCC	2	Ta	Negative

Specimen	Pathological diagnosis			
	Type	Grade[a]	Stage[b]	Immunofluorescence
Biopsy	Papillary TCC	2	Ta	Negative
Excision	Papillary TCC	2	Ta	Negative
Cystectomy	Papillary TCC	2	Ta	Negative
Cystectomy	Papillary TCC	2	Ta	Negative
Biopsy	Papillary TCC	1	Ta	Moderately positive
Biopsy	Papillary TCC	1	Ta	Negative
Biopsy	Papillary TCC	1	Ta	Negative
Biopsy	Papillary TCC	1	Ta	Negative
Biopsy	Papillary TCC	1	Ta	Negative
Biopsy	Papillary TCC	1	Ta	Negative
Biopsy	Papillary TCC	1	Ta	Negative
Biopsy	Papillary TCC	1	Ta	Negative
Excision	Papillary TCC	1	Ta	Negative
Biopsy	Slight atypia	0	T0	Negative
Cystectomy	Chronic cystitis	0	T0	Negative
Cystectomy	Chronic cystitis with ulcerations	0	T0	Negative
Barbotage	Severe atypia	0	T0	Moderately positive
Barbotage	Normal bladder	0	T0	Negative
Barbotage	Normal bladder	0	T0	Negative
Barbotage	Normal bladder	0	T0	Negative
Barbotage	Normal bladder	0	T0	Negative
Biopsy	Chronic inflammation	0	T0	Negative
Biopsy	No malignancy	0	T0	Negative
Biopsy	No malignancy	0	T0	Negative
Biopsy	Normal	0	T0	Negative
Ureter	No malignancy	0	T0	Negative

[a] Bladder tumors were graded according to Koss (24) by the increasing degree of cellular atypia and nuclear abnormalities and number of cells in mitosis where Grade 0 corresponds to no tumor; Grade 1, well differentiated cells approximating the normal transitional cell layers, although in a papillary arrangement; Grade 2, moderately differentiated cells in a papillary arrangement with epithelium thicker than in Grade 1 and cells are in varying degrees of anaplasia; and Grade 3, poorly differentiated cells which occur in sheets with considerable cell pleomorphism and are more invasive than the papillary carcinoma.

[b] Bladder tumors were staged according to the tumors-nodules-metastasis system (35) where T0 designates no evidence of primary tumor; Ta, papillary noninvasive carcinoma; T1, invasion within lamina propia; T2, invasion of superficial muscle; T3a, invasion of deep muscle and T3b, invasion into perivesical fat; T4a, invasion of neighboring structures and T4b, fixed or infiltrating abdominal wall; and Tis, carcinoma *in situ*.

of 9 specimens with Grades 2 and 1 TCC, respectively. Normal bladder or bladder specimens with cystitis or urothelial atypia also did not stain.

Table 3 lists the results of immunofluorescence staining with 3G2-C6 on other urological tissue samples. Grade 3 renal cell carcinoma and TCC and stained positively and no staining was evident with normal and benign specimens, low grade renal cell carcinoma, or adenocarcinoma. 3G2-C6 also stained 5 of 8 testicular specimens with seminoma or mixed testicular tumors.

Preliminary Characterization of the 3G2-C6 Antigen. Fig. 2 shows the results of polyacrylamide-gel electrophoresis and autoradiography of the immunoprecipitated surface membrane antigen from MGH-U1, -U2, and -U4 cells. When the developed autoradiographs were compared with molecular weight standards a specific band of M_r 90,000 was apparent in the lanes with both MGH-U1 and -U2 precipitated material. No similar band was evident in the lane containing the material derived from -U4 cells, a cell line that was negative in ELISA and immunofluorescence studies for 3G2-C6.

DISCUSSION

Four monoclonal antibodies have been produced which react in varying degrees to bladder tumors and other tumors of urological origin. One McAb, 3G2-C6, exhibiting specificity for six of the bladder cell lines was characterized more extensively. Preliminary analysis of 3G2-C6 ascites by high pressure liquid chromatography (data not shown) revealed an IgG peak comprising about 10% of the ascites total protein with a concentration of 1.2 mg/ml thus explaining the relatively low titer observed with dilutions of 3G2-C6 ascites in binding with reactive bladder cell lines. Other investigators have reported concentrations as high as 7 mg antibody protein/ml ascites fluid.

Although malignant cells *in vitro* may exhibit phenotypic and genotypic variations from the original tumor it is interesting to note that 3G2-C6 reacted preferentially with TCC cell lines derived from poorly differentiated, highly invasive primary tumors (MGH-U1, -U2, T-24, HT-1376, HT-1197, FL-16, and AB) while hardly at all with cell lines derived from well differentiated low grade papillary carcinomas (RT-4, MGH-U3, -U4, and FL-12). It also reacted to some extent with kidney lines PN and SV, established in our lab from high grade renal cell carcinomas, two seminoma lines, and one melanoma and breast cancer cell line. Presently the significance of these cross-reactions is not apparent but many investigators have reported tumor reactive monoclonal antibodies which bind to other tumor types or tissues as well (7, 14, 23, 28). Therefore our McAbs may react with other types of tumors and cells not examined by this study It should be kept in mind also that due to the heterogeniety of tumors results from a few representative cell lines in one tumor category do not necessarily indicate that other cells or tissues of the same type will react in the same manner.

Indirect immunofluorescence staining of surgical specimens

Table 3

Reaction of McAb 3G2-C6 with nonbladder urological tissue specimens by indirect immunofluorescence

The indirect immunofluorescence assay method is described in Table 2. The same designation for levels of positive cells is also used.

Specimen	Pathological diagnosis			Immunofluorescence
	Type	Grade[a]	Stage[b]	
Kidney				
Nephrectomy	TCC (renal pelvis)	3	T3	Highly positive
Paracaval lymph node	TCC (renal pelvis)	3	T3	Moderately positive
Nephrectomy	Renal cell carcinoma	3	T2	Highly positive
Nephrectomy	Renal cell carcinoma	2	T2	Negative
Nephrectomy	Renal cell carcinoma	2	T2	Negative
Nephrectomy	Renal cell carcinoma	1	T3a	Negative
Nephrectomy	Normal area	0	T0	Negative
Nephrectomy	Lipogranuloma	0	T0	Negative
Nephrectomy	Multiple angiomyolipoma	0	T0	Negative
Testis				
Orchiectomy	Embryonal carcinoma and seminoma		T1	Highly positive
Orchiectomy	Seminoma		T1	Highly positive
Orchiectomy	Embryonal with teratoma and seminoma		T1	Moderately positive
Orchiectomy	Teratoma and seminoma *in situ*		T1	Moderately positive
Orchiectomy	Mixed, mostly embryonal carcinoma		T4a	Moderately positive
Orchiectomy	Hemorrhagic infarction		T0	Negative
Orchiectomy	Teratoma		T1	Negative
Orchiectomy	Embryonal carcinoma		T4a	Negative
Prostate				
Biopsy	TCC, metastatic to prostate	2	T4	Highly positive
Biopsy	Benign prostatic hyperplasia	0	T0	Negative
Biopsy	Adenocarcinoma	3	T3a	Negative
Prostatectomy	Adenocarcinoma	1	T1-T2	Negative
Prostatectomy	Benign prostatic hyperplasia	0	T0	Negative
Erythrocytes				
Peripheral RBC	Normal, type A			Negative
Peripheral RBC	Normal, type B			Negative
Peripheral RBC	Normal, type O			Negative

[a] Grading of kidney and prostate tumor specimens is similar to that of bladder tumors described in Table 2, where Grade 1 is the least anaplastic and Grade 3 shows the greatest degree of anaplasia. Testicular tumors are not graded but are classified according to one or a combination of 4 types, seminoma, embryonal, teratoma, and choriocarcinoma.

[b] As with bladder carcinoma described in Table 2 (Footnote *b*) tumors are staged according to the tumors-nodules-metastasis system (35).

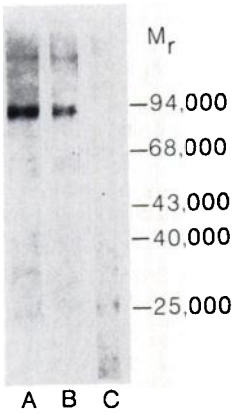

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Fig. 2. Autoradiogram after SDS-gel electrophoresis of ^{125}I-labeled cultured bladder tumor cells immunoprecipitated by 3G2-C6. Radiolabeling, immunoprecipitation, electrophoresis, and autoradiography are described in "Materials and Methods." Immunoprecipitates of ^{125}I-MGH-U1 (*A*), ^{125}I-MGH-U2 (*B*), and ^{125}I-MGH-U4 (*C*) cells. Standards are: phosphorylase A, M_r 94,000; bovine serum albumin, M_r 68,000; ovalbumin, M_r 43,000; aldolase, M_r 40,000; and chymotrypsin, M_r 25,000.

revealed that 3G2-C6 predominantly reacted with higher grades of TCC. Interestingly seminoma or mixed testicular tumors containing seminoma also reacted with 3G2-C6. These findings warrant further investigation as currently there is no useful marker for detection of seminoma. Another antibody, 2H3, displayed a pattern of reactivity to the bladder lines similar to 3G2-C6. It is possible that this McAb is either detecting a different epitope on the same antigen or binding to the same antigen but with a different affinity from 3G2-C6. Further studies with cross-absorptions and competitive binding might clarify the nature of each antigen and determine if the two are distinct.

One major problem usually encountered in the production and isolation of tumor specific MaAbs is the availability of a suitable normal cell counterpart. Normal cells are needed for the screening and characterization procedures to identify relevant McAbs. We have resolved this problem by using the collagen gel technique of Reznikoff *et al.* (40) to grow normal urothelial cells from ureter and bladder epithelium. Although epithelial cultures could be successfully passaged three of four times only cells from primary cultures of the initial explant outgrowth were used in our study. In this manner the cells would more probably represent the original histopathological status of the specimen and not be altered considerably by extended exposure to tissue culture conditions. It is possible, however, that the culture technique selects a subpopulation of epithelial cells capable of *in vitro* growth not representative of all cell types comprising the normal adult urothelium. Binding by the four McAbs in these studies was not detected at any significant level in over 30 such urothelial cultures. Other normal cell populations such as skin fibroblasts, peripheral blood lymphocytes, fetal lung cells, and normal erythrocytes also did not react significantly with these antibodies even though it is not uncommon to find normal cell antigens expressed to greater extent on malignant cells (23, 28, 53, 55). Depending

on the eventual applications of these McAbs further testing with appropriate normal tissues will be required.

Visualization of McAb 3G2-C6 binding by indirect immunofluorescence and precipitation of the antigen from ^{125}I-labeled bladder tumor cell membranes indicated that 3G2-C6 reacted with a membrane surface antigen. It is interesting to note that the immunogen for McAb 3G2-C6 was the highly passaged bladder tumor cell line MGH-U1 originated from a highly invasive TCC. The fact that McAb 3G2-C6 reacted against established TCC cell lines as well as fresh tumor cells implies that this antigen may be consistently expressed in advanced bladder cancer and preserved under *in vitro* conditions. The finding that the antigen detected by 3G2-C6 can be expressed both *in vitro* and *in vivo* also supports the value of using cultured cells for raising antitumor antibodies.

From immunofluorescence studies it was observed that positive staining tumors contained both reactive and nonreactive cells and negative specimens possessed almost no reactive cells. Perhaps a more sensitive assay method would be able to detect the presence of some 3G2-C6 antigen on nonreactive cells such as those which occur in low grade bladder tumors. Preliminary studies with flow cytometry (data not shown) indicate that fluorescent cells can be detected in bladder barbotages and biopsy specimens. A suspension of MGH-U1 cells for example contains 92% fluorescent cells. The differences in 3G2-C6 expression which occur within a single specimen, however, are consistent with the heterogeneous nature of tumors.

Recently other investigators have produced McAbs which react with high grade bladder TCC (13, 16, 30, 48) and with papillomas and low grade papillary carcinomas (13, 30) but none of these antibodies display patterns of reactivity analogous to 3G2-C6 and their corresponding antigens have not been identified. Masuko *et al.* (29), however, have characterized several antigens from TCC with McAbs, all with different molecular weights from the antigen recognized by 3G2-C6. They have also isolated three antibodies which react with T-24 and not with MGH-U1. In this study McAb 3G2-C6 was found to precipitate a M_r 90,000 membrane component from cultured bladder tumor cells and react to cells in urine and bladder barbotages from patients with high grade bladder cancer as indicated by immunofluorescence. Since works by others have indicated the presence of other bladder cancer associated antigens in urine (4, 9) it would also be interesting to determine if the 3G2-C6 antigen is shed from bladder tumor cells, if it is detectable in culture supernatants or urine specimens, and if its presence could be used as an indicator of bladder cancer.

The fact that 3G2-C6 reacts with tumor cells in the advanced stages of cancer may also provide an opportunity to approach the mechanism of antigen changes on the cell surface during malignant transformation. The analysis of cell surface antigens has recently begun to take on great significance in the prediction and diagnosis of human bladder cancer. Many studies document differences in antigen expression between normal and malignant bladder cells (39, 49, 56). For example the loss of normal blood group isoantigens ABH and the appearance of the Thomsen-Friedenreich or T-antigen in TCC have been correlated with the subsequent invasiveness of the disease and poor prognosis (51). In this study the antigen recognized by 3G2-C6 was not detected on normal erythrocytes of any type. The ability of McAb 3G2-C6 to bind primarily to advanced TCC may reflect a change in the cell surface which is manifested during malignant progression.

In summary we have produced four McAbs; one, 3G2-C6, detects a tumor associated M_r 90,000 cell surface protein present in high grade TCC of the bladder and the others react in varying amounts with bladder tumor cells. These McAbs may prove to have potential for detection, treatment, and study of the mechanism of malignant progression of human bladder cancer.

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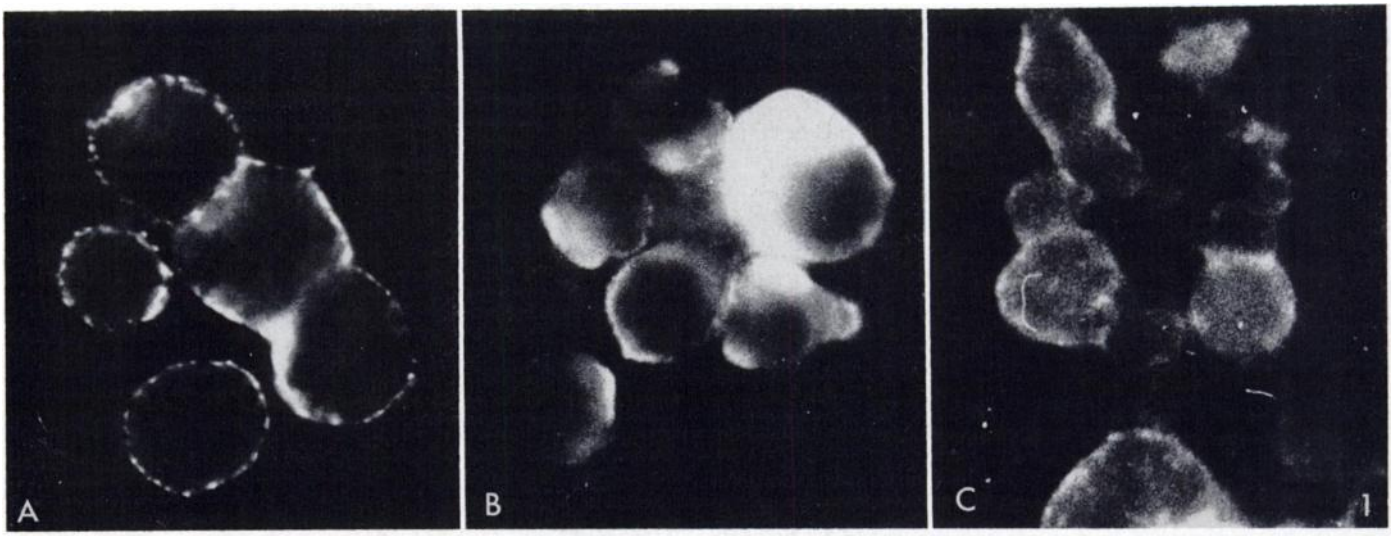

Fig. 1. Indirect immunofluorescent staining of human bladder tumor cells and tumor specimens with 3G2-C6. Cells were stained as described in Table 2. *A*, MGH-U1 bladder tumor cell line; *B*, a bladder tumor specimen with Grade 3 transitional cell carcinoma; *C*, a testicular tumor from patient with seminoma.