**ABSTRACT**

Two murine monoclonal antibodies (Mabs), 3G2-C6 and C3, which react to surface components on human bladder carcinoma cells, were produced using cultured human bladder tumor cells as immunogens. The expression of these antigens is highly restricted to malignant cells, and as such these Mabs are potentially useful for cancer diagnosis and treatment. We report here the immunochemical characterizations and molecular size determinations of these two Mab-reacting bladder tumor-associated antigens.

The 3G2-C6 antigen has a molecular weight of 92,000, while the C3 antigen is a macromolecule with a molecular weight of about 600,000, consisting of 4 subunits of identical size, as determined by high pressure liquid chromatography gel filtration and sodium dodecyl sulfate and gradient-gel electrophoresis. The apparent affinity constants for the binding of these two Mabs and the number of antigenic determinants per cell were determined in 4 human bladder tumor cell lines (MGH-U1 through MGH-U4) with different degrees of malignancy. The apparent affinity constants for 3G2-C6 ranged from $1.8 \times 10^{-7}$ to $1.7 \times 10^{-8}$ M, while those for C3 ranged from $1.9 \times 10^{-7}$ to $4.4 \times 10^{-8}$ M. The number of antigenic determinants per cell ranged from $3 \times 10^6$ to $2.9 \times 10^7$ for 3G2-C6 and from $2 \times 10^6$ to $1.2 \times 10^7$ for C3. This coincides with our earlier observation that bladder tumor cells of higher malignancy tend to express higher numbers of determinants of these antigens, particularly of 3G2-C6. Very low levels of both of the antigens were released from the cells; less than 10% of the antigens could be detected in 3-day spent culture medium. Radioactively labeled Mabs were used to assess the stability of the antibody bound to MGH-U1 cells. More than 70% of 3G2-C6 remained bound to the cell after 24 h, whereas more than 60% of C3 was lost from the cell and recovered in the culture medium as small fragments.

This information may be useful for the clinical applications of these Mabs, including the improvement of *in vitro* detection of bladder cancer through identification of exfoliated tumor cells and determining the potential utilities of these Mabs in *in vivo* localization of *in situ* and metastatic bladder tumors.

**INTRODUCTION**

A number of Mabs to human bladder carcinoma-associated antigens have been produced (1–12). The availability of these Mabs offers numerous potential clinical applications. One of the examples is the detection of bladder carcinoma through the identification of tumor cells in urinary specimens using Mabs to tumor cell surface antigens. The biological nature of this cancer and the inadequacy of the current cytological method offer a particular advantage for this application. Urothelial cancer cells have a high tendency to detach from the mucosa, and the exfoliated cells present in the urinary specimen provide an opportunity for their detection. Yet, the current standard method of identifying these cells, based on morphological criteria, is very ineffective in detecting low-grade, well-differentiated tumor cells (13, 14). Immunological methods, using Mabs to tumor-associated cell surface antigens, thus should provide the needed improvement. Several studies have already demonstrated the superiority of Mab methods over the conventional cytological method in detecting tumor cells in barbotage specimens from bladder cancer patients (15, 16).

Other potential applications for bladder carcinoma-reacting Mabs include *in vivo* tumor localization, either for carcinoma *in situ* on the bladder urothelium or metastatic tumor outside the bladder, and tumor therapy. The *in situ* detection application is particularly advantageous due to the fact that the bladder is an isolated organ with limited cell types present, thereby reducing the need for highly specific Mabs and the difficulty in detecting this type of tumor by the standard cystoscopy and biopsy procedures. However, none of these applications have been fully explored at the present time. This may be due to the fact that the immunological and immunochemical properties for many of these Mabs have not been adequately characterized to allow the assessment of their applications in these areas.

In our laboratory, we have generated several mouse Mabs to human bladder tumor-associated antigens. The production and immunological characterization of these Mabs have been reported previously (10). Two of these Mabs, 3G2-C6 and C3, produced by using cultured bladder tumor cells as immunogens, are both IgG1 and reacting to surface components of human bladder carcinoma cells. The presence of these antigens is highly restricted to malignant cells; thus these Mabs are potentially useful for cancer diagnosis and treatment. Recently, using Mab 3G2-C6 with an immunofluorescent method, we (16) were able to detect positive cells in 87% (56 of 64) of bladder washing specimens from patients with bladder carcinoma, including 18 of 19 patients with grade 1 carcinoma, in comparison with 37.5% (24 of 64) by the standard cytological method. However, further improvements are needed to enhance the detection of low-grade tumor cells and reduce the nonspecific reactions before the Mab method can be used for routine clinical diagnosis. In the present study, we report results of immunochromical characterizations and binding properties of these Mabs with their respective antigens. This information may be useful in further improvement of the *in vivo* detection method. It also suggests potential applications of these Mabs in *in vivo* localization of *in situ* and metastatic bladder carcinomas.

**MATERIALS AND METHODS**

**Cell Lines.** Human bladder tumor cell lines, MGH-U1, -U2, -U3, and -U4, used in this study have been established in our laboratory (17). These cell lines represent bladder transitional carcinomas with different degrees of malignancy indicated by their characteristics and origins. MGH-U1 and -U2 are sublines of T-24, a cell line derived from a poorly differentiated transitional cell carcinoma (18). MGH-U3 was derived from a well-differentiated grade 1 carcinoma and MGH-U4 from a patient with severe atypia of the urothelium which is considered to be a premalignant abnormality. MGH-U1 and -U2 were cultured in...
Mab C3 was removed by washing with 9 ml of 0.1 M glycine buffer, pH 2.8. Fractions of 1 ml were collected, and the activity. The unreacted glutaraldehyde was removed by washing the column with 0.1 M PBS, pH 8.1, before the radiolabeled cell membrane lysate was applied. Purification of C3 Antigen. C3 antigen was purified from cultured human bladder carcinoma cells (MGH-U1) by affinity chromatography. Cell surface proteins were radiolabeled with lactoperoxidase and NaI from the iodinated protein. The iodination protocol yielded I25I-C3 which was used to locate the antigen by immunoblotting. The reaction, the mixture was applied to a Sephadex G-25 column (PD-10; Pharmacia Chemical Co., Piscataway, NJ) to separate the free NaI25I from the iodinated protein. The iodination protocol yielded 125I-labeled 3G2-C6 and 125I-labeled C3 with specific activities of 0.82 and 0.85 GBq/mg, respectively, with NaI25I (Du Pont-New England Nuclear, Boston, MA) according to the procedure of Markwell (21) using Iodo-Beads (Pierce Chemical Co., Rockford, IL). Briefly, 100 µg of purified IgG in 0.5 ml PBS were mixed with 2.5 mCi of NaI25I. The Iodo-Beads were added and the mixture was incubated at room temperature for 15 min. At the end of the reaction, the mixture was applied to a Sephadex G-25 column (PD-10; Pharmacia Chemical Co., Piscataway, NJ) to separate the free NaI25I from the iodinated protein. Purification of C3 Antigen. C3 antigen was purified from cultured human bladder carcinoma cells (MGH-U1) by affinity chromatography. Cell surface proteins were radiolabeled with lactoperoxidase and NaI25I as described previously (10). Radiolabeled cells were washed in PBS and solubilized by 0.5% NP-40 with 1 mM phenylmethysulfonyl fluoride at 0°C for 30 min. The membrane lysate was centrifuged at 250,000 × g at 4°C for 1 h, and purification of the antigen from the solubilized radiolabeled lysate was accomplished by affinity chromatography using a glutaraldehyde cross-linked Affi-Gel-Mab C3 column prepared according to the method of Braesco-Andersen et al. (22). Briefly, a column was packed with 3 ml Affi-Gel (Sepharose-protein A; Bio-Rad) and equilibrated with 0.1 M PBS at pH 8.1. One mg of purified Mab C3 in 1 ml of PBS was added to the column, which was washed with 0.1 M PBS, pH 8.1. Then 0.05% glutaraldehyde was added to cross-link protein A with Mab C3. The total reaction time was held to less than 5 min to minimize the destruction of antibody activity. The unreacted glutaraldehyde was removed by washing the column with 3 ml of PBS followed by 25 ml of 2% glycer in PBS. Un-cross-linked Mab C3 was removed by washing with 9 ml of 0.1 M glycine buffer, pH 2.8, and the column was then reequilibrated with PBS, pH 8.1, before the radiolabeled cell membrane lysate was applied. The column was washed with 0.1 M PBS, pH 8.1, until the radioactivity of the eluate reached a minimum. The bound antigen was eluted with 0.1 M glycine buffer, pH 2.8. Fractions of 1 ml were collected, and the radioactivity was determined to locate the antigen peak. Fractions containing radioactivity were pooled, adjusted to pH 7.4, and concentrated by centrifugation in Centricron tubes (Amicon, Lexington, MA). Radiimmunoassay. A direct radioimmunoassay procedure was used to quantitate antibody-antigen binding. Culture human bladder tumor cells were used as the antigen source. The assay was performed in 96-well immunofiltration plates (V & P Scientific, Inc., San Diego, CA) which have a small filter disc located at the bottom of each well to retain cells and allow proteins to pass through. For the assay, cells were detached from culture with 0.1% EDTA in PBS, washed with PBS, resuspended in PBS buffer (10% FBS-1% bovine serum albumin-0.3% gelatin in PBS), and placed into the wells which had been precoated with the FBS buffer at 5 × 10^5 cells/well. After 3 washings with gelatin buffer (0.3% gelatin in PBS), 50 µl of diluted 125I-labeled Mab were added. The reaction was allowed to proceed at room temperature for 2 h with shaking. At the end of the reaction, each well was washed 3 times with the gelatin buffer. The filter disc was dried by vacuum and removed from the well, and the radioactivity was measured with a gamma counter.

Binding of the Mabs to Cells after Prolonged Incubation. To investigate the persistence of Mab binding to different bladder tumor cells in culture conditions, MGH-U1 and -U3 were placed in the culture dishes at 1 × 10^6 cells/well and incubated overnight to permit cell attachment. The cells were then incubated with 200 µl of diluted 125I-labeled Mab 3G2-C6 or C3 for 30 min at 37°C. Cells were then washed 3 times with PBS to remove unbound antibodies and incubated in the medium without antibodies. The culture medium was removed daily for 5 days and the amount of 125I-labeled Mab remained bound to the cells was determined with a gamma counter. The nature of the antibody released to the medium was also examined by applying the culture medium to a gel filtration column using HPLC. Gamma counting was used to locate the antibody eluted from the gel filtration column.

Immu-no-Dot. A microfiltration Immuno-Dot unit (Bio-Rad) was used to detect the presence of solubilized antigen. To attach the proteins to a nitrocellulose membrane, 100 µl of sample solution were applied to the membrane and permitted to soak through. The blocking solution (1% bovine serum albumin in 20 mM Tris with 0.5 mM NaCl, pH 7.5) was added and allowed to filter through the membrane. Following washing with Tween 20 washing buffer (20 mM Tris, 0.5 mM NaCl, and 0.05% Tween 20, pH 7.5), the diluted 125I-labeled Mab was added to the membrane and permitted to pass through. Unbound Mab was removed by washing with Tween 20 washing buffer, and the membrane was cut and the radioactivity counted.

Electrophoresis. Polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (20). Protein bands were visualized with Coomassie blue and radiolabeled proteins were visualized by autoradiographic analysis of the dried gels. Samples were extracted with 0.125 M Tris-HCl containing 1.25% SDS or 0.25% NP-40 by incubating at room temperature. Reduction of proteins, when performed, was done by 5% 2-mercaptoethanol. In the present study, 7.5% SDS-gel and 4-25% gradient gels with SDS or NP-40 were used. For gradient gels, both the running and stacking gels contained 0.1% SDS or NP-40. Gels were run at a constant voltage for 24 h in Tris-glycine buffer (pH 8.3) containing either 0.04% SDS or 0.1% NP-40. For SDS gels, myosin (M, 200,000), β-galactosidase (M, 116,000), phosphorylase b (M, 92,000), bovine serum albumin (M, 66,000), and ovalbumin (M, 45,000) were used as molecular weight standards. For NP-40 gradient gels, thyroglobulin (M, 670,000), ferritin (M, 450,000), phosphorylase a (M, 370,000), leucine aminopeptidase (M, 300,000), and catalase (M, 240,000) were used as standards.

Immunoblotting and Autoradiography. Proteins separated by SDS-gel electrophoresis were electrotransferred to a nitrocellulose membrane at 4°C for 5 days and the amount of I25I-labeled Mab remained bound to the membrane was determined by 5% 2-mercaptoethanol. In the present study, 7.5% SDS-gel and 4-25% gradient gels with SDS or NP-40 were used. For gradient gels, both the running and stacking gels contained 0.1% SDS or NP-40. Gels were run at a constant voltage for 24 h in Tris-glycine buffer (pH 8.3) containing either 0.04% SDS or 0.1% NP-40. For SDS gels, myosin (M, 200,000), β-galactosidase (M, 116,000), phosphorylase b (M, 92,000), bovine serum albumin (M, 66,000), and ovalbumin (M, 45,000) were used as molecular weight standards. For NP-40 gradient gels, thyroglobulin (M, 670,000), ferritin (M, 450,000), phosphorylase a (M, 370,000), leucine aminopeptidase (M, 300,000), and catalase (M, 240,000) were used as standards.

Determination of Affinity Constants and Binding Sites per Cell. The direct radioimmunoassay procedure described above was used to determine affinity constants for the binding of the Mabs to their respective antigens and the number of antibody-binding sites per cell. Briefly, serial dilutions of an 125I-labeled Mab were reacted with a constant number of tumor cells in a 96-well immunofiltration plate at room temperature for 2 h to allow the reaction to reach equilibrium. The unbound antibody was removed and amounts of bound antibody were...
measured by gamma counting. The concentrations of the bound and unbound antibody were calculated from the specific activity of the labeled antibody. Scatchard double-reciprocal plots of bound versus unbound antibody to different cell lines were obtained for each Mab. Affinity constants and the number of antigen determinants per cell were determined from the intercepts on the abscissa and ordinate (25, 26).

Detection and Quantitation of Shed Antigen. The amount of antigen shed by the cells in culture was determined by a competitive binding assay using spent culture medium as the source of shed antigen. MGH-U1 cells were grown in McCoy's 5A medium with 5% FBS. At the end of 3 days, spent medium was removed and the cell number was determined. The spent medium was centrifuged at 200,000 × g for 1 h to remove dead cells and debris, and the supernatant was concentrated 10-fold by ultrafiltration in a Centricon tube. For the assay, different volumes of the concentrated spent medium were mixed with a constant amount of 125I-labeled Mab. These were allowed to react for 3.5 h at room temperature before being added to immunofiltration wells each containing 2.3 × 10^5 MGH-U1 cells and allowed to bind competitively for 2 h at room temperature. A 10-fold concentrated fresh medium was used as the control. Reductions in binding of the antibody to the cell due to the presence of shed antigen in the medium was determined, and the percentage of antigen shed to the medium during the 3-day culture period was calculated from the number of cells in the competitive binding assay and number of cells in the source culture.

Molecular Weight Determination of the C3 Antigen. C3 antigen was radiolabeled with 125I as part of the cell surface proteins and then purified by antibody affinity chromatography as described above. The molecular weight of the antigen was determined by electrophoresis in 4–25% gradient gels containing NP-40 under nonreducing conditions and by HPLC gel filtration. For the latter, a Bio-Sil TSK-250 column (300 × 7.5 mm) (Bio-Rad) was used. The column was eluted with 0.1 M sodium phosphate buffer at pH 6.8. Successive 4-drop fractions were collected and gamma counting was used to locate the radiolabeled antigen. Thyroglobulin (M, 670,000), galactosidase (M, 520,000), apoferritin (M, 480,000), ferritin (M, 450,000), catalase (M, 240,000), IgG (M, 160,000), ovalbumin (M, 45,000), and myoglobin (M, 17,000) were used as molecular weight standards. To verify the identity of the antigen peak obtained from the HPLC column, NP-40 membrane extract from MGH-U1 cells was applied to the column, successive 4-drop fractions were collected, and their C3 antigen activity was assayed by immuno-dot analysis using 125I-labeled Mab C3. The number of subunits of the C3 antigen and their molecular weights were determined by applying the affinity-purified radiolabeled antigen to SDS-gel electrophoresis under reducing conditions. Radioautography of the dried gel was then used to detect the antigen subunits.

RESULTS

Specificity of 3G2-C6 and C3 Binding to Bladder Carcinoma Cells. Competitive radioimmunoassay was used to assess whether these two Mabs recognize the same or separate epitopes. The assay was performed by measuring the binding of 125I-labeled 3G2-C6 or C3 to MGH-U2 cells in excess amounts of unlabeled C3 or 3G2-C6, respectively. First, excess purified C3 (2.31 μg) was added to 5 × 10^4 cells and binding permitted to proceed at room temperature for 30 min. Different amounts of 125I-labeled 3G2-C6, ranging from 1.8 to 18 ng, were then added to the cells and incubated for 2 h at room temperature. The cells were washed with gelatin buffer and the amount of bound 125I-3G2-C6 was determined in a gamma counter. For comparison, the binding characteristics of different amounts of 125I-3G2-C6 added to MGH-U2 in the absence of C3 or in the presence of 3G2-C6 were also obtained. A similar competitive experiment was carried out using excess 3G2-C6 and different amounts of 125I-C3. The results (Fig. 1) indicated that there was no competition between the binding properties of the two Mabs, and thus they appeared to recognize two distinct antigenic determinants.

Fig. 1. Competitive binding between 3G2-C6 and C3 to cell surface antigens. In A, 5 × 10^4 MGH-U2 cells were incubated with 1.8 to 18 ng of 125I-3G2-C6 alone (+) and in the presence of 2.3 μg of C3 (■) or 12.5 μg of 3G2-C6 (▲). In B, 5 × 10^4 MGH-U2 cells were incubated with 3 to 30 ng of 125I-C3 alone (●) and in the presence of 30 ng of 3G2-C6 (■) or 4.6 μg of C3 (▲). Experiments were conducted in triplicates. Bars, SD.

Affinity Constants and Quantitation of Binding Sites. Direct radioimmunoassay was carried out to determine the affinity constants for binding of the two Mabs to four human bladder tumor cell lines. This was done by determining the binding of increasing amounts of 125I-labeled 3G2-C6 or C3 to a constant number of the cells. Double reciprocal plots obtained for all combinations of antibodies and cell lines were linear as expected for homogeneous monoclonal antibodies (Figs. 2 and 3). This enabled us to determine the affinity constants (Ks) from the intercepts at the negative extension of the abscissa and the number of binding sites per cell from the intercepts of the ordinates (26). Table 1 summarizes the results of these determinations. The bindings of 3G2-C6 and C3 to antigens on the four tumor cell lines all had high affinity, with Ks ranging from 1.8 × 10^-10 to 1.7 × 10^-8 M for 3G2-C6 and 1.9 × 10^-9 to 4.4 × 10^-8 M for C3.

There are significant differences in the numbers of 3G2-C6 antigen sites present among the cell lines examined. The poorly differentiated cells (MGH-U1 and -U2, 3.0 and 2.2 × 10^3 sites/cell, respectively) expressed about 100-fold higher amounts of 3G2-C6 than the well-differentiated cells (MGH-U3 and -U4, 2.9 and 5.4 × 10^2 sites/cell, respectively). The expression of the C3 antigen was less differentiation dependent. There was only a 10-fold difference between the poorly differ-

Fig. 2. Double-reciprocal plots of the binding of 3G2-C6 to four bladder tumor cell lines.
entiated (1.3 and $2.1 \times 10^6$) and the well-differentiated (1.2 and $1.4 \times 10^6$) cells. Also, compared to 3G2-C6, the C3 antigen was present at higher numbers among all the cell lines studied, ranging from 4- to 9.5-fold higher for MGH-U1 and -U2 cells to 41- and 26-fold higher for MGH-U3 and -U4 cells, respectively.

Detection of Shed Antigens in Spent Culture Medium. Antigen activities of 3G2-C6 and C3 in the spent culture medium of MGH-U1 cells were determined by a competitive inhibition assay. The results (Table 2) indicate that the antigens present in the 3-day spent media, as percentages of total cellular antigen, were quite low, at 10.8 ± 3.7% (SD) for 3G2-C6 and 6.2 ± 3.6% for C3 antigens. These amounts of antigen present in the spent medium were likely derived from the degradation of dead cells during the 3 days of culturing rather than from shedding of antigen from viable cells.

Binding of the Mabs to Cells after Prolonged Culturing. Fig. 4 shows the percentages of Mabs 3G2-C6 and C3 that remained bound to MGH-U3 and -U4 cells after various culture periods. The amount of cell-bound Mabs decreased continuously over the 5-day period, but the reductions occurred at different rates. The higher binding of 3G2-C6 over C3 was maintained in both cell lines, and the difference was greater in MGH-U3 compared to MGH-U1 cells. About 70% of 3G2-C6 remained attached to the cells after 24 h, while only about 20-40% of C3 remained. Further, loss of Mab from the MGH-U3 cells was greater than from the -U1 cells, suggesting a difference between these two cell lines with respect to their response to Mab binding. The nature of the antibody released into the spent medium was examined by HPLC-gel filtration. In all of the spent media obtained from both cell lines treated with both 125I-Mabs, materials eluted with radioactivity from the column were found in those fractions with a molecular weight of around 1000, indicating that these were degraded products of the antibody.

Molecular Weight of 3G2-C6 Antigen. The subunit molecular weight of the 3G2-C6 antigen has been previously determined to be about 90,000 (10). In the present study, the size of the antigen molecule and its number of subunits were determined by reducing and nonreducing SDS electrophoresis of radiolabeled cell membrane extracts. Western blotting and autoradiography were used to identify the antigen and its subunits on the gels. A distinct $M_r$, 92,000 component was detected with electrophoresis performed under either reducing or nonreducing conditions. This indicates that 3G2-C6 antigen consists mainly of a single polypeptide chain with a molecular weight of about 92,000. A minor component with a molecular weight of 88,000 was also present; this was detected only upon prolonged exposure as required for autoradiography (Fig. 5).

Molecular Weight and Subunits of C3 Antigen. Purification of the C3 antigen from 125I-labeled membrane extract using antibody affinity chromatography yielded a single peak of radioactivity when the column was eluted with glycine buffer to release the bound antigen. Determinations of the molecular weight of the purified C3 antigen were performed by nonreducing gradient gel electrophoresis, containing either 0.1% NP-40 or SDS, and by HPLC gel filtration. The result of NP-40 gradient gel electrophoresis revealed a single radioactive band at a molecular weight of $5.7 \pm 0.6 \times 10^3$ (Fig. 6A). Electrophoresis of the antigen in SDS-gradient gel and in 7.5% SDS-gel both under reducing conditions yielded a single band with a molecular weight of $1.4 \pm 0.1 \times 10^4$ (Fig. 6B and C). These results indicate that the C3 antigen is a $M_r$, 570,000 macromolecule composed of four identical $M_r$, 140,000 subunits.

A similar result was obtained from HPLC gel filtration of the purified antigen. The antigen peak, identified by its radioactivity, had a retention time of $6.6 \pm 0.12$ min (Fig. 7A), corresponding to a molecular weight of 640,000 (Fig. 7B). Further evidence that this component was indeed the C3 antigen was obtained from HPLC gel filtration of the unlabeled membrane extract of MGH-U1 cells. The binding activity of the eluted fractions to Mab C3 was determined by the Immuno-Dot method using 125I-labeled C3. Results shown in Fig. 7C indicate that peak C3 binding activity has a retention time of $6.4 \pm 0.1$ min, corresponding closely to that obtained for the radiolabeled, purified C3 antigen.

**DISCUSSION**

Table 3 summarizes the molecular characteristics of the antigens to 3G2-C6 and C3, as well as the immunochemical properties of interactions with their respective Mabs. It appears that the two Mabs have separate epitopes which are likely to be located on different antigens. Evidence of a separate antigen for each of the two Mabs includes (a) differences in molecular weights and number of subunits, (b) different numbers of antigenic determinants among different cultured bladder tumor cells, and (c) the results of an earlier study (10) which showed

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**Table 3. Affinity constants and antigen sites/cell of 3G2-C6 and C3 in bladder tumor cells with different degrees of malignancy**

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>3G2-C6</th>
<th>C3</th>
<th>Antigen sites/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGH-U1</td>
<td>8.5 ± 0.4 x 10^-10</td>
<td>6.8 ± 1.3 x 10^-10</td>
<td>3.0 ± 0.5 x 10^0</td>
</tr>
<tr>
<td>MGH-U2</td>
<td>8.5 ± 2.4 x 10^-18</td>
<td>1.2 ± 0.2 x 10^-10</td>
<td>2.2 ± 1.0 x 10^0</td>
</tr>
<tr>
<td>MGH-U3</td>
<td>1.8 ± 0.3 x 10^-19</td>
<td>4.4 ± 0.6 x 10^-10</td>
<td>2.9 ± 0.7 x 10^0</td>
</tr>
<tr>
<td>MGH-U4</td>
<td>1.7 ± 0.8 x 10^-19</td>
<td>1.9 ± 0.2 x 10^-10</td>
<td>5.4 ± 1.5 x 10^0</td>
</tr>
<tr>
<td>C3</td>
<td>1.4 ± 0.1 x 10^4</td>
<td>1.2 ± 0.2 x 10^4</td>
<td></td>
</tr>
</tbody>
</table>

*Mean ± SD.*
Table 2 Determinations of 3G2-C6 and C3 antigens in spent media of MGH-U1 cells after 3 days in culture

The assays were carried out by determining the inhibition of binding by various amounts of concentrated spent medium to antigen on 2.3 x 10^6 cells. The assays were carried out by determining the inhibition of binding by various amounts of concentrated spent medium to antigen on 2.3 x 10^6 cells.

<table>
<thead>
<tr>
<th>(A) Vol. of conc. spent medium added (μl)</th>
<th>(B) Cell equivalence (×10⁶)</th>
<th>(C) Antibody bound to cells (%)</th>
<th>(D) Inhibition of binding</th>
<th>(E) Shed antigen in spent medium* (10^⁶ cells)</th>
<th>(F) Shed antigen* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>3.6</td>
<td>79 ± 2.8</td>
<td>0.26 ± 0.04</td>
<td>0.6 ± 0.09</td>
<td>16.7 ± 2.5</td>
</tr>
<tr>
<td>50</td>
<td>7.2</td>
<td>76 ± 5.3</td>
<td>0.31 ± 0.07</td>
<td>0.7 ± 0.16</td>
<td>9.9 ± 2.2</td>
</tr>
<tr>
<td>75</td>
<td>10.8</td>
<td>66 ± 5.4</td>
<td>0.51 ± 0.09</td>
<td>1.2 ± 0.18</td>
<td>10.8 ± 1.7</td>
</tr>
<tr>
<td>100</td>
<td>14.4</td>
<td>61 ± 2.8</td>
<td>0.63 ± 0.05</td>
<td>1.5 ± 0.12</td>
<td>10.1 ± 0.8</td>
</tr>
<tr>
<td>120</td>
<td>17.4</td>
<td>67 ± 6.1</td>
<td>0.49 ± 0.09</td>
<td>1.1 ± 0.21</td>
<td>6.5 ± 1.2</td>
</tr>
</tbody>
</table>

3G2-C6 antigen

25                                     | 3.6                         | 88 ± 7.5                      | 0.14 ± 0.09              | 0.3 ± 0.20                                    | 8.6 ± 5.6           |
| 50                                     | 7.2                         | 85 ± 1.1                      | 0.34 ± 0.01              | 0.8 ± 0.02                                    | 10.8 ± 0.3          |
| 75                                     | 10.8                        | 80 ± 5.4                      | 0.25 ± 0.07              | 0.6 ± 0.16                                    | 5.4 ± 1.5           |
| 100                                    | 14.4                        | 78 ± 3.2                      | 0.28 ± 0.04              | 0.6 ± 0.09                                    | 4.4 ± 0.6           |
| 120                                    | 17.4                        | 88 ± 3.5                      | 0.13 ± 0.04              | 0.3 ± 0.09                                    | 1.7 ± 0.5           |

C3 antigen

25                                     | 3.6                         | 88 ± 7.5                      | 0.14 ± 0.09              | 0.3 ± 0.20                                    | 8.6 ± 5.6           |
| 50                                     | 7.2                         | 85 ± 1.1                      | 0.34 ± 0.01              | 0.8 ± 0.02                                    | 10.8 ± 0.3          |
| 75                                     | 10.8                        | 80 ± 5.4                      | 0.25 ± 0.07              | 0.6 ± 0.16                                    | 5.4 ± 1.5           |
| 100                                    | 14.4                        | 78 ± 3.2                      | 0.28 ± 0.04              | 0.6 ± 0.09                                    | 4.4 ± 0.6           |
| 120                                    | 17.4                        | 88 ± 3.5                      | 0.13 ± 0.04              | 0.3 ± 0.09                                    | 1.7 ± 0.5           |

* Number of cells from which the spent medium was obtained. Calculations are based upon 760 μl of concentrated spent medium obtained from 1.1 x 10^⁷ cells.

** Ratio between percentage of reduction of antibody bound to cell versus percentage of bound antibody bound to cells, calculated as

\[ \text{Inhibition of binding} = \frac{100\% - C}{C} \]

† Amount of antigen present in spent medium added, calculated from: (D) x 2.3 x 10^⁶ cells.

‡ Determined by

\[ \left( \frac{E}{B} \right) \times 100\% \]

§ Mean ± SD.

Fig. 4. Binding of the Mabs to bladder tumor cells, MGH-U1 and MGH-U3, after prolonged culturing. Cells, 1 x 10^⁶ cells/60-mm dish, were incubated with 125I-labeled Mabs, 3G2-C6 or C3, for 30 min at 37°C. Cells were then washed 3 times with PBS to remove unbound antibodies and incubated in the medium without antibodies. Cultured media were removed daily for 5 days and the amount of 125I-labeled Mab remaining bound to the cells was determined with gamma counting. Results are the average of three separate determinations. Bars, SD.

different reaction patterns for the two Mabs with respect to a large panel of tumor cells. Studies with tissue sections of human bladder tumors of different grades (27) also indicate that the expression of 3G2-C6 is relatively more differentiation dependent, with poorly differentiated tumor cells expressing higher levels of the antigen, while the expression of the C3 antigen is less differentiation dependent.

Both of these Mabs react with high affinity to their respective antigens. The apparent affinity constants are on the order of 10^-9 M, similar to several other tumor-associated antibody-antigen systems (26, 28-30). Levels of antigens detected in spent medium of the bladder tumor cells in culture were very low. This finding indicates that either the antigens are shed at very low levels or the shed antigens have lost their binding activity to the antibodies. Since the antigen activity detected in the 3-day spent media was about 10% of total antigen activity of the cells, it is likely that most of this activity was derived from the degradation of dead cells. This finding suggests that the approach for applying these Mabs to cancer diagnosis should be the detection of antigens on the cell surface, rather...
The complex, shedding of the complex (31), antigen modulation which this could occur include dissociation of antibody from antibody-antigen complex from the cells. Processes through prolonged culturing must be due to the release of Mabs or and inside, the disappearance of bound Mabs from cells upon quantitate Mabs remaining with the cell, both on the surface the reduction seems to depend on both antibody and cell type. than the search for soluble antigens in urine or sera.

Prolonged incubation of tumor cells with bound Mabs resulted in a progressive reduction of bound Mabs. The rate of the reduction seems to depend on both antibody and cell type. In the present study, since radiolabeled antibodies were used to quantitate Mabs remaining with the cell, both on the surface and inside, the disappearance of bound Mabs from cells upon prolonged culturing must be due to the release of Mabs or antibody-antigen complex from the cells. Processes through which this could occur include dissociation of antibody from the complex, shedding of the complex (31), antigen modulation (32), and cell death. Since the released antibody was found to be in the form of small degraded components, a process other than simple antibody-antigen dissociation is likely involved. In both cell lines, the cell-bound Mab C3 was reduced at significantly higher rates than the Mab 3G2-C6. Differences in antibody affinities in interactions with antigens on different cells may be partially responsible. However, the extent of this is unknown. Thus, it appears that different cells can behave quite differently in response to the same antibody binding to its antigenic determinant. Similar observations have been reported for the binding of Mabs to other tumor-associated antigens (26, 31).

There were a number of technical problems associated with the determination of molecular weight for C3 antigen due to its large size and association with the cell membrane. Thus, it was difficult to distinguish the antigen from membrane aggregates and undissociated membrane components. We also encountered considerable difficulty in trying to maintain C3 in the unaggregated form. The use of NP-40 proved to be effective for solubilizing the antigen from the membrane, maintaining it in an unaggregated state, and preserving its antibody binding activity; however, use of this agent may block the subsequent protein transfer onto the nitrocellulose membrane, thereby limiting the use of Western blotting for antigen identification. Electrophoresis on gradient gels and gel filtration with HPLC proved to be effective for determining the molecular weight of the macromolecule, although there was a 13% difference in results between the two methods. An unexpected finding was the dissociation of C3 antigen into subunits upon SDS-gel electrophoresis under nonreducing conditions (Fig. 7B). However, there have been several reports indicating that treatment by SDS under nonreducing conditions can dissociate holoproteins into their monomer subunits (33–35).

The chemical nature of the antibody binding sites for these two antigens has yet to be defined. Preliminary experimental data have indicated that the binding activity can be destroyed by heat, organic solvents, and fixatives such as acetone and formaldehyde, suggesting that they probably consisted of peptide components. For the C3 antigen, the fact that its dissociation into subunits resulted in the loss of antibody binding activity suggests a steric requirement for this interaction and implies that the binding site may be composed of several antibody-interacting loci that are not situated continuously on the same polypeptide chain.

Although many bladder carcinoma-associated antigens have been identified by Mabs (3–11), only a few have been characterized in terms of their molecular nature and immunological properties. Braesch-Anderson et al. (22) have isolated and characterized two bladder carcinoma-associated antigens. One, defined by Mab S2C6, has a molecular weight of 59,000, and another, defined by Mab 7E9, consists of two polypeptides with molecular weights of 29,000 and 23,000. Since the molecular properties of most of the other antigens have yet to be defined, it is difficult to assess whether the two described in our study are different from those previously reported. However, based on the different reaction patterns to different tumor cells and normal tissues, the antigens described in these reports appear to be distinct from ours.

Other Mabs have been described which detect tumor-associated antigens with molecular weights in the 90,000 range, similar to that of the 3G2-C6. These include SP-2 (36), B6.2 (37, 38), L3 (39, 40), and a large number of Mabs which recognize the melanoma-associated antigens gp87, gp95, or p97. The last three were later found to be the same antigen (41–43). Mab SP-2 was produced using proteins released into

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**Fig. 7.** Molecular weight determinations of C3 antigen by HPLC gel filtration. A, HPLC gel filtration chromatography of affinity-purified radiolabeled C3 antigen of MGH-U1 cells in a Bio-Sil TSK-250, 300 x 7.5-mm column (Bio-Rad). The retention time from 5 determinations was 6.64 ± 0.12 min. B, molecular weight determination of the C3 antigen by HPLC gel filtration by comparing the retention time of C3 with those obtained with molecular standards. Thyroglobulin (M, 670,000), galactosidase (M, 520,000), apoferritin (M, 480,000), ferritin (M, 450,000), catalase (M, 240,000), IgG (M, 160,000), ovalbumin (M, 45,000), and myoglobin (M, 17,000) were used as the standards. C, binding activity of HPLC fractions to Mab C3. Unpurified NP-40 membrane extract from MGH-U1 cells was applied to the column and fractions were assayed for C3 binding activity by an Immuno-Dot assay. Results of 5 separate experiments indicated that the peak binding activity was located at a retention time of 6.4 ± 0.1 min.

**Table 3** Summary of molecular and immunological characteristics for 3G2-C6 and C3 antigens

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Molecule</th>
<th>Molecular Weight</th>
<th>No. of Subunits</th>
<th>Molecular Weight</th>
<th>Antibody Isoform</th>
</tr>
</thead>
<tbody>
<tr>
<td>3G2-C6</td>
<td>MGH-U1</td>
<td>90,000</td>
<td>1</td>
<td>600,000</td>
<td>IgG1s</td>
</tr>
<tr>
<td>C3</td>
<td>MGH-U2</td>
<td>89,000</td>
<td>4 identical</td>
<td>59,000</td>
<td>IgG1a</td>
</tr>
</tbody>
</table>

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tissue culture fluid by a human breast cancer cell line as the immunogen. The antigen appears to be a proteolipid expressed in a significant number of breast cancer tumors and can be detected in the sera of patients with the cancer (36). However, the fact that the antigen is not localized on the cell surface and is readily shed by the cells indicates that it is not identical to 3G2-C6. The antigen recognized by Mab B6.2 is a cell surface glycoprotein. The epitope for the Mab is stable to formalin. The expression of B6.2 antigen is highly restricted to breast carcinoma cells and is cell cycle dependent. Anti-lung tumor Mab L3 recognizes the same melanoma-associated cytoplasmic antigen identified by Mab 465.12S (37, 38). The antigen is a glycoprotein and is localized in the cytoplasm as well as on the cell surface. It is sensitive to SDS, is shed by the cells, and can be found in serum and medium of cultured tumor cells. The melanoma antigen identified as p97, gp87, and gp95 by different investigators is structurally related to transferrin and is an integral protein of the cell membrane (44). The antigen is also shed by the cell and is present in culture fluids. In this regard, it is different from 3G2-C6. Another melanoma antigen, FD, is also a glycoprotein with a molecular weight of 90,000 (45, 46). However, the expression of this antigen is restricted to a very limited number of cells and it has been shown that anti-serum to the antigen does not react with bladder carcinoma cells including T24, the parent cell line of MGH-U1 (45). The separate identity of antigen 3G2-C6 with respect to some of these other antigens cannot be entirely ruled out until direct blocking experiments with different Mabs (41), are performed. A large number of tumor-associated macromolecular antigens has been reported with molecular sizes ranging from 2.5 × 10^5 to over 10^6. Several of these (28, 47–51) are in the molecular weight range of C3. These include CA-3 (700,000), NCRC-11 (>400,000), KA-32 (>500,000), CA 125 (200,000–1,000,000), O1.95-45 (500,000) and, 225.28S (700,000). Due to their large sizes, the molecular weights reported for these antigens are less precise and detailed characterizations for their molecular nature are still lacking. Therefore, the possible overlapping identity of C3 with any of these antigens requires further investigation.

Although many Mabs have been produced with varying reactivity and specificity to bladder carcinoma (3–12), only a few have demonstrated clinical utilities. This may be due to the fact that a great majority of these have not been sufficiently characterized immunologically and immunochemically to allow the assessment of their clinical potentials. The two Mabs described in the current study have been shown to react selectively to certain populations of bladder tumor cells and the expression of their reacting antigens in malignant bladder tumor cells is about 2 orders of magnitude greater than in normal urothelial cells (10). Mab 3G2-C6 has also been shown to have potential in urinary cytological detection of bladder carcinoma (16). Information from current study indicates that this Mab may be suitable for in vivo detection and localization of in situ or metastatic bladder tumors. For either of these tumors, there is no effective means of detection available currently. The high affinity binding, low shedding of the antigen, and stable binding of the antibody to cells should favor a high degree and more specific binding between the antibody and the antigen and a low level of circulating antigen, thus a low background noise of detection. On the other hand, Mab C3, due to its relative uniform expression among different bladder tumors, may have potential utility in therapy. Further evaluations on distributions of these antigens in normal tissues will be needed to substantiate these prospects. 

REFERENCES

TWO BLADDER-ASSOCIATED Mab-REACTING ANTIGENS


Immunochemical and Biochemical Characterizations of Two Monoclonal Antibody-reacting Antigens Associated with Human Bladder Carcinoma

Duan-sun Zhang and Chi-Wei Lin