Characterization of the Shed Form of the Human Tumor-associated Glycoprotein (TAG-72) from Serous Effusions of Patients with Different Types of Carcinomas

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ABSTRACT

Monoclonal antibody (MAb) B72.3 binds a high molecular weight tumor-associated glycoprotein designated TAG-72. This study reports the isolation and characterization of secreted TAG-72 directly from effusions of ovarian, colorectal, pancreatic, and endometrial carcinoma patients and compares them to TAG-72 derived from the LS-174T colon carcinoma xenograft. The B72.3-reactive antigen, TAG-72, was used as immunogen to produce second generation anti-TAG-72 MAb. One of these second generation MAb, CC49, had a higher affinity than that of B72.3 and was utilized as an affinity reagent in a procedure to purify the TAG-72 present in the serous effusions of carcinoma patients. A three-step purification procedure, utilizing heat extraction, CC49 antibody affinity chromatography, and gel filtration chromatography, resulted in a 1000- to 4400-fold purifications of the TAG-72 derived from effusions, as analyzed using a double-determinant radioimmunoassay. Radiolabeled TAG-72 from each of the effusions demonstrated similar high molecular weight bands on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Similar results from the various effusions were also obtained in Western blotting analyses. Analyses of TAG-72 from the different effusions in radioimmunoassay using five different anti-TAG-72 MAb revealed similar binding patterns. The results of these studies thus indicate that TAG-72 obtained directly from patients with ovarian, colorectal, endometrial, and pancreatic carcinomas and the LS-174T xenograft are highly similar in terms of immunochemical properties and antigenic profile.

INTRODUCTION

The human tumor-associated antigen defined by the murine MAb B72.3, designated TAG-72 (1-9), is a high molecular weight glycoprotein with the characteristics of a mucin (8, 9). MAB B72.3 was prepared using a membrane-enriched fraction from a human metastatic breast carcinoma as an antigen (1). Other MAbS, generated by immunizations with tumors derived from different sites, have also been reported to react with high molecular weight or mucin-like glycoproteins (10-19) but have been shown to be distinct from B72.3 (20). Immunoperoxidase studies have demonstrated that MAB B72.3 reacts with a broad spectrum of epithelial malignancies, including the majority of colon adenocarcinomas, invasive ductal carcinomas of the breast, non-small cell lung carcinomas, and ovarian, gastric, and pancreatic carcinomas (2-7). MAB B72.3 shows little or no reactivity to most normal adult tissues but does react strongly with secretory phase, but not proliferative or resting, endometrium (21). The TAG-72 antigen has been shown to be shed into the serum of patients with carcinomas (22-24). Approximately 40% of patients with gastrointestinal malignancies (55% of patients with advanced disease), 36% of patients with lung adenocarcinomas, and 24% of patients with ovarian carcinomas (53% of stage IV patients) had serum TAG-72 levels elevated above 6 units/ml, as opposed to only 3.5% of a control population and 6.7% of patients with benign gastrointestinal diseases (22).

Affinity-purified TAG-72 antigen from the human colon carcinoma xenograft LS-174T was utilized as an immunogen, and second generation anti-TAG-72 MABs were produced and characterized (25). All of the second generation antibodies gave immunostaining patterns virtually identical with those of B72.3 in Western blotting procedures (25). One of the second generation MAbS, designated CC49, has been utilized with MAB B72.3 in a DRIA to detect the presence of reactive antigen in the sera of patients with various carcinomas (22). MAB CC49 was also selected for utilization in an affinity chromatography step of a larger scale purification because of its higher binding affinity than B72.3 (16.2 x 10^6 M^-1 versus 2.5 x 10^9 M^-1) (9, 25).

MAB B72.3 has been reported to react with the O-linked disaccharide, N-acetylenuraminic acid-a2-6-N-acetylgalactosamine-α1→O-Ser/Thr (sialosyl-Tn structure) (26, 27). Competitive binding studies show that some of the epitopes recognized by the panel of second generation anti-TAG-72 antibodies appear to be structurally or spatially related to each other, but none appear to be identical (28). Mucins are a diverse class of molecules, and mucins derived from different types of carcinomas have been shown to express different tumor-associated and blood group epitopes and to differ in their overall biochemical characteristics (8-20). A tumor-associated mucin polypeptide has been shown to undergo altered glycosylation and present different epitopes when expressed in different tissues (11, 12, 15, 16). A carcinoma-associated ganglioside epitope has also been shown to reside on mucins in the sera of carcinoma patients (13). The overall goal of this study therefore was to determine whether TAG-72 expressed by different types of carcinomas and secreted forms of TAG-72 might differ immunochemically from the previously characterized TAG-72 isolated from colon carcinoma tissue (8, 9).

The purpose of the present investigation was 2-fold: (a) to develop a method to isolate the shed form of TAG-72 from malignant effusions of patients and (b) to make immunochemical comparisons between the TAG-72 derived from malignant effusions of different carcinoma types and the TAG-72 derived from the LS-174T colon carcinoma xenograft.

MATERIALS AND METHODS

Generation and Source of MAbS. MAB B72.3, CC15, CC49, CC83, and CC92 were prepared as previously described (1, 25). MAB CC15 is an IgG2b; the remainder of the MAbS are of the IgG1 isotype.

Purification of MAbS. MAb IgG were purified from ascitic fluids by ammonium sulfate precipitation followed by anion-exchange chromatography or by protein A-Sepharose CL-4B chromatography (29). The
TAG-72 FROM PATIENTS' EFFUSIONS

Table 1  TAG-72 levels in patients' effusions and in LS-174T cell line grown as a xenograft in athymic mice

<table>
<thead>
<tr>
<th>Source</th>
<th>Patient</th>
<th>Site of primary tumor</th>
<th>Activity (units/ml)</th>
<th>Protein (mg/ml)</th>
<th>Specific activity (units/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascites effusion</td>
<td>1</td>
<td>Ovarian</td>
<td>255</td>
<td>46.0</td>
<td>5.5</td>
</tr>
<tr>
<td>Ascites effusion</td>
<td>2</td>
<td>Colorectal</td>
<td>357</td>
<td>32.4</td>
<td>11.0</td>
</tr>
<tr>
<td>Pleural effusion</td>
<td>3</td>
<td>Pancreatic</td>
<td>614</td>
<td>32.5</td>
<td>18.9</td>
</tr>
<tr>
<td>Ascites effusion</td>
<td>4</td>
<td>Endometrial</td>
<td>690</td>
<td>98.2</td>
<td>7.0</td>
</tr>
<tr>
<td>Human colon carcinoma xenograft LS-174T</td>
<td></td>
<td></td>
<td>10,950</td>
<td>10.32</td>
<td>1061.0</td>
</tr>
</tbody>
</table>

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protein concentrations of the purified IgG were estimated using a bicinchoninic acid assay procedure (30).

Isolation of TAG-72. The following TAG-72-containing serous effusions from patients were obtained from Drs. W. W. Johnston and C. A. Szpak (Department of Pathology, Duke University Medical Center): a moderately differentiated grade II adenocarcinoma of the colon (Dukes stage C-2); an adenocarcinoma of the pancreas; a well-differentiated grade I, stage 1A papillary adenocarcinoma of the endometrium; and a moderately differentiated stage 4 papillary adenocarcinoma of the ovary. All tissue was stored at −20°C until used. Fifty ml from the pancreatic and endometrial carcinoma patients and 25 ml from the colorectal adenocarcinoma patients and 25 ml from the pancreatic and endometrial carcinoma patients were used as starting material for the purification procedure. Effusions were thawed and clarified by centrifugation at 10,000 and 100,000 × g for 40 min and diluted with extraction buffer (50 mM Tris, pH 8.5-150 mM NaCl) to a final protein concentration of 10 mg/ml.

LS-174T, a human colon adenocarcinoma cell line (31), was obtained from the American Type Culture Collection (Rockville, MD). Tumor cells were inoculated s.c. into athymic mice as described previously (8). After 2 or 3 weeks, tumors were harvested and frozen. Tumors and the soluble fraction were reduced with 50 mM dithiothreitol and then alkylated with 150 mM iodoacetamide in the same buffer. The fraction was then dialyzed against 10 mM NH4HCO3 and concentrated by lyophilization.

Immunoadsorbent was prepared by coupling 40 mg of purified Mab CC49 to 20 ml of 2-fluoro-1-methylpyridinium toluenesulfonate-activated Avid- Gel F (Bioprobe International, Inc., Tustin, CA) (32). The immunoadsorbent was equilibrated with PBS and packed in a column (1.5 x 10 cm). The heat-extracted material was applied to the column and washed successively with three buffers (0.5 M NaCl + 0.1 M NaCH3COO, pH 4.0; 0.5 M NaCl + 0.1 M NaB4O7, pH 8.0; and PBS) until the absorbence of the washes was <0.01 at 280 nm. Elution of the antigen from the adsorbent was carried out with 3 M NaCl in PBS. The eluted antigen fractions were dialyzed against 10 mM NH4HCO3 and concentrated by lyophilization.

The affinity-purified fraction was dissolved in 2.5 ml 6 mM guanidine HCl, clarified by centrifugation, and applied to a Superose-6 gel filtration column (1.6 x 50 cm) (Pharmacia, Uppsala, Sweden) equilibrated in 6 mM guanidine HCl. Elution was carried out with the equilibration buffer at a flow rate of 0.5 ml/min. The immunoreactive material was detected by ELISA as described below.

Estimation of Protein. Protein concentration at each purification step was estimated using a bicinchoninic acid assay (30) (Pierce, Rockford, IL). Since the efficiency of these procedures for detection of highly glycosylated polypeptides may be less than that for nonglycosylated proteins, protein concentrations in purified fractions (Superose-6, zone I) were estimated by amino acid analysis (33), and the values obtained by the bicinchoninic acid were corrected accordingly.

For amino acid analysis, samples were hydrolyzed with 6N HCl + 0.2% 2-mercaptoethanol in evacuated tubes at 110°C for 24–48 h. Analysis was carried out by ion-exchange chromatography on a Beckman 121 MB amino acid analyzer or by reversed phase chromatography following o-phthalaldehyde conversion (33).

Radioimmunoassay. Quantitative measurement of antigenic reactivity was carried out using the CA 72-4 double-determinant assay (Centocor, Malvern, PA) as previously described (22). TAG-72 specifically bound to immobilized MAb CC49 was quantitated using 125I-labeled B72.3 antibody. One unit of antigen is arbitrarily defined as the amount of TAG-72 reactivity found in 1 µg of protein from a standard crude extract of LS-174T xenograft.

ELISA. ELISAs were carried out using antigens immobilized on 96-well polystyrene microtiter plates (34). Two µl of Superose-6 column fractions or 1 unit of purified TAG-72 was used as target antigen. The final volume was adjusted to 50 µl with PBS and dried overnight. Nonspecific protein absorption was blocked with 5% BSA in PBS for 1 h at 37°C, and hybridoma supernatants or purified Mabs (50 µl) were added. After incubation at 37°C for 1 h, the unbound IgG was removed by washing the plates with 1% BSA in PBS. Horseradish peroxidase-labeled, affinity-purified goat anti-mouse IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was then added. After an additional 1-h incubation at 37°C, the unbound second antibody was removed by washing the plates with 1% BSA in PBS, and the substrate, 0.8 mg/ml o-phenylenediamine (Sigma Chemical Co., St. Louis, MO) + 0.0125% H2O2 + 0.1% sodium citrate, pH 5.0, was added for 10 min at room temperature. Absorbance at 490 nm was determined.

Radiolabeling of TAG-72. Purified preparations of TAG-72 were labeled with Na125I using iodogen (35) (Pierce, Rockford, IL). Approximately 5 x 106 cpm of samples was analyzed by SDS-PAGE (36) in 3–12% gels under reducing conditions and visualized by autoradiography.

Western Blotting Analysis. Proteins separated by SDS-PAGE (36) in
TAG-72 FROM PATIENTS' EFFUSIONS

TAG-72 antigen was purified by a three-step procedure consisting of extraction of supernatant at 100°C, followed by CC49 antibody-affinity chromatography and Superose-6 gel filtration chromatography. At each step, TAG-72 was quantitated by DDRIA assay (22) and the protein concentration by bicinchoninic acid assay (30).

RESULTS

Isolation of TAG-72. Forty-three effusions from patients with various carcinomas were screened for TAG-72 antigen level, and effusions with relatively high antigen levels (>250 units/ml) were selected for further purification (Table 1). For comparative purposes, TAG-72 derived from LS-174T (8, 9) was also used. The activity in the effusions ranged from 255-690 units/ml and from 5.5-18.9 units/mg protein, whereas extracts of LS-174T xenografts had an initial specific activity of 1061 units/ml and from 5.5-18.9 units/mg protein, whereas extracts of LS-174T xenografts had an initial specific activity of 1061 units/ml and from 5.5-18.9 units/mg protein. A major consideration in the design of a purification scheme was the limited quantity and low specific activity of TAG-72 in the samples. Isolation of TAG-72 from effusions is shown in the flow chart (Fig. 1).

Effusion supernatants and extracts of LS-174T xenografts were initially subjected to heat extraction, reduction, and alkyl-}


tation. This treatment did not alter the immunoreactivity of TAG-72 to MAbs CC49 and B72.3 and resulted in a 12- to 15-fold purification of the TAG-72 antigen with yields of 62-121% of the starting material. Isolation of TAG-72 from effusions is shown in the flow chart (Fig. 1).

Affinity chromatography of the reduced and alkylated extracts, utilizing immobilized CC49, resulted in the recovery of TAG-72 activity in the range of 44-121% of the starting material. Cumulative purifications of >160-fold were achieved at this point (Table 2).

Gel filtration chromatography of the CC49 antibody affinity-purified effusion and LS-174T fractions on a Superose-6 column gave the elution patterns shown in Fig. 2. Six molar guanidine HCl was included in the elution buffer to minimize nonspecific aggregation of the contaminants with the TAG-72. The absorbence patterns at 280 nm comprise a well-separated minor peak coeluting with the antigenic activity at the void volume followed by two major protein peaks without activity. These results suggest that the high molecular weight TAG-72 contributes relatively little to the total protein absorbence pattern and Superose-6 is an excellent choice to separate high molecular weight antigen from the residual contaminants such as serum albumin, globulins, and immunoglobulin of mouse origin. Superose-6, zone I, fractions (Fig. 2) obtained at this step did not contain immunologically detectable mouse IgG. Zone II fractions contained lower molecular weight contaminants and were not used for further immunochemical characterization. Affinity-purified colorectal and pancreatic effusions (not shown) gave gel filtration patterns quite similar to those of the ovarian effusion (Fig. 2A). The overall yield of TAG-72 for the entire procedure was in the range of 20-29% with purifications of 750- to 4400-fold (Table 2).

Analysis of Radiolabeled Proteins. Material from the excluded fractions of the Superose-6 column, zone I, was radioiodinated and analyzed under reducing conditions by SDS-PAGE. Superose-6, zone I, material derived from ovarian, colorectal, pancreatic, and endometrial effusions migrated as a broad high molecular weight band after iodination (Fig. 3, lanes A–D). Lower molecular weight bands (less than approximately 200,000) were not visualized, indicating that the preparations are free of lower molecular weight contaminants.

Western Blotting Analysis. Pooled immunoreactive material from the excluded fractions (zone I) of the Superose-6 column (Fig. 2) was subjected to SDS-PAGE and Western blot analysis

<table>
<thead>
<tr>
<th>Step</th>
<th>Total (units)</th>
<th>Total protein (mg)</th>
<th>Specific activity (units/mg)</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovarian effusion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supernatant</td>
<td>12,750</td>
<td>2300</td>
<td>5.5</td>
<td>1</td>
</tr>
<tr>
<td>Heat extract</td>
<td>9,438</td>
<td>146</td>
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<td>12.3</td>
</tr>
<tr>
<td>CC49 affinity</td>
<td>10,724</td>
<td>5.34</td>
<td>2,008.0</td>
<td>365.0</td>
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<tr>
<td>Superose-6 (zone I)</td>
<td>3,037</td>
<td>0.135°</td>
<td>22,512.0</td>
<td>4,093.0</td>
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<tr>
<td>Colorectal effusion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supernatant</td>
<td>17,850</td>
<td>1620</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
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<td>16,710</td>
<td>115</td>
<td>145</td>
<td>13.2</td>
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<tr>
<td>CC49 affinity</td>
<td>19,094</td>
<td>6.2</td>
<td>3,080</td>
<td>280.0</td>
</tr>
<tr>
<td>Superose-6 (zone I)</td>
<td>4,634</td>
<td>0.152°</td>
<td>30,500</td>
<td>2,772.0</td>
</tr>
<tr>
<td>Pancreatic effusion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supernatant</td>
<td>15,350</td>
<td>813</td>
<td>18.9</td>
<td>1</td>
</tr>
<tr>
<td>Heat extract</td>
<td>13,472</td>
<td>58</td>
<td>234.3</td>
<td>12.4</td>
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<tr>
<td>CC49 affinity</td>
<td>18,534</td>
<td>3.7</td>
<td>5,099.0</td>
<td>265.0</td>
</tr>
<tr>
<td>Superose-6 (zone I)</td>
<td>3,630</td>
<td>0.193°</td>
<td>18,810.0</td>
<td>995.0</td>
</tr>
<tr>
<td>Endometrial effusion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supernatant</td>
<td>17,250</td>
<td>2455</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>Heat extract</td>
<td>16,989</td>
<td>160</td>
<td>105.9</td>
<td>15.1</td>
</tr>
<tr>
<td>CC49 affinity</td>
<td>12,251</td>
<td>2.2</td>
<td>5,594</td>
<td>797.0</td>
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<tr>
<td>Superose-6 (zone I)</td>
<td>5,017</td>
<td>0.162°</td>
<td>30,960</td>
<td>4,423.0</td>
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<tr>
<td>LS-174T xenograft</td>
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<tr>
<td>Supernatant</td>
<td>360,499</td>
<td>339.6</td>
<td>1,061</td>
<td>1</td>
</tr>
<tr>
<td>Heat extract</td>
<td>225,000</td>
<td>30.4</td>
<td>7440</td>
<td>7</td>
</tr>
<tr>
<td>CC49 affinity</td>
<td>159,750</td>
<td>9.2</td>
<td>173,641</td>
<td>164</td>
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<tr>
<td>Superose-6 (zone I)</td>
<td>70,544</td>
<td>0.888°</td>
<td>801,000</td>
<td>755</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, % recovery.  
° Protein content estimated by amino acid analysis.
Fig. 2. Gel filtration chromatograms of affinity-purified TAG-72 on Superose-6 column (1.6 x 50 cm) equilibrated with PBS containing 6 M guanidine HCl. A, affinity-purified fraction from malignant ovarian effusion; B, affinity-purified fraction from malignant endometrial effusion; C, affinity-purified fraction from LS-174T xenograft. O, the TAG-72 antigen profile as measured by ELISA.

(Fig. 4). We have shown previously the specificity of CC49 in Western blotting, using an isotype-identical negative control MAb (MOPC-21) (25). The latter control was used in the present studies and again demonstrated no reactivity (not shown). CC49 stained a high molecular weight band in the Superose-6, zone I, material derived from ovarian, colorectal, pancreatic, and endometrial effusions and from LS-174T xenografts (Fig. 4, lanes A–E).

Reactivities of MAb with TAG-72. TAG-72 isolated from ovarian, colorectal, pancreatic, and endometrial effusions and from LS-174T xenografts was tested for reactivity with MAbs B72.3 (Fig. 5A), CC92 (Fig. 5B), CC83 (Fig. 5C), CC49 (Fig. 5D), and CC15 (data not shown). Each of these MAbs has previously been shown to react with a different epitope (28). Each MAb showed similar reactivity with 1 unit of TAG-72 regardless of the source. These results suggest that the serological profile of shed TAG-72 is highly similar on preparations derived from different tumor types.

DISCUSSION

The TAG-72 antigen has been isolated from malignant effusions of four different carcinoma types. The purification scheme utilized in this paper was designed to work with clinical specimens containing relatively low titers of the antigen. Our rationale for the isolation of TAG-72 from these effusions was to determine the extent to which the physical and immunochemical properties of TAG-72 derived from various malignancies would resemble that of the previously characterized TAG-72 from the long-term established LS-174T colon carcinoma xenograft line.
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Fig. 4. Western blot analysis of purified TAG-72 (Superose-6, zone I) after SDS-PAGE (3–12% gradient gel) analysis under reducing conditions. MAb CC49 was utilized for detection. TAG-72 was derived from the following: lane A, malignant ovarian effusion; lane B, malignant colorectal effusion; lane C, malignant pancreatic effusion; lane D, malignant endometrial effusion; lane E, LS-174T xenograft. Ordinate, M, in thousands.

Fig. 5. Reactivities of purified MAb B72.3 and CC MAbs to purified TAG-72 preparations (Superose-6, zone I) using ELISA. Dilutions of MAb B72.3 (A), CC92 (B), CC83 (C), and CC49 (D) were tested with one DDR1A unit of TAG-72 isolated from malignant ovarian (●), colorectal (○), pancreatic (●), and endometrial effusions (□) and the LS-174T xenograft.

REFERENCES


due to the removal of interfering proteins from the preparations.

Another major increase in the specific activity of the TAG-72 preparations occurred after immunoaffinity chromatography. However, elution of the bound TAG-72 also resulted in the release of some IgG from the column. This IgG and most other remaining contaminating proteins were removed by gel filtration chromatography on a Superose-6 column in 6 M guanidine HCl. This particular matrix optimized the separation of the lower molecular weight proteins from the mucin.

It is of interest that the Western blotting analyses showed very similar broad, high molecular weight bands from the TAG-72 isolated from the various malignant effusions and the LS-174T xenografts. These results were also very similar to those obtained by direct radioiodination and SDS-PAGE/autoradiography of the TAG-72 derived from the malignant effusions. Radioiodinated TAG-72 derived from the LS-174T xenograft has also shown a similar high molecular weight band on SDS-PAGE analysis (25). These results suggest that the overall molecular size of the TAG-72 isolated from the various malignant effusions and LS-174T xenografts is very similar and that the TAG-72 prepared from these sources is relatively free of lower molecular weight contaminants.

TAG-72 derived from the malignant effusions and from LS-174T was tested with several anti-TAG-72 MAbs. Each MAb tested reacted in a very similar manner regardless of the source of TAG-72. These results suggest that the profile of B72.3 and CC epitope expression and the overall immunochemical properties of TAG-72 preparations isolated from different types of malignancies are very similar.
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