Analysis of a Human Tumor-associated Glycoprotein (TAG-72) Identified by Monoclonal Antibody B72.3

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ABSTRACT

Monoclonal antibody B72.3 binds a high-molecular-weight tumor-associated glycoprotein identified as TAG-72. This study reports the partial purification and characterization of TAG-72 from a xenograft of a human carcinoma cell line, LS-174T, which expresses high levels of this antigen. The tumor homogenate was initially fractionated by Sepharose CL-4B chromatography. The high-molecular-weight TAG-72, found in the exclusion volume, was then subjected to two sequential passages through B72.3 antibody affinity columns. At each step of the procedure, TAG-72 content was quantitated using a competition radioimmunoassay, and the degree of purification was expressed as the ratio of antigen in units to total protein. The three-step procedure produced a purification of TAG-72 with minimal contamination by other proteins as shown by polyacrylamide gel electrophoresis, followed by staining with Coomassie Blue or periodic acid/Schiff reagent. The density of affinity-purified TAG-72, as determined by cesium chloride gradient ultracentrifugation, was found to be 1.45 g/ml. This density determination, together with the high molecular weight of TAG-72, its resistance to Chondroitinase digestion, the presence of blood group-related oligosaccharides, and sensitivity to shearing into lower-molecular-weight forms suggest that TAG-72 is a mucin-like molecule.

INTRODUCTION

Monoclonal antibody B72.3 was generated using a membrane-enriched fraction from a mammary carcinoma metastasis (1). Immunoperoxidase studies have demonstrated that B72.3 reacts with approximately 50% of breast carcinomas (2) and to greater than 80% of colon carcinomas (3) but shows no significant reactivity with normal adult liver, spleen, heart, breast, uterus, bone marrow, colon, stomach, salivary gland, lymph node, or kidney (2, 3). Monoclonal antibody B72.3 is useful in RIAs to detect the presence of the antigen in the serum of patients with colon carcinomas (4) and to monitor antigenic expression in human carcinoma xenografts (4, 5). Monoclonal antibody B72.3 has also been used in immunocytochemical studies to detect occult tumor cells in cytological preparations of effusions (6, 7). Clinical trials are now ongoing using B72.3 to detect colon carcinoma lesions in situ. The antigen detected by B72.3 is a high-molecular-weight, tumor-associated glycoprotein designated as TAG-72 (8).

Mucins are ambiguously classified as glycoproteins of high molecular weight which contain large amounts of carbohydrate joined in O-glycosidic linkages to serine and threonine residues (9, 10). Biologically, mucins are usually viscous secretions whose main function is lubrication and protection (11). Important changes have been found to take place in mucins and heavily glycosylated glycoproteins during transformation (12–14). Many of the tumor-associated antigens identified by monoclonal antibodies have been characterized as mucins. For example, monoclonal antibody 19-9 has been used to detect a mucin in the serum of gastrointestinal and pancreatic cancer patients (15). Similarly, Du-Pan-2 (16) detects a mucin-like antigen isolated from a human pancreatic adenocarcinoma, and OC125 (17, 18), MOv1, and MOv2 (19) monoclonal antibodies react with mucin-like molecules in ovarian carcinomas.

The purpose of the present study was to purify and characterize the TAG-72 molecule(s). The breast tumor metastasis used to generate monoclonal antibody B72.3 provided a rich source of TAG-72, but limited availability necessitated the identification of an alternate and reproducible source for TAG-72 purification studies. We have recently shown that, although B72.3 reacts with a high percentage of breast and colon carcinomas (2, 3), it reacts with only 1 of 18 colon carcinoma cell lines and 1 of 25 breast carcinoma cell lines (8). LS-174T, a colon carcinoma cell line (20), expresses low levels of TAG-72 when grown in culture. However, the three-dimensional spatial configuration of the carcinoma cell line has been shown to be important for the expression of TAG-72 (8). Consequently, the LS-174T cells, when grown as a xenograft in athymic mice, exhibit a 100-fold increase in antigen expression to levels comparable to those seen in metastatic tumor masses from patients (8). The immunohistological evaluation of LS-174T xenografts identifies it as a well-differentiated adenocarcinoma demonstrating monoclonal antibody B72.3 reactivity with both extracellular mucin and intracytoplasmic antigen. This antigenic distribution is similar to that found in mucinous adenocarcinomas of the human colon. Because of the ease of production and reproducibility of this tumor, LS-174T xenografts were chosen as the source of TAG-72 for this study.

MATERIALS AND METHODS

Antibody Preparation. Monoclonal antibody B72.3 was generated using a membrane-enriched fraction of cells from a mammary carcinoma metastasis as described elsewhere (1). The antibody was purified from ascitic fluid by ammonium sulfate precipitation followed by ion-exchange
chromatography (2, 4).

B7.23 (IgG1) and goat anti-mouse IgG heavy chain were labeled with Na125I using lodogen (Pierce, Rockford, IL) as described previously (5). MOPC-21, a myeloma IgG1 (Litton Bionetics, Inc., Rockville, MD) was used as a control immunoglobulin to demonstrate the specificity of B7.23 antibody binding.

Tumor Preparation. LS-174T, a human colon adenocarcinoma cell line (20), was obtained from the American Type Culture Collection and grown in Eagle’s minimal essential medium with nonessential amino acids, supplemented with 10% fetal calf serum and gentamicin (50 μg/ml). Cells were removed from culture flasks with 0.1% trypsin containing 0.5 mM EDTA and washed twice with culture medium without serum.

Four-wk-old female, athymic mice were obtained from Charles River (Kingston, NY). Mice were inoculated s.c. with 1 x 10⁶ cells in 0.1 ml of PBS. Tumors were harvested when they reached approximately 1 cm in diameter (15-20 days after implantation), quick frozen in liquid nitrogen, and stored at -70°C. Larger tumors were not used due to necrosis.

A breast tumor metastasis to the liver, which was used as the immunogen for the production of B7.23, was also used in this study.

Tumor Extraction. Tumor extracts were prepared by three different methods. (a) The first method used nitrogen decomposition and sonication. Tumors were finely minced in 3 volumes (w/v) of TBS containing 0.1% phenylmethylsulfonyl fluoride, 1.0 mM ε-aminocaproic acid, and 1% aprotinin (0.2 trypsin inhibitor units/ml). The tumor was homogenized for 2 min (Silverson homogenizer, top speed) and then subjected to nitrogen pressure at 1000 psi (Parr Instrument Co., Moline, IL). The homogenate was centrifuged for 15 min at 10,000 x g, and the resultant supernatant was sonicated (Heat Systems Ultrasonics, Plainview, NY) for 1 min at the maximum setting. After sonication, the homogenate was further clarified by centrifugation at 10,000 x g for 15 min, and the supernatant was used for further studies. (b) The second method was Omni-mix homogenization. Tumors, ranging in size from 3-5 g, were minced in TBS containing protease inhibitors as described above and homogenized using the Sorvall Omni-mixer (Sorvall Instruments, Wilmingon, DE) at maximum speed for 1 min at 4°C. This was followed by centrifugation at 1000 x g for 15 min. The supernatant was further clarified by centrifugation at 10,000 x g for 15 min, and the supernatant was used for further studies. (c) Disaggregation with frosted glass slides was the third method used to prepare tumor extracts. LS-174T tumors are characterized by large pools of extracellular antigen as shown by immunoperoxidase analysis. In an attempt to remove this extracellular antigen by gentle extraction procedures, tumors were disaggregated between frosted glass microscope slides, and the resulting cellular suspension was washed in TBS. The majority of the cells were left intact by this method, and these intact cells, as well as cellular debris, were removed by centrifugation as described above, leaving the extracellular antigen in the supernatant.

Protein concentration was determined by the method of Lowry et al. (21) using BSA as a standard. Tumor extracts were aliquoted and stored frozen at -20°C.

Antigen Purification. LS-174T tumors were homogenized using the Omni-mixer. All steps of the purification were carried out at 4°C. The tumor homogenate (75 mg) was resuspended in 10 ml of TBS and loaded onto a Sepharose CL-4B column (5.5 x 25 cm), previously equilibrated in TBS. The column was eluted with TBS, and 7.5-ml fractions were collected. Absorbance at 280 nm was determined, and an aliquot from each fraction was diluted 1:100 in H2O for use in a SPRIA.

Fractions containing TAG-72 were pooled, concentrated using Aquacide II (Calbiochem, San Diego, CA), and dialyzed against TBS. The pooled peak was then loaded onto a B27.2 affinity column prepared using 1.1-carboxydimidazole-activated affinity matrix Reacti-Gel HW-65F (Pierce, Rockford, IL). One hundred ml of packed gel were coupled with 200 mg of B27.2 according to the method of Hearn et al. (22). The column was washed with TBS, and the bound antigen was eluted with 3 ml NaI in TBS. Fractions (5.0 ml) were collected, and absorbance at 280 nm was determined. A small aliquot from each fraction was removed and diluted 1:100 in H2O, and the presence of antigen was determined by SPRIA as described below. The fractions containing the antigen were pooled, dialyzed extensively in TBS to remove NaI, concentrated by lyophilization, and then treated. The bound peak from the first affinity column was then loaded onto a second B7.23 affinity column consisting of 20 ml of HW 65F affinity matrix coupled to 40 mg of B27.2. Chromatography was carried out as described above.

Aliquots were saved at each step of the purification procedure, and the protein concentration was determined by the method of Lowry et al. (21). Quantitation of the antigen at each step was done using a competition radioimmunoassay.

SPRIA. Samples to be tested in a SPRIA (50 μl) were dried in 96-well polyvinyl chloride microtiter plates. When appropriate, antigen-coated wells were incubated with modifying agents such as 0.1 M NaOH (50 μl) for 30 min at room temperature in the dark. To minimize nonspecific protein absorption, microtiter wells were treated with 100 μl of 5% BSA (w/v) in PBS and incubated for 1 h at 37°C. The BSA was removed, and 125I-labeled B7.23 was added (75,000 cpm in 25 μl). After an overnight incubation at 4°C, unbound antibody was removed by washing with 1% BSA (w/v) in PBS. The bound 125I-B7.23 was detected by cutting individual wells from the plate and measuring the radioactivity in a gamma counter.

Solid-phase radioimmunoassay and immunostaining of thin-layer chromatograms of glycolipids were performed as previously described (23).

Competition Radioimmunoassay. The amount of TAG-72 was quantified using a competition RIA. Dilutions of samples to be tested, as well as those for the standard curve, were incubated with 100 μl of 125I-B7.23 (100,000 cpm) in 250-μl polyethylene microtiter tubes (Brinkman Instruments, Inc., Westbury, NY) at 4°C overnight. The tubes were centrifuged at 8000 x g for 5 min, and 50 μl of the supernatant were transferred in triplicate to 96-well polyvinyl chloride microtiter plates that were pre-coated with 5 μg of LS-174T tumor extract per well. Following an overnight incubation at 4°C, the plates were washed with 1% BSA (w/v) in PBS, and the radioactivity was counted. Competition curves were plotted for each sample tested as the percentage of bound radioactivity as a function of antigen competitor protein concentration. The linear portion of the competition curve was compared to a standard extract containing TAG-72. This standard is based on an extract of the breast tumor metastasis that was used to generate the B7.23 monoclonal antibody. One unit of TAG-72 is defined as the amount of TAG-72 found in 1 μg of the breast tumor metastasis extract.

Double Determinant Assay. Specific carbohydrate sequences were detected on TAG-72 by monoclonal antibodies using a double determinant assay similar to that previously described (15). Monoclonal antibody B27.3 (50 μg/ml) in PBS containing 0.1% NaCl was added to the wells of a microtiter plate and incubated overnight at 4°C. The plates were washed and incubated with TBS containing 1% BSA and 0.1% NaN3 at pH 8.0 for 2 h at 22°C. This was followed by an incubation under similar conditions with affinity-purified TAG-72 dilute in Tris-BSA buffer. The material that specifically bound to the wells coated with B27.3 was assayed for binding by monoclonal antibodies specific for carbohydrate sequences by the solid-phase radioimmunoassay described above. The second monoclonal antibodies used in this assay are specific for blood group oligosaccharides and are all of the IgM isotype. Antibody AH6-252 binds Lea oligosaccharides (Chembiomed, Edmonton, Alberta, Canada); antibody 10c17 binds Leb oligosaccharide (24); F-8 binds Leb oligosaccharide (Chembiomed, Edmonton, Alberta, Canada); antibody AH6-252 binds Leb oligosaccharides (Monocarb, Inc., Lund, Sweden); anti-B antibody binds blood type B oligosaccharides (Monocarb, Inc.); antibody 10b2 binds blood type H 2 oligosaccharides (26); and antibody CSLEX1 is specific for sialylated Lea oligosaccharides (27). Binding of the second antibody was detected using 125I-labeled goat anti-mouse IgM antibody. Monoclonal antibody 19-9 (15) was also used to detect the presence of sialylated Lea oligosaccharide. Since antibody 19-9 is an IgG1, a direct binding assay, as...
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described above, was used rather than a double determinant assay.

SDS:PAGE. Electrophoresis was carried out in 3–10% (w/v) polyacrylamide gels (14 x 12.5 x 1.5 or 3.0 mm) according to the method of Laemmli (28) using a stacking gel of 3% (w/v) acrylamide.

Proteins in gels were visualized by staining with Coomassie Blue R250 (29). Gels were stained for carbohydrate according to the procedure of Fairbanks et al. (30) using the PAS reagent.

Western Blotting Procedures. Proteins separated by SDS:PAGE were transferred to nitrocellulose paper (Schleicher and Schuell, Keene, NH) at 0.35 amp for 2 h according to the method of Towbin et al. (31). Nitrocellulose paper was incubated with 50 mM Tris-HCl:150 mM NaCl:5 mM EDTA:5% BSA (w/v):0.05% Nonidet P-40 (v/v), pH 7.2, to minimize nonspecific adsorption. The nitrocellulose paper was then incubated with B27.3 (10 μg/ml) followed by 125I-conjugated goat anti-mouse IgG heavy chain (5 x 106 cpm/ml). These antibodies were diluted in the buffer described above. Incubation of each antibody was for 1.5 h at 37°C with constant rocking. After incubation with each antibody, the nitrocellulose paper was extensively washed with 50 mM Tris-HCl (pH 7.2):150 mM NaCl:5 mM EDTA:1% BSA (w/v):0.5% Triton X-100 (v/v):0.1% SDS (w/v). The Western blots were air dried and exposed to Kodak XAR X-ray film at −70°C for 4–16 h using intensifying screens.

Density Gradient Ultracentrifugation. Ultracentrifugation of affinity-purified TAG-72 and LS-174T tumor extract was carried out in 5 ml of cesium chloride isopyknic density gradients. The antigen was dissolved in a CsCl solution with a starting density of 1.42 g/ml, and the gradients were formed by centrifugation in a Beckman SW 50.1 rotor at 150,000 x g for 72 h at 5°C. Fractions (0.2 ml) were collected, the density was determined by refractive index, and an aliquot was diluted 1:10 for SPRIA to localize the antigen.

Chondroitinase Digestion. Affinity-purified TAG-72 and LS-174T tumor extract were dialyzed overnight in enriched Tris buffer (250 mM Tris-HCl:176 mM NaCl:250 mM NaCl, pH 8.0) (32). Chondroitinase ABC (Sigma, St. Louis, MO) was dissolved in the buffer described above at a concentration of 5 units/ml. The substrate (50 μg) was incubated with 0.5 unit of Chondroitin ABC at 37°C for 1.5 h. Activity of the enzyme was confirmed using chondroitin sulfate type A and type C (Sigma) as substrate according to the method of Yamagata et al. (33).

Neuraminidase Digestion. Neuraminidase type X (Sigma) was dissolved in 50 mM sodium acetate buffer, pH 5.1. Digestion was carried out at 37°C for 1.5 h at an enzyme concentration of 0.04 unit/mg of protein. Digestion was stopped by adding an equal volume of SDS:PAGE sample buffer and heating at 100°C for 2 min. Samples were analyzed by Western blotting procedures as described above.

Protease Digestions. Affinity-purified TAG-72 was digested with trypsin, chymotrypsin type VII, papain, and Pronase (all from Sigma) or Staphylococcus aureus V-8 protease (Miles Laboratories, Naperville, IL). Digestions were carried out at 37°C for 1 h, using 50 mM CaCl2 in 40 mM Tris-HCl, pH 8.1, for trypsin digestion; 10 mM CaCl2 in 40 mM Tris-HCl, pH 8.1, for chymotrypsin digestion; 2 mM EDTA in 5 mM cysteine-HCl, pH 6.2, for papain digestion; 40 mM Tris-HCl, pH 7.8, for V-8 protease digestion; and 0.39 mM NaCl:11 mM KCl:5 mM CaCl2:3.6 mM MgSO4 in 60 mM Tris-HCl, pH 7.6, for Pronase digestion.

After digestion, an aliquot was removed, mixed with an equal volume of SDS:PAGE sample buffer, and separated by electrophoresis, followed by Western blotting techniques. A sample was also heat inactivated at 100°C for 2 min, and TAG-72 was quantitated by competition RIA. Heating TAG-72 at 100°C for 2 min did not significantly reduce the binding of B27.3.

To confirm that the antibody used for the competition RIA was not affected by any residual protease activity present after heat inactivation, 125I-B27.3 was incubated with heat-inactivated proteases and separated by SDS:PAGE, followed by autoradiography. The 125I-B27.3 appeared intact.

RESULTS

Identification of a Source for TAG-72. Monoclonal antibody B72.3 has been shown to react with an Mr 220,000–400,000 glycoprotein complex (34) found in a breast tumor metastasis to the liver which was used as the immunogen for the production of B72.3. This antigen complex has been termed tumor-associated glycoprotein 72 or TAG-72 (8). Because of the limited availability of this breast tumor metastasis, an alternate source of TAG-72 was needed for the purification and characterization of the antigen. LS-174T xenografts in athymic mice were chosen for this purpose. It was found that these xenografts exhibit antigen levels comparable to those seen in metastatic tumor masses from patients, and they demonstrate an identical standard curve in competition RIA to the breast metastasis used as the original immunogen, thus indicating a similar amount of TAG-72 per gram of tumor (8). Immuno-peroxidase analysis of LS-174T xenografts using monoclonal antibody B72.3 on formalin-fixed sections reveals cell-associated antigen as well as large extracellular mucinous pools of antigen. This is similar to the antigenic distribution observed in mucinous adenocarcinoma of the human colon and provides a sufficient source of antigen.

Effect of Different Methods of Extraction on TAG-72. TAG-72 identified by Western blotting techniques from LS-174T xenografts shows a range of apparent molecular weights ranging from 200,000 to greater than 1 million (Fig. 1, Lane A). We recognize that the molecular weight determined by SDS:PAGE...
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is only an approximation since the migration of heavily glycosylated proteins is very different from that of the protein molecular weight markers. The range of molecular weight of TAG-72 from LS-174T tumors is higher than that found in the breast tumor metastasis used as immunogen to generate monoclonal antibody B72.3 (Fig. 1, Lane A). However, this higher molecular weight range observed for TAG-72 from LS-174T xenografts compares very well with that observed from other breast and colon tumors extracted by a similar method from recently obtained, snap frozen material (data not shown).

Large molecules, especially mucins, are subject to shearing (9), resulting in breakage of an original high-molecular-weight form into a variety of lower-molecular-weight fragments. Since the extraction protocol for the antigen shown in Fig. 1, Lane B, consisted of nitrogen decompression followed by sonication, the different molecular weights observed may be due to fragmentation of an original high-molecular-weight molecule. To test this hypothesis, more gentle extraction methods were used, such as homogenization with the Omni-mixer or gentle disruption of the tumor with frosted glass microscope slides. After extraction, the proteins were separated by SDS-PAGE on 3–10% polyacrylamide gels, and the antigens were analyzed by Western blotting procedures.

Dissociation of the tumor with frosted glass microscope slides (Fig. 1, Lane D) appeared to be the least disruptive technique utilized. This technique produced a high-molecular-weight band and an absence of lower-molecular-weight forms. If the high-molecular-weight antigen produced by this method is then sonicated, a range of lower-molecular-weight forms is generated similar to that seen with nitrogen decompression and sonication (data not shown). This demonstrates that TAG-72 fragments with harsh treatments, such as sonication.

Some shearing was evident in the Omni-mix extract (Fig. 1, Lane C), but this method of homogenization appeared to be less destructive to the antigen than nitrogen decompression followed by sonication. Despite some shearing of TAG-72, homogenization with the Omni-mixer was chosen as the initial step for purification, because it allowed easy and reproducible handling of large quantities of tumors.

Purification of TAG-72. Purification of TAG-72 from LS-174T xenografts was performed using a three-step procedure consisting of passing the tumor homogenate through a Sepharose CL-4B column followed by two sequential passages through B72.3 affinity columns. At each step of the purification, the total protein was determined, and the antigen was quantitated by a competition RIA (Fig. 2). Purification was expressed as units of TAG-72 per µg of total protein.

LS-174T tumors were homogenized using the Omni-mixer and centrifuged to remove nuclei and mitochondria. The supernatant was chromatographed on a Sepharose CL-4B column. Essentially all of the TAG-72 was found in the void volume of the column (Fig. 3A), resulting in a 14-fold purification (Table 1, Fig. 2). The exclusion of TAG-72 from the Sepharose CL-4B column is indicative of its high molecular weight (>1 x 10^6). The antigen peak from the Sepharose column was pooled and chromatographed on a B72.3 affinity column (Fig. 3B). Bound TAG-72 was eluted with 3 M NaI, which was found to be the best reagent to desorb the antigen with minimal loss in immunoreactivity. A small amount of TAG-72 (7%) was present in the flow-through of this column (Fig. 3B). The bound peak was further purified by passage through a second B72.3 affinity column.

Sequential passage through a second affinity column gave an increased level of purification. A 21-fold purification was achieved with a single affinity column and was increased to a 47-fold
human tumor-associated glycoprotein (TAG-72)

TAG-72 was purified from LS-174T tumors by a three-step procedure consisting of chromatographic separation by Sepharose CL-4B followed by two sequential passages through B72.3 affinity columns. At each step, the protein was analyzed by the method of Lowry et al., and the TAG-72 was quantitated (as units) in a competition RIA.

**Table 1**

Purification of TAG-72 from LS-174T xenografts

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<th>Affinity peak</th>
<th>Total units</th>
<th>Recovery (%)</th>
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<td>Tumor extract</td>
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<tr>
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<tr>
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purification after the second affinity column (Table 1, Fig. 2). Although the use of two affinity columns reduced the total recovery of TAG-72 from 42 to 15%, the second affinity column was necessary to achieve the increased degree of purification.

Purification procedures did not appear to change the molecular weight range of TAG-72. When equal units of TAG-72 before and after purification (Fig. 4, Lanes 1 and 2) were compared using SDS-PAGE and Western blotting procedures, identical patterns were observed. The purification procedures did not appear to produce significant shearing of the antigen into lower-molecular-weight components. When the starting material was compared to the purified TAG-72 on the basis of equal protein (Fig. 4, Lanes 3 and 4), the purified TAG-72 appeared to have a more disperse molecular weight-range. Since the purification produced a 47-fold increase in the units of TAG-72 relative to the total protein, the range in molecular weight found in Lane 4 is believed to result from the antigen overload of the polyacrylamide gel.

Coomassie Blue does not stain components in the same molecular weight range as TAG-72 (Fig. 4, Lanes 5 and 6). This is not surprising, since heavily glycosylated glycoproteins often have Coomassie-binding sites masked by carbohydrate. However, Coomassie Blue staining can be used to evaluate the purity of the TAG-72 produced by the three-step procedure. Equal protein of starting tumor homogenate and purified TAG-72 separated by SDS-PAGE and stained by Coomassie Blue shows very different staining profiles. Coomassie-stained components in the purified TAG-72 preparation are essentially absent.

Periodic acid/Schiff reagent does stain a band in the same molecular weight range as TAG-72 identified by Western blots (Fig. 4, Lanes 7 and 8). If equal amounts of protein of the starting LS-174T tumor homogenate and purified TAG-72 are separated by SDS-PAGE and stained with PAS reagent, there appears to be insufficient antigen in the total tumor homogenate to stain (Fig. 4, Lane 7). The 47-fold purification of TAG-72 was sufficient to increase the amount of antigen relative to the protein to produce staining with PAS (Fig. 4, Lane 8).

**Analysis of Purified TAG-72 by Density Gradient Ultracentrifugation.** The density of affinity-purified TAG-72 was determined by equilibrium ultracentrifugation in CsCl as shown in Fig. 5A. The gradient extended from 1.25 g/ml-1.63 g/ml and was analyzed for TAG-72 content in a SPRIA. The highest antigenic activity was detected at a density of 1.45 g/ml, typical of that found for heavily glycosylated glycoproteins such as mucins (11, 35).

The total LS-174T tumor extract was similarly analyzed by density gradient ultracentrifugation. The TAG-72 in the total extract produced essentially an identical density profile, indicating that the TAG-72 molecule isolated by our purification methods did not represent a subset of the original antigen (Fig. 5B).

**Fig. 4.** Purification of TAG-72 from LS-174T xenografts. LS-174T tumor homogenate (Lanes 1, 3, 5, and 7) and TAG-72, purified by the three-step procedure described here (Lanes 2, 4, 6, and 8), were separated by SDS-PAGE on 3-10% polyacrylamide gels followed by Western blotting procedures (Lanes 1, 2, 3, and 4) or by staining with Coomassie Blue (Lanes 5 and 6) or periodic acid/Schiff reagent (Lanes 7 and 8). Equal antigen (10 units) was added to Lanes 7 and 2. Equal protein (10 μg) was added to Lanes 3 to 8.

**Fig. 5.** Density gradient ultracentrifugation of TAG-72. Affinity-purified TAG-72 (A) and LS-174T tumor homogenate (B) were subjected to equilibrium ultracentrifugation in cesium chloride. Fractions obtained were monitored for the presence of TAG-72 by SPRIA (•), and density was determined by refractive index measurements (O).
Identification of Oligosaccharide Sequences on TAG-72. The presence of specific oligosaccharide sequences on the affinity-purified TAG-72 molecule(s) was demonstrated using monoclonal antibodies to specific blood group oligosaccharides in a double determinant assay. Using this assay, it was found that the molecule containing the B72.3 epitope also contained Le\(^\alpha\), Le\(^\beta\), H type 2, and sialylated Le\(^\alpha\) oligosaccharide structures (Table 2). The affinity-purified TAG-72 molecule also bound monoclonal antibody 19-9, and it can therefore be postulated that the molecule containing the TAG-72 determinant also contains sialylated Le\(^\beta\) oligosaccharide. However, since the binding of monoclonal antibody 19-9 was determined by direct binding SPRIA using purified TAG-72 rather than by a double determinant assay used for the detection of the other blood group-related oligosaccharides, this binding may be to a contaminant present in the purified TAG-72 preparation. Further work addressing this question is in progress.

Antibody B72.3 does not bind purified glycolipid containing any of the blood group oligosaccharides tested (Le\(^\alpha\), Le\(^\beta\), A and B blood groups, H type 2, sialylated Le\(^\beta\), and sialylated Le\(^\alpha\)). Similarly, antibody B72.3 does not bind glycolipids extracted from a wide variety of human tumors and tumor cell lines as determined by immunostaining thin-layer chromatograms (data not shown). These include the LS-174T xenograft and the breast tumor metastasis used as immunogen in the production of B72.3. These properties of monoclonal antibody B72.3 clearly distinguish it from that of monoclonal antibody 19-9.

Analysis of TAG-72 by Enzymatic Digestions. Digestion of affinity-purified TAG-72 by Chondroitinase ABC was used to determine whether the TAG-72 epitope is carried by a proteoglycan or a mucin; chondroitin sulfate proteoglycans are sensitive to Chondroitinase ABC digestion, but mucins are not. After 1.5 h of digestion, the TAG-72 epitope remained unaffected when observed by Western blotting procedures (Fig. 6a). This finding, along with the density determinations and double determinant assay, indicates that TAG-72 has characteristics consistent with that of a mucin.

Neuraminidase digestion greatly decreased the ability of B72.3 to bind the antigen (Fig. 6b). The sensitivity of TAG-72 to neuraminidase suggests that either sialic acid forms part of the antigenic determinant or is necessary for its conformational integrity.

<table>
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<th>Antibody</th>
<th>Antigen</th>
<th>Neuraminidase</th>
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</table>

In addition to an overall reduction in antibody binding, removal of sialic acid with neuraminidase appeared to decrease the electrophoretic mobility of TAG-72. Although it is impossible to distinguish between a loss of antigen binding in the lower-molecular-weight forms from a decrease in electrophoretic mobility, the latter is believed to be the case since similar anomalous behavior in which removal of sialic acid leads to an increase in apparent molecular weight has been observed following neuraminidase treatment of other glycoproteins such as glycophorin (36). The presence of large quantities of bound SDS effectively abolishes the native charge on most proteins and glycoproteins resulting in a migration that correlates with the molecular weight. However, it is believed that high levels of sialic acid contribute a significant additional negative charge to the SDS:protein complex (36). As a result, migration of these heavily sialylated glycoproteins is greater than would be expected. The mobilities of desialylated proteins therefore are reduced, reflecting the loss of the additional negative charge contributed by sialic acid.

To investigate the sensitivity of TAG-72 to proteolytic enzymes, affinity-purified TAG-72 was incubated for 1 h with trypsin, chymotrypsin, papain, Pronase, or S. aureus V-8 protease. The sensitivity of TAG-72 to digestion was analyzed by Western blotting procedures. A competition RIA was used to quantitate the effect of proteolytic digestion on the antibody-binding sites and to determine whether the complete TAG-72 molecule was cleaved into small units that retained immunoreactivity but were undetectable by Western blotting techniques.

As shown in the Western blots (Fig. 6c), all the proteases tested exhibited some alterations in the molecular weight of the molecule carrying the TAG-72 epitope. Digestion by trypsin, chymotrypsin, and staphylococcal V-8 protease altered the pattern of bands observed in Western blots, indicating that the overall TAG-72 molecule was sensitive to these proteases (Fig. 6c, Lanes 2, 3, and 6). However, no reduction in antibody-antigen binding was detected by competition RIA (Fig. 6d), indicating that the actual epitope on TAG-72 recognized by B72.3 was unaffected by these three proteases.

Digestion by papain and Pronase appeared to be the most destructive (Fig. 6c, Lanes 4 and 5). Competition RIA confirmed that approximately 75% of the antigenic determinants recognized by B72.3 were destroyed by either papain or Pronase digestion (Fig. 6d). The sensitivity of TAG-72 to these proteases suggests that either protein is directly involved in the antibody-binding site or is necessary for the conformational integrity of the epitope recognized by B72.3.

DISCUSSION

Monoclonal antibody B72.3 is directed against a high-molecular-weight determinant called TAG-72. TAG-72 precipitated from the breast tumor metastasis used as immunogen was found to have an apparent molecular weight of approximately 220,000-400,000 by SDS-PAGE (Ref. 34; Fig. 1A). However, the work reported here provides evidence that this molecular weight range may reflect a shearing of the native high-molecular-weight antigen caused by the sonication step used in the initial extraction. When LS-174T xenografts or snap-frozen colon and breast carcinoma biopsy material was prepared without sonication, the TAG-72 identified in Western blots exhibited a higher molecular weight without such an apparent heterogeneity in size. This antigen is found both in the stacking gel and at the top of a 3-
Fig. 6. Enzymatic digestion of TAG-72. Affinity-purified TAG-72 (a, c, d) or LS-174T tumor extract (b) was digested with Chondroitinase ABC (a), neuraminidase (b), or a variety of proteases (c, d) as described in “Materials and Methods.” After digestion, proteins (10 μg/lane) were separated by SDS-PAGE on 3–10% polyacrylamide gels and analyzed using Western blotting procedures. The effect of the various protease digestions on TAG-72 was quantitated by competition RIA (d) and expressed as units/ml, a, Lane 1, affinity-purified TAG-72 control; Lane 2, Chondroitinase ABC-digested TAG-72; b, Lane 1, LS-174T tumor extract control; Lane 2, neuraminidase-digested LS-174T tumor extract. c and d, Lane 1, affinity-purified TAG-72 control; Lane 2, trypsin digestion (1.0 unit/2.5 μg TAG-72); Lane 3, chymotryptic digestion (1.0 unit/2.5 μg TAG-72); Lane 4, papain digestion (0.1 unit/2.5 μg TAG-72); Lane 5, Pronase digestion (0.1 unit/2.5 μg TAG-72); Lane 6, staphylococcal V-8 protease digestion (5.0 units/2.5 μg TAG-72).

10% polyacrylamide separating gel after SDS-PAGE. It is difficult to assign a molecular weight to heavily glycosylated glycoproteins on the basis of SDS-PAGE. However, the high molecular weight (>1 × 10^6) is apparent, since it is found in the void after Sepharose CL-4B chromatography.

TAG-72 was chromatographed on a Sepharose CL-4B column in the presence of 6 M guanidine-HCl. The antigen profile obtained in SPRIA was similar to that obtained in the absence of guanidine-HCl, thus indicating that the molecular weight observed was not due to the aggregation of low-molecular-weight forms (data not shown). These results were confirmed by Western blotting procedures.

TAG-72 was purified by a three-step procedure involving Sepharose CL-4B chromatography, followed by two sequential passages of the extract through B72.3 affinity columns. The Sepharose column was useful as an initial step to remove many of the contaminating lower-molecular-weight murine proteins, such as albumin and murine immunoglobulin. This column alone produced a 14-fold purification of the antigen. Passage through a series of two B72.3 affinity columns was able to increase the purification of TAG-72 to 47-fold.

The 47-fold purification obtained by this three-step procedure may be deceptively low. The LS-174T xenograft has large extracellular pools of antigen in addition to cell-associated antigen. These extracellular pools result in an unusually high concentration of the antigen. The competition RIA is based on an arbitrary quantitation of 1 unit of antigen as the amount of antigen corresponding to 1 μg of protein in the LS-174T tumor. If this starting assumption is based on a tumor with extremely high levels of antigen per gram of tumor, the degree of purification obtained may be misleading relative to other nonmucinous tumors or tissue culture cells which lack the high antigen concentration present in extracellular pools. However, since we feel that the LS-174T xenograft is representative of typical mucinous adenocarcinomas seen in colon carcinoma patients, we feel that our choice of both antigen source and the definition of 1 unit of TAG-72 is justified.

The exact nature of the epitope on the TAG-72 molecule detected by monoclonal antibody B72.3 is unknown at this time. However, neuraminidase digestion of TAG-72 greatly reduces antibody binding, thus providing evidence that sialic acid is either involved in the antigenic determinant or responsible for the conformational integrity of the antibody binding site. Periodate oxidation also reduces antibody binding, suggesting further evidence that carbohydrate is involved in the epitope. Whether protein, in addition to carbohydrate, forms part of the TAG-72 antigenic determinant remains unresolved. Papain and Pronase digestion reduced antibody-antigen binding by approximately 75% as shown by competition RIA. Although this provides evidence that the peptide portion of the molecule is involved in the epitope or affects its conformational integrity, we cannot rule out contaminating glycosidase activity in the protease sample. Further work to elucidate the nature of the TAG-72 epitope is in progress.

Monoclonal antibodies often recognize carbohydrate determinants found on both glycoproteins and glycolipids. Monoclonal antibody 19-9 provides an example of this (15, 23). It appears that the TAG-72 epitope is not found on glycolipids. This clearly distinguishes the TAG-72 epitope from the determinant recognized by monoclonal antibody 19-9.

Data are presented here to suggest that the TAG-72 molecule is a mucin. This conclusion is based on the following observations: (a) TAG-72 has a high molecular weight (>1 × 10^6) as shown by its exclusion from a Sepharose CL-4B column; (b) the density of TAG-72 determined by equilibrium centrifugation in CsCl was 1.45 g/ml, indicating a heavily glycosylated glycoprotein (11, 35); (c) TAG-72 demonstrates a change in migration after neuraminidase digestion, indicating that it is a heavily sialylated molecule with an abundance of O-glycosidically linked oligosaccharides (36) characteristic of mucins; (d) blood group antigens commonly found on mucins are found on affinity-purified TAG-72; and (e) Chondroitinase ABC digestion had no effect on TAG-72, thus demonstrating that the TAG-72 epitope is not expressed on a chondroitin sulfate proteoglycan.

The purification of TAG-72 from LS-174T as described in this paper should enable further characterization of both the mucin-like molecule and epitope defined by monoclonal antibody B72.3. This purification procedure will also allow one to compare mole-
TAG-72 molecule purified in these studies can now be used toward the production of "second generation" monoclonal antibodies reactive with different determinants on the TAG-72 molecule. These antibodies should subsequently aid in the further characterization of this tumor-associated antigen.

**REFERENCES**

Analysis of a Human Tumor-associated Glycoprotein (TAG-72) Identified by Monoclonal Antibody B72.3

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