Serological Mapping of the TAG-72 Tumor-associated Antigen Using 19 Distinct Monoclonal Antibodies

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ABSTRACT

Monoclonal antibody (MAb) B72.3 has been shown to be of potential utility in the management of human carcinoma via its use in (a) the targeting of carcinoma lesions in colorectal and ovarian cancer patients, (b) immunohistochemical analyses of biopsies and effusions, and (c) serum assays to help define the presence of carcinoma. The B72.3-reactive antigen, designated tumor-associated glycoprotein 72 (TAG-72), has been characterized as a high molecular weight glycoprotein with the properties of a mucin. We report here the utilization of MAb B72.3 and 18 second generation MAbs (generated using purified TAG-72 obtained from a colon carcinoma xenograft as immunogen) to construct a serological map of the TAG-72 molecule. The generation and initial characterization of 10 of the second generation MAbs have been described previously; in addition, eight previously unreported MAbs were used. All 19 MAbs produced immune precipitate lines against purified TAG-72 in double immunodiffusion, indicating that each epitope recognized by a single MAb is present at least twice on the TAG-72 molecule. Immunodepletion analyses utilizing 11 of the anti-TAG-72 MAbs indicated that each recognizes the same molecule or population of molecules.

INTRODUCTION

MAb$^2$ B72.3 was generated using a membrane-enriched fraction of a human carcinoma metastasis to the liver as immunogen (1) and has been shown to have selective reactivity for a wide range of human adenocarcinomas of the colon, stomach, pancreas, breast, ovary, lung (non-small cell), and endometrium (2–5). B72.3 has also been shown to be nonreactive or only weakly reactive to most normal adult tissues (2, 6) with the exception of secretory endometrium (7) and transitional colonic epithelium (8, 9). The B72.3-reactive antigen, designated TAG-72 (10), is also expressed in some human fetal tissues, including fetal gut (2).

TAG-72 has been partially purified from the LS-174T human colon cancer xenograft via several chromatographic procedures, including sequential passages through B72.3 affinity columns, and has been characterized as a high molecular weight glycoprotein with the characteristics of a mucin (10). The B72.3-reactive epitope has been reported to contain or be an $O$-linked sialyl-$L$-$	ext{6ac}$-N-acetylgalactosaminyl structure (11, 12).

MAB B72.3 has been utilized successfully to (a) study the biology of human carcinoma cell populations (13–15), (b) detect occult carcinoma by immunohistological and immunocytochemical analyses of cell-associated TAG-72 in fine needle aspiration biopsies and human effusions (16–27), (c) detect TAG-72 in sera of carcinoma patients via RIA (28, 29), and (d) target carcinoma lesions in situ using radiolabeled MAb B72.3 IgG (30–35). Therapy trials using MAb B72.3 are in progress.

In view of the selective expression of the TAG-72 antigen in a wide range of human carcinomas versus normal adult tissues and the potential applications of MAB B72.3 in the management of human carcinomas, we prepared a second generation of MAbs to the TAG-72 antigen using purified TAG-72 from a colon carcinoma xenograft as immunogen. We have recently reported on the production of 28 such MAbs, designated CC (since the source of the immunogen was a colon carcinoma). Muraro et al. (36) have previously shown that the CC MAbs also have selective reactivity for carcinomas versus normal adult tissues. Nine CC MAbs were selected for further characterization. They were shown to react with purified TAG-72 via Western blotting and have higher $K_v$ values for TAG-72 than MAB B72.3, with MAbs CC92, 49, and 83 having $K_v$ values of 14.3, 16.2, and 27.7 $\times$ 10$^6$ M$^{-1}$, respectively, versus 2.5 $\times$ 10$^6$ M$^{-1}$ for MAB B72.3.

In this study, we report the use of MAB B72.3, 10 CC MAbs previously described, and 8 CC MAbs not previously characterized to help define a serological map and further characterize the TAG-72 antigen.

MATERIALS AND METHODS

Generation and Source of MAbs. MAB B72.3 was generated as previously described (1). Ten IgG CC MAbs, CC11, 15, 29, 30, 40, 46, 49, 50, 83, and 92, were prepared as previously described (36). MAB CC11 is an IgG2b, and the remainder are IgG1. TAG-72, purified from xenografts generated from the LS-174T cell line by the method of Johnson et al. (10), was used as immunogen to prepare second generation MAbs CC101, 102, 109, 111, 112, 115, 117, and 118. Members of this latter group are of the IgM isotype. To prepare these MAbs, three 4-week-old BALB/c mice were immunized by i.p. inoculation of 10 $\mu$g TAG-72 in complete Freund's adjuvant. After 80 days, the mice received booster doses of 50 $\mu$g TAG-72 in incomplete Freund's adjuvant. Seven days later, the mice received 10 $\mu$g TAG-72 in saline i.v. Spleen cells were harvested 3 days later for cell fusion. Hybridomas were prepared using a modification of the method of Herzenberg and Milstein (37). Two thousand cultures were assayed for reactivity to TAG-72 using anti-IgM reagents; 110 were found to produce antibodies binding to TAG-72. Further cloning and assaying via SPRIA, as described below, yielded 34 cultures that produced antibodies which bound to a colon carcinoma extract and to purified TAG-72 but not to a normal liver extract. These were further cloned by limiting dilution into 3264 wells, and approximately 20 clones each of the original 34
cultures were assayed in a SPRIA for binding to TAG-72. This yielded 23 clones that produced antibodies with binding specificity for TAG-72. The 23 clones were subsequently grown for further study, and culture supernatants were assayed in SPRIA for binding to a metastatic breast carcinoma extract and to TAG-72 and for lack of binding to normal spleen and normal liver. This yielded 15 clones that produced antibodies which bind to the carcinoma extract and to TAG-72 but not to the normal extracts. These cultures were then recloned and reassayed in SPRIA to yield MAb CC101, 102, 109, 111, 112, 115, 117, and 118. Ascitic fluids containing the various MAbs were generated as reported elsewhere (36).

Additional MAbs used as controls in this study include (a) anti-CEA MAbs B1.1 and COL-1 (38, 39); (b) MAbs F36-96 and F4-11, prepared using purified CEA as immunogen (40) and known to be reactive with a protein epitope and a carbohydrate epitope of the CEA molecule, respectively (40, 41); and (c) MAb BL-3, an anti-IgM idiotype (30) and myeloma proteins MOPC 21 (IgG1), UPC 10 (IgG2a), and MOPC 104E (IgM), which were used as negative controls (42, 43) (Organon Teknika, Durham, NC).

Purification of MAbs. MAb IgGs were purified from ascitic fluids by ammonium sulfate precipitation followed by ion-exchange chromatography or by protein A-Sepharose CL-4B chromatography as previously described (44, 45). IgMs were precipitated by dialysis against deionized and distilled water at 4°C (46). The precipitated IgM was dissolved in a small volume of PBS, applied to a Sephadex G-200 column (1.6 x 90 cm), and eluted with PBS. The IgM was excluded from the gel and recovered in the first elution peak (46). Column fractions were analyzed by SDS-PAGE, and the protein concentrations of the purified IgG and IgM were determined by the method of Lowry et al. (47).

Preparation of Tissue Extracts. Neoplastic and normal human tissues were obtained from the Departments of Surgery and Pathology of the George Washington University, Washington, DC. LS-174T (48), a human colon adenocarcinoma cell line, was obtained from Dr. Philip Noguchi (Bureau of Biologics, Food and Drug Administration). Tissue extracts were prepared by a method used by Colcher et al. (49), and the protein contents were determined by the method of Lowry et al. (47).

Immunoperoxidase Studies. Reactivity of hybridoma tissue culture supernatants with formalin-fixed or frozen 5-µm tissue sections was determined using a modification of the avidin-biotin-peroxidase complex (Vectastain ABC kit; Vector Laboratories, Inc., Burlingame, CA) method (2).

SDS-PAGE. Electrophoresis was carried out in 3-12% polyacrylamide gels according to the method of Laemmli (50). Proteins in the gels were visualized by staining with Coomassie blue R250 or by Western blotting (see below).

Western Blotting Analyses. Proteins separated by SDS-PAGE were transferred to nitrocellulose filter paper at 4°C for 4 h at 30 V as previously described (10). The blots were then treated with 5% BSA in PBS, washed with PBS containing 0.05% Tween-20, and incubated with each purified MAb at a concentration of 10 µg/ml for 2 h at room temperature with gentle agitation. After washing, the blots were incubated for 1 h at room temperature with 5 x 10^4 cpm/ml 125I-labeled goat anti-mouse IgG or anti-mouse IgM. The filters were then extensively washed overnight and exposed to Kodak XAR X-ray film.

Purification of TAG-72 and CEA. TAG-72 was purified from xenografts generated from the LS-174T cell line. Modifications of the methods of Johnson et al. (10) and Sheer et al. (51) yielded a large amount of pure antigen suitable for the cross-completion and immunodiffusion studies. All steps were carried out at 4°C. LS-174T tumors were minced in 20 volumes of PBS containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM e-aminoacaproic acid, 1% aprotinin, 1 µg/ml pepstatin, 0.1 mM leupeptin, 100 µg/ml bacitracin) and homogenized using the Tekmar Tissuemizer (Tekmar Co., Cincinnati, OH). The homogenate was centrifuged at 15,000 x g for 30 min, and the supernatant was then treated with perchloric acid (0.6 M final concentration) to precipitate the proteins. The mixture was centrifuged, and the supernatant, including the acid-soluble glycopolypeptides, was immediately dialyzed against several changes of distilled water and then against PBS. The supernatant was mixed with an immunoabsorbent containing 168 mg of purified MAB CC49, which was prepared using 50 ml of the 1,1'-carbonyldimidazole-activated affinity matrix Reactigel HW-65F (Pierce, Rockford, IL) (10). The mixture was incubated overnight at 4°C while gently shaking. A batch method using a Buchner-type filtering funnel with fritted disc (150 ml; Fisher Scientific, Pittsburgh, PA) was utilized for the washing and elution of the antigen from the adsorbent. The adsorbent was sequentially washed with 0.1 M acetic acid, pH 4.0, + 0.5 M NaCl; 0.1 M sodium borate, pH 8.0, + 0.5 M NaCl; and PBS until the absorbance of the wash buffers 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 solution was centrifuged to separate fine particulates derived from the adsorbent, dialyzed against several changes of PBS, concentrated, and applied to a Sepharose CL-2B column (1.6 x 90 cm). This molecular sieving chromatography was done using PBS containing 6 M guanidine-HCl. Two peaks with antigenic reactivity were seen, although the trough between the peaks was not always clear. The fractions containing the high molecular weight TAG-72 were pooled, concentrated, and applied to a second Sepharose CL-2B column.

Aliquots were saved at each step of the purification procedure, and the protein concentration was determined by the method of Lowry et al. (47). Quantitation of TAG-72 was performed with a double determinant RIA (Centocor, Malvern, PA) as previously described (29). One unit of antigen is arbitrarily defined as the amount of TAG-72 reactivity found in 1 µg protein from a standard crude extract of a breast carcinoma metastasis (28). The overall recovery of TAG-72 antigen and total protein after the final step was 27.6 and 0.006% of the starting material, respectively, with a 4325-fold purification resulting in a final specific activity of 2638 units/µg of protein.

TAG-72 was partially purified for utilization in the immunodepletion analyses as follows. LS-174T xenografts were minced and homogenized on ice in 10 volumes of 50 mM Tris, pH 8.5, + 150 mM NaCl + 2 mM phenylmethylsulfonyl fluoride and centrifuged for 15 min at 15,000 x g. The supernatant was decanted and centrifuged at 100,000 x g for 40 min at 4°C. Dithiothreitol was added to 50 mM and the supernatant was heated to 100°C for 20 min, cooled to ambient temperature, and centrifuged at 15,000 x g for 15 min. The supernatant was decanted and centrifuged at 100,000 x g for 40 min, iodoacetamide was added to 150 mM, and the pH was adjusted to 8.5. The reduced and alkylated supernatant was dialyzed against two changes of 10 mM NH4HCO3, lyophilized, redissolved in 6 µg guanidine-HCl, and applied to a Superox 6 column (2.5 x 100 cm) (Pharmacia, Uppsala, Sweden). Excluded fractions were pooled, dialyzed against PBS, and assayed for TAG-72 using a double determinant RIA (Centocor) (29).

CEA was highly purified from a liver metastasis of human colon carcinoma as previously described (40).

Immunodiffusion Test. A microscopic double immunodiffusion test was performed in a 1.0% agarose layer in PBS on a microscope slide (41). The antigens and antibodies were allowed to react overnight at room temperature. Immunoprecipitation lines were stained on dried gels using Coomassie blue R250.

Radiolabeling of Proteins. Affinity-purified goat anti-mouse IgG and goat anti-mouse IgM antibodies, protein A, and purified TAG-72 were labeled with Na2125I by the Iodogen (Pierce) method (52).

SPRIAs. SPIAs were performed using antigens immobilized on 96-well polyvinyl chloride microtiter plates (Dynatech, Alexandria, VA) as previously described (13). Samples (50 µl) to be tested (tissue extracts, TAG-72, CEA, or chromatography fractions) were dripped in each well overnight at 37°C. Nonspecific protein absorption was blocked with 5% BSA in PBS at 37°C for 1 h. Hybridoma supernatants or varying amounts of purified MAbs (in 50 µl) were then added. After a 1-h incubation at 37°C, unbound IgG was removed by washing the plates with 1% BSA in PBS. Radiolabeled goat anti-mouse IgG or goat anti-mouse IgM was added (75,000 cpm in 25 µl of 1% BSA in PBS); after an additional 1-h incubation at 37°C, unbound radiolabeled second antibody was removed by washing, and the radioactive counts bound in individual wells were determined in a gamma counter. IgM MAbs CC101-CC118 were assayed as described above, except that, to detect binding of antibody, 50 µl of rabbit anti-mouse IgM (Cooper Biomedical, Malvern, PA) were added to each well. The plates were incubated for 1 h at 37°C and washed, and 125I-labeled protein A (Pharmacia) at 4873
50,000 cpm in 25 μl was added to each well and incubated at 37°C for 1 h. The unbound protein A was removed by extensive washing with 1% BSA in PBS.

To determine the isotype of MAbs, a RIA was developed using an F(ab')2 fragment of an anti-murine IgG (heavy and light chain specific) dried on each well as previously described (36). The MAb captured on the well was detected by incubation with isotype-specific rabbit anti-mouse immunoglobulin antisera followed by 125I-protein A.

ELISA. ELISAs were carried out using antigens immobilized on 96-well polyvinyl chloride microtiter plates. Partially purified TAG-72 in 50 μl PBS was dried overnight at 37°C and used as target antigen. 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 in 0.1 M sodium citrate, pH 5.0, was added for 10 min at room temperature. Absorbance at 490 nm was determined.

Cross-competition assays among MAbs for TAG-72 binding were performed by a SPRIA according to a modification of a method of Legrain et al. (53). To each well of 96-well plates previously coated with a given MAb, 10,000 cpm of 125I-labeled TAG-72 in 50 μl and increasing amounts of competitor MAbs in 50 μl of 1% BSA in PBS were added. After a 2-h incubation at 37°C, each well was washed with 1% BSA in PBS, and binding of 125I-labeled TAG-72 was determined in a gamma counter. The amounts of competitor antibodies required to inhibit binding of 125I-labeled TAG-72 by 50% were calculated.

Immunodepletion Analyses. Monoclonal antibodies BL-3 (30), B72.3, CC1, CC46, CC49, CC83, and CC92 were diazyed against 50 mm NaHCO3 and coupled to Avidigel F (Bioprobe International, Tustin, CA) (54) at 2.5 mg/ml packed volume following the manufacturer's directions. Coupling efficiencies of >95% were routinely achieved.

Immunodepletions utilizing the solid-phase antibodies were carried out as follows. Aliquots (20 μl) of the solid-phase antibodies (containing 50 μg of bound immunoglobulin) were blocked with 0.5 ml 5% BSA/PBS for 1 h at 37°C on a rotator and washed 3 times with PBS. Aliquots of TAG-72 containing 600 units of activity in 0.5 ml PBS were immunodepleted by incubation with the blocked and washed 20-μl aliquots of the solid-phase antibodies for 1 h on a rotator at room temperature, followed by removal of the solid-phase antibodies by low speed centrifugation. This immunodepletion step was carried out a total of three times on each aliquot of TAG-72. Remaining activity was then evaluated in solid-phase ELISA.

RESULTS

Reactivity of anti-TAG-72 MAbs with Carcinoma and Normal Tissue Extracts. IgM MAbs CC101, 102, 109, 111, 112, 115, 117, and 118 were all tested in SPRIA with an extract of the LS-174T colon carcinoma cell line and extracts of biopsies of five colon carcinomas and five ovarian carcinomas. MAbs of the IgM isotype were analyzed because of the possibility that they would reveal epitopes not identified by the IgG MAbs. As seen in Table 1, all of these MAbs were reactive with the LS-174T extract and with four of the five colon carcinoma extracts. The reactivity of the IgM MAbs with the ovarian carcinoma extracts varied from two to five of five tumors tested (Table 1); moreover, the CC MAbs differed as to which tumors they reacted with. For example, CC102 reacted with tumors D85 and D106, CC111 with V43 and D85, and CC118 with V45 and D85.

MAbs CC101, 102, 109, 111, 112, 115, 117, and 118 were also tested against extracts of 34 biopsies from a variety of normal adult human tissues. As with reactivity to tumor, 500 cpm was arbitrarily chosen as a “positive” reaction. All of these MAbs were nonreactive (<500 cpm) with biopsies of liver (n = 4), spleen (n = 8), kidney (n = 2), bone marrow (n = 1), and thyroid (n = 1). Some of the MAbs showed some reactivity to some normal tissues. All the MAbs were tested for reactivity with 11 different preparations of erythrocytes from different blood groups and all were negative with the following exceptions: CC101 reacted with 1 of 11 preparations (517 cpm), CC115 reacted with 3 of 11 (579–736 cpm), and CC117 reacted with 1 of 11 (579 cpm). When tested for reactivity with 5 histologically normal colon specimens, and lung and stomach specimens, the following positive reactions were seen: CC101 reacted with stomach (605 cpm) and 5 of 5 normal colon (614–4468 cpm); CC102 reacted with stomach (8008 cpm), lung (609 cpm), and 2 of 5 colon (694 and 806 cpm); CC109 reacted with 3 of 5 normal colon (645–1143 cpm); CC111 reacted with stomach (3961 cpm) and 2 of 5 colon (524 and 619 cpm); CC112 reacted with stomach (533 cpm) and 2 of 5 colon (620 and 704 cpm); CC115 reacted with stomach (1,147 cpm) and 1 of 5 colon (568 cpm); CC117 and CC118 reacted only with 1 of 5 colon (578 and 621 cpm, respectively).

The IgM MAbs CC101, 102, 109, 111, 112, 115, 117, and 118 were evaluated in immunoperoxidase assays with formalin-fixed sections of five different colon carcinomas and a wide range of normal tissues. All of the MAbs reacted to the colon carcinomas in a manner similar to that reported for MAb B72.3 with regard to the distribution of reactivity and intensity of staining. MAbs 102, 112, and 118, however, showed greater reactivity in terms of percentage of positive tumor cells. MAbs CC111, 115, and 117 reacted with a higher percentage of tumor cells but also gave some nonspecific background staining of muscle and fat. Normal tissues evaluated included colon, appendix, small bowel, stomach, esophagus, gall bladder, liver, pancreas, salivary gland, kidney, bladder, ovary, uterus, endocervix, testis, spleen, lymph node, bone marrow, thyroid, adrenal, lung, breast, brain, and heart. We have previously reported in detail the reactivity of MAb B72.3 with these normal tissues (2). All of the IgM CC MAbs were similar to B72.3 in that they were negative when tested against appendix, esophagus, gall bladder, liver, pancreas, ovary, lymph node, bone marrow, thyroid, adrenal, brain, and heart. They were also similar to B72.3 in that they showed minimal activity to the following tissues: superficial epithelium of small bowel (10–15% of cells), Leydig cells of the testis (10–20%), endocervical glandular epithelium (10–40%), respiratory epithelium of the lung (5–20%), secretory endometrium (5–15%), and superficial epithelium of the stomach (5–20%, except for CC109 and 118 which were negative). There were, however, some differences in the reactivity of the IgM CC MAbs compared with that of B72.3. There was minimal reactivity of CC102 and 115 to goblet cells of normal colon (<5%); CC102, 109, 111, 112, 115, 117, and 118 showed weak reactivity to salivary gland duct epithelium (1%); CC118 to transitional epithelium of the bladder (10%); and CC111 to renal tubules (1%). A more detailed description of MAb CC112 has recently been reported (6).

Western Blotting. We have previously described the reactivity of the IgG MAbs to TAG-72 in Western blotting (36). All of the IgM MAbs were analyzed using Western blotting methodology for reactivity with the TAG-72 antigen using a crude extract of the human colon carcinoma xenograft, LS-174T. The results of five IgM MAbs, (CC109, 112, 115, 117, and 118), CC49, and MAb B72.3 are shown in Fig. 1. As previously described (36), B72.3 and all of the IgG MAbs recognize a very
Table 1 Reactivity of IgM CC MAbs in SPRIA with carcinoma biopsies

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* The extract of a human colon adenocarcinoma cell line LS-174T was obtained from xenografts grown in athymic mice.
* Neg, negative. All extracts listed as negative (Neg) displayed cpm <500.

Fig. 1. Immunodetection of TAG-72 in a crude extract of LS-174T as recognized by IgM CC MAbs. Immunoactivity of the extract was determined by a double determinant assay (28), the crude extract was separated by SDS-PAGE on 3–12% polyacrylamide gels, and TAG-72 was visualized using Western blotting procedures (10) (see “Materials and Methods”). Equal amounts of antigen (10 units) were added to all lanes. MAbs used for detection were as follows: lane A, CC109; lane B, CC112; lane C, CC115; lane D, CC117; lane E, CC118; lane F, CC49; and lane G, B72.3. Vertical scale, molecular weight markers (x10^-3).

Fig. 2. SDS-PAGE analysis of purified TAG-72. The purified TAG-72 was separated by SDS-PAGE in 3–12% polyacrylamide gels and detected by Western blotting procedures (10). In Lane A, purified TAG-72 was first radiiodinated by the Iodogen method. About 50,000 cpm of labeled TAG-72 was added to the lane. After electrophoresis, the TAG-72 was transferred to nitrocellulose filter paper. In Lane B, purified TAG-72 (10 units) was electrophoresed and transblotted to nitrocellulose filter paper. The blotted TAG-72 was incubated with MAb CC49 and then with a radiolabeled goat anti-mouse IgG antibody (5 x 10^6 cpm/ml). The filters were then extensively washed and exposed to Kodak XAR X-ray film. Lane M contained molecular weight markers (x10^-3).
nodiffusion and the cross-competition studies.

**Double Immunodiffusion Test.** The 19 anti-TAG-72 MAb s (11 IgG MAb s including B72.3 and 8 IgM MAb s) were tested in immunodiffusion against purified TAG-72. All MAb s produced immune precipitate lines, indicating that each epitope recognized by each MAb is present at least twice on the TAG-72 molecule (55).

Cross-Competition RIAs among MAb s for TAG-72 Binding. To determine relationships between epitopes recognized by these MAb s, cross-competition analyses between antibodies for TAG-72 binding were performed using a series of SPRIs. All 19 MAb s were separately immobilized in wells of polystyrene plates and were assayed for the binding of 125I-labeled TAG-72 in competition with increasing amounts of 19 competitor MAb s, including each homologous MAb.

In an attempt to quantify the inhibitory effect of each MAb, the amounts of competitor MAb s required to inhibit the binding of 125I-labeled TAG-72 to each MAb dried on wells by 50% were determined from each respective inhibition curve (Table 2). To normalize for the relationships between the MAb s, the relative competition values of MAb s for 50% competition were calculated from those data given in Table 2 by dividing the values obtained for each MAb coated on the plates by the value obtained for 50% inhibition with each homologous MAb. These relative values are graphically represented in Fig. 3. This presentation allows the comparison of the ability of each MAb to inhibit the binding of other MAb s to TAG-72 with the reciprocal competition of each MAb binding to TAG-72 by the other MAb s. In an attempt to classify the relative abilities of each of these anti-TAG-72 MAb s to compete for epitopes recognized by the other 18 MAb s, a "competition index" was defined as the number of anti-TAG-72 MAb s with which a MAb could achieve 50% competition using ≤10 times the amount of homologous antibody required for 50% inhibition. Thus, as seen in the last column of Fig. 3, CC92, which competes only with itself, has a competition index of 1; CC115, which acts as a competitor for the binding of all of the anti-TAG-72 MAb s, has a competition index of 19. Note that no differences were observed in the distribution of the IgM MAb s, i.e., CC101, 102, 109, 111, 112, 115, 117, and the IgG MAb s, as far as competition indices were observed. Differences in patterns of competition could also not be explained on the basis of Ks values. For example, CC92 has been shown (36) to have a higher Ks than CC46 or B72.3. Furthermore, CC49 and CC83 have similar Ks values but compete quite differently.

MAb s CC115 and 112 were the most broadly inhibitory. MAb CC115 was partially competed for by only two MAb s other than itself, but it in turn acted as a competitor for binding of all of the other MAb s, with strong competition of 16 of 19 MAb s. This suggests that the epitope for this antibody is distinct from the other 18 MAb s but may be either structurally or spatially related to all the other MAb s. In a competition RIA for the epitope recognized by CC112, only CC112 and 115 competed. However, CC112 also acted as an efficient competitor in the majority of competition RIAs for the other MAb s. MAb CC112 may recognize a similar epitope to CC115, because it was competed for only by itself and CC115, and it also showed a broad spectrum of inhibitory reactivities. However, the pattern of CC112 in reciprocal inhibition assays can clearly be distinguished from that of CC115 (Fig. 3).

A different pattern of competitive reactivities was seen with MAb s CC92, 29, 102, and B72.3. These were competed for by many other MAb s tested, but they in turn competed only for binding of one or two MAb s. This suggests that they bind to

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**Table 2.** Cross-competition analysis among anti-TAG-72 MAb s for 50% inhibition.

<table>
<thead>
<tr>
<th>Competitor MAb</th>
<th>CC115</th>
<th>CC112</th>
<th>CC92</th>
<th>29</th>
<th>102</th>
<th>B72.3</th>
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<tr>
<td>B72.3</td>
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<tr>
<td>B72.3</td>
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distinct epitopes that are structurally or spatially related to ones recognized by other antibodies. These four MAbs, however, showed poor reciprocal inhibition to one another, suggesting that the epitopes recognized by them are not related to one another.

MAbs CC15, 40, 11, 50, and 30 appeared to form another group in that they were competed for by a group of six or seven MAbs but showed inhibitory reactivities against many MAbs. These MAbs also showed more or less reciprocal inhibitions to each other. This suggested that the epitopes recognized by these five MAbs may be spatially close to one another or structurally similar, but as can be seen in Fig. 3, none of them were identical to one another.

MAbs CC101 and 117 showed similar inhibitory reactivities against 6 MAbs, and their competition patterns by 14 or 15 MAbs were also similar to each other with few exceptions. These two MAbs also showed partial reciprocal inhibition. These results suggest that these two MAbs bind epitopes that are structurally or spatially related to one another but are clearly not identical.

The remaining MAbs, CC46, 111, 83, 118, 49, and 109, were competed for by 6 to 13 MAbs and showed inhibitory reactivities against 4 to 12 MAbs, but no remarkable similarities of their reactivity patterns among or between them and the MAbs mentioned above were seen. Each appeared to be distinct.

In summary, the patterns of cross-competition between the 18 second generation CC MAbs and B72.3 indicate the presence of a complex array of epitopes on the TAG-72 molecule. All of the MAbs tested seemed to recognize epitopes that are more or less structurally or spatially related to one another. None of them, however, appear to recognize identical epitopes. The spectrum of inhibitory reactivities of these MAbs for TAG-72 binding varied from extremely restricted inhibition (CC92 and CC9) to more broad inhibition (CC115 and 112). In general, there was an approximately reciprocal relationship between the ability of a MAb to compete for binding with the other anti-TAG-72 MAbs and the tendency of that MAb to be inhibited by the binding of other MAbs, but none of the anti-TAG-72 MAbs failed to either inhibit or be inhibited by the binding of other anti-TAG-72 MAbs.

Immunodepletion Analyses. Studies were carried out to determine whether various epitopes of TAG-72 were carried on different molecules. Saline extracts of LS-174T xenograft were heat extracted, size fractionated, and subjected to immunodepletions utilizing immobilized MAbs B72.3, CC11, CC49, CC83, and CC92 as described in “Materials and Methods.” MAb BL-3, an isotype-matched antidiotypic (30), was also immobilized and utilized as a negative control. The immunodepleted extracts were subsequently evaluated for remaining reactivity in a solid-phase ELISA. As shown in Fig. 4, immunodepletions utilizing MAbs B72.3, CC11, CC46, CC49, CC83, or CC92 all resulted in the virtually complete removal of reactivity in ELISA with MAbs B72.3, CC11, CC15, CC29, CC30, CC40, CC46, CC49, CC50, CC83, and CC92. These results strongly suggest that the epitopes recognized by B72.3 and the other IgG anti-TAG-72 MAbs tested, although distinct, are all expressed on the same molecule or on the same population of molecules.

DISCUSSION

Our rationale for the preparation of the second generation of MAbs against the MAb B72.3-defined antigen, TAG-72, was that additional anti-TAG-72 MAbs could be useful in combi-
nation with B72.3, or perhaps more useful than B72.3, in pathology and clinical applications. In a recent study (36), we generated and partially characterized 28 second generation IgG CC MAbs to TAG-72. Those MAbs were selected to be only of IgG isotypes. In the present study, we described the generation and partial characterization of 8 IgM CC MAbs against TAG-72.

As shown in Table 1, and as previously described (36), the second generation CC MAbs showed differential reactivities with biopsy extracts of five different colon carcinomas and five different ovarian carcinomas. Similar findings in analyses of mucin glycoproteins have also been reported. Recently, Linsley et al. (56) extensively characterized 14 new MAbs directed to mucin glycoproteins expressed by human breast carcinomas and found that the new MAbs recognized mucins that bound their original MAAb1 but that the expression of epitopes for the new MAbs varied between different sources of mucins. Burchell et al. (57) also characterized two MAbs against human milk fat globule and showed that the relative levels of binding of the two MAbs varied between cell lines from normal and malignant breast epithelium, with one epitope being expressed more strongly on tumor cells lines. In a recent study, Bara et al. (58) generated seven MAbs against gastric mucins and observed some differences in the reactivities of the MAbs in subepithelial, submucosal, and endocervical mucus cells. These observations indicate that mucins from different sources may vary antigenically.

To determine whether one or several of these second generation MAbs against TAG-72 can be used either as a more efficient substitute for, or in combination with, B72.3, 11 IgG MAbs (including B72.3) and 8 IgM MAbs were selected for further serological mapping of TAG-72. As shown in Fig. 1 and as previously described (36), all 19 of these MAbs reacted predominantly with a high molecular weight glycoprotein in a crude extract of LS-174T. The additional bands recognized by MAbs CC112, 115, and 118 appear to represent fragmented TAG-72 molecules. Fragmentation of TAG-72 molecules has been shown to occur during the purification procedure. The apparently selective detection of these fragments by MAbs CC112, 115, and 118 is most likely related to sensitivity, since these MAbs gave the strongest staining reactions in Western blots.

By immunodiffusion, all 19 MAbs tested produced precipitate lines against purified TAG-72, indicating the existence of multiple epitopes for each MAb (55), as originally suggested for MAbs B72.3 from results using a sandwich-type, double determinant RIA procedure (59). Precipitate lines have also been observed with other MAbs that recognize carbohydrate epitopes on the glycoprotein molecules (58, 60). When tested with immunoelectrophoresis using the MAb CC112, a broad migration pattern of TAG-72 was seen in α- to β-globulin region (data not shown).

The results of the immuno depletion analyses strongly suggest that epitopes recognized by the anti-TAG-72 MAbs tested all reside on the same molecule or population of molecules. The antigenic complexity of the TAG-72 molecule is further demonstrated by the results of the 19 cross-competition assays between the anti-TAG-72 MAbs (Table 2 and Fig. 3). To determine the relationships between the epitopes recognized by the 19 anti-TAG-72 MAbs, cross-competition SPRIAs utilizing purified radiolabeled TAG-72 were used. This method alleviated the problem of labeling all antibodies to be tested and is convenient for mapping analysis of many MAbs if the corresponding purified antigen is available. Cross-competition assays between the anti-TAG-72 MAbs revealed a complex array of epitopes on the TAG-72 molecule. As shown in Fig. 3, many antibodies competed for, or were competed by, other antibodies in nonreciprocal fashion. Although the results for the nonreciprocal patterns of cross-competition are unclear, several factors, such as affinity constants, the molecular size of the MAbs (i.e., IgG versus IgM), the size and/or number of the epitopes recognized by each MAb, and the structural and/or steric relationshipship between the epitopes, may be considered as possible explanations. Steric hindrance alone probably cannot account for all of the nonreciprocal results, since 5 of 8 IgM MAbs used only competed for less than 6 of 19 MAbs tested, and in turn, the same 5 IgM MAbs were competed significantly by more than 12 MAbs, including several IgG MAbs. Similarly, differences in affinity constants alone cannot explain all of the nonreciprocal inhibition patterns. This is shown by the finding that MAb CC92, which has a fairly high Ks of 14.3 × 106 M⁻¹ (36), only competed for itself but was significantly competed by 17 of 19 MAbs tested (Fig. 3). This can be contrasted with the results obtained with MAb CC15 which has a lower Ks (9.13 × 106 M⁻¹) (36) and yet competes much better in competition RIAs than CC92 (Fig. 3). Nonreciprocal cross-competition could result from (a) steric hindrance of an epitope by a second antibody bound to a different site, (b) recognition by an antibody of several structurally related sites, only some of which may be recognized by other antibodies, or (c) conformational change in the antigen molecule by binding of one antibody, which may affect binding of the second antibody.

These studies thus further characterize the TAG-72 molecule, reveal an array of distinct tumor-associated epitopes, and may provide a rational basis for the selection of new MAbs or combinations of MAbs for potential clinical applications.

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Serological Mapping of the TAG-72 Tumor-associated Antigen Using 19 Distinct Monoclonal Antibodies

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