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ISOLATION AND CHARACTERIZATION OF A CARCINOMA-ASSOCIATED ANTIGEN

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Summary. GA733 is a murine IgG_{2a} monoclonal antibody (MAb) against human gastric carcinoma and is highly tumoricidal in nude mice. The GA733 antigen is a cell surface protein with two subunits of 30,000 and 40,000 daltons. The antigen isolated by immunoaffinity chromatography consists mainly of the 30,000-dalton subunit which bears the GA733 epitope. This subunit displayed several isoelectric points between 6.9 and 7.7. Anti-colon carcinoma MAb 17-1A also detects this antigen, but probably binds to a different epitope.

We have demonstrated that murine IgG_{2a} monoclonal antibodies (MAbs) have a potent tumoricidal effect in nude mice (1). As a result of these studies, therapeutic trials were begun in humans using the IgG_{2a} anti-colorectal carcinoma MAb 17-1A (2). Beneficial effects of this treatment might be due to the activation of human effector cells or to the induction of anti-idiotypes and anti-anti-idiotypes, thereby producing a long-term immune response to the tumor (3-4). Recently, the trials have been extended to include IgG_{2a} MAb GA733, which detects a glycoprotein with 30,000- and 40,000-dalton subunits (5). In this study we have isolated and biochemically characterized the GA733 antigen. We find that MAbs GA733 and 17-1A detect the same protein although, based on competition studies and anti-idiotype studies, they most likely bind to different epitopes.

MATERIALS AND METHODS

Cell lines and tumors. Colorectal carcinoma cell line SW948 was grown in culture using Eagle's minimal essential medium supplemented with 10% heat-inactivated fetal calf serum. For growth of SW948 tumors, 5 X 10^6 cells were injected subcutaneously into nude mice. The resulting tumors (5-20 mm) were excised about 8 weeks later and stored at -70°C.

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MAbs and immunoaffinity matrices. The properties of 17-1A and GA733 (IgG_{2a}) MAbs have been described (5-6). Culture supernatant from hybridoma P3X63Ag8 which secretes MAb MOPC21 (IgG_1) was used as a nonspecific control. In some cases BR15-6A MAb (IgG_{2a}, anti-Y carbohydrate determinant) was used as a negative control. IgG was purified from ascites fluid by protein A Sepharose chromatography. MAbs dissolved in 0.15 M NaCl, 10 mM sodium phosphate, pH 7.4, were coupled to cyanogen bromide-activated Sepharose at 4°C.

Isolation of GA733 antigen. The tumors were thawed, minced into fine pieces, and extruded through a wire screen. As judged by trypan blue staining, this preparation contained cell fragments and no intact cells. The preparation was centrifuged at 105,000xg for 60 min and the cell fragments in the pellet were extracted with 5 volumes of NP-40 buffer (0.5% NP-40, 140 mM NaCl, 10 mM NaF, 10 mM Tris, 5 mM EDTA, 100 Kallikrein IU/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, pH 7.5) for 16 hr. The extract was clarified by centrifugation at 105,000xg for 60 min and filtered through a 0.45-μm filter.

The extract from about 15 grams of SW948 tumors was passed through a bovine serum albumin (BSA)-Sepharose column (3 ml of 10 mg/ml) equilibrated with 0.2% NP-40, 20 mM Tris, pH 7.5. The extract was then passed twice through a GA733-IgG column (2 ml of 5 mg/ml). The column was then washed with 40 ml of the same buffer and eluted with 0.2% NP-40, 50 mM diethylamine, pH 11.0. The receiving tube for each 8 ml fraction contained 0.1 ml, 1 M Tris, pH 6.0. The purified protein was stored at -20°C.

Immunoprecipitation, gel electrophoresis, and Western blotting. Immunoprecipitations were carried out using agarose derivatized with goat antibody directed against mouse immunoglobulin (IgG) (7). One-dimensional gel electrophoresis was performed according to Laemmli (8). Two-dimensional gel equilibrium electrophoresis was by the method of O'Farrell (9) using pH 3.5-10 ampholines. Western blotting was performed according to Towbin et al. (10).

Radioiodination of GA733 antigen. The antigen was radioiodinated on ice with 5 mM chloramine-T using 0.4 mCi $Na^{125}I$ per sample. After 2 min the reaction was stopped with 14 mM sodium metabisulfite and then applied to a PD-10 column equilibrated with 0.1% BSA, 0.15 M NaCl, 10 mM sodium phosphate, pH 7.4. The fractions at the excluded volume of the column were pooled and stored at -20°C.

Binding of GA733 antigen to bound antibody. Solid-phase microtiter plates (Costar Plastics) were incubated with IgG (1 μg/well) in phosphate-buffered saline (PBS) (0.15 M NaCl, 10 mM sodium phosphate, 0.02% NaN_3, pH 7.5) for 16 hr at 4°C. Wells were blocked with PBS + 2% BSA for 3 hr at room temperature, and were washed with PBS + 2% gamma globulin-free horse serum. Radioiodinated GA733 antigen (25,000 cpm/well) and in some cases competing MAb were incubated at 4°C in the wells. After 16 hr, the wells were washed 3 times with the horse serum buffer and eluted with Laemmli sodium dodecyl sulfate (SDS) sample buffer for 30 min at 37°C. Samples were boiled, assayed for radioactivity in a gamma counter, and further analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography.

Anti-idiotypic antibodies (anti-Ids). Anti-Ids were prepared against MAbs GA733 and 17-1A as described and were shown to react with the combining sites of the corresponding MAbs (11). These anti-Ids were used in direct binding assays with various MAbs as targets and in binding inhibition assays in which binding of MAb to either colon carcinoma target cells or homologous anti-Ids was inhibited by homologous or heterologous anti-Ids as described in detail elsewhere (11).

RESULTS AND DISCUSSION

Isolation and molecular characterization of GA733 antigen. Cell fragments from SW948 tumors were solubilized with NP-40 buffer, clarified by

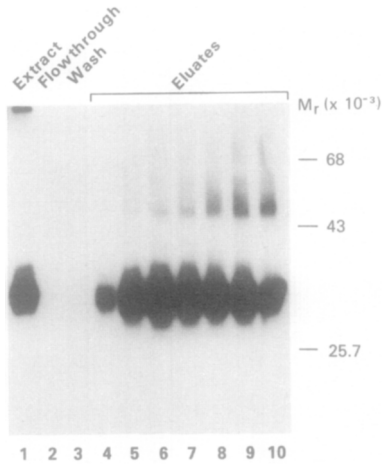

Figure 1. Analysis of GA733 immunoaffinity chromatography by Western blotting. Cell extract (10 ml) was applied to a GA733 IgG Sepharose column, washed with 40 ml of 0.2% NP-40, 20 mM Tris, pH 7.5, and eluted 8 times with 8 ml of 0.2% NP-40, 50 mM diethylamine, pH 11.0. Twenty-five µl of each fraction was mixed with SDS-PAGE sample buffer lacking mercaptoethanol, boiled and subjected to SDS-PAGE. The proteins present in the gel were electrophoretically transferred to nitrocellulose, and the GA733 antigen was detected with GA733 MAb and [^{125}I]goat anti-mouse IgG by the Western blot procedure.

ultracentrifugation, and applied to a GA733 immunoaffinity column. After thorough washing, the protein bound to the column was eluted with mild base. Analysis of the resulting fractions by Western blotting demonstrated that most of the antigen was bound by the column (Fig. 1) and was released from the column by the base. Binding of the GA733 MAb to antigen is probably dependent on the tertiary conformation of the protein since samples treated with mercaptoethanol gave no bands in Western blots.

The eluate was dialyzed against 0.1% SDS, 50 mM NH_4HCO_3 and lyophilized. This material was redissolved and iodinated with chloramine-T as described in Materials and Methods. The nonradioactive and the iodinated GA733 antigens were analyzed by SDS-PAGE and found to be nearly homogeneous by silver staining and autoradiography, respectively (Fig. 2A and B). Furthermore, the [^{125}I]GA733 antigen was immunoprecipitated by MAb GA733 but not by a control MAb, confirming that the purified protein is the GA733 protein. The [^{125}I]GA733 antigen was further characterized by two-dimensional gel

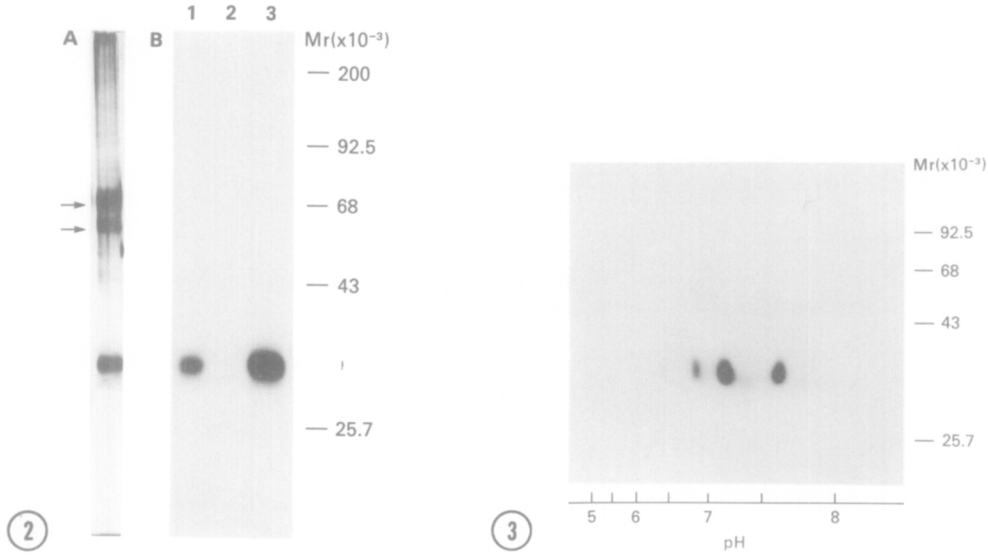

Figure 2. Analysis of purified GA733 antigen by SDS-PAGE. Panel A. Purified antigen was electrophoresed on a 10% polyacrylamide gel and detected by silver staining. Two nonspecific bands which appear even in lanes to which no sample was applied are marked with arrows. Panel B. [^{125}I] GA733 antigen was either analyzed directly by SDS-PAGE (lane 3) or first immunoprecipitated with GA733 MAb (lane 1) or with P3X63Ag8 (lane 2), a nonspecific control.

Figure 3. Charge heterogeneity of GA733 antigen. Radioiodinated GA733 antigen was analyzed by two-dimensional gel electrophoresis using ampholines in the pH 3.0-10.0 range.

electrophoresis (Fig. 3). Three major species were detected with pIs between 6.9 and 7.7

Binding of GA733 antigen by MAb 17-1A. GA733 MAb completely inhibited the binding of [^{125}I]17-1A MAb to SW948 cells while 17-1A MAb partially inhibited [^{125}I]GA733 MAb binding (not shown). To determine whether these MAbs detect the same antigen, microtiter plates were coated with either GA733, 17-1A, ME75-29, or BR15-6A IgG, the wells were blocked and incubated with [^{125}I]GA733 antigen, and washed. Bound radiolabeled protein was eluted with Laemmli sample buffer and analyzed by SDS-PAGE and autoradiography. Fig. 4A shows that both GA733 and 17-1A MAbs but not ME75-29 or BR15-6A bound [^{125}I]GA733 antigen. Between 5 and 15% of added cpm was bound by GA733 MAb, depending upon the batch of [^{125}I]GA733 antigen used. We then tested whether GA733 or 17-1A MAb would inhibit each other in binding to ^{125}I-labeled puri-

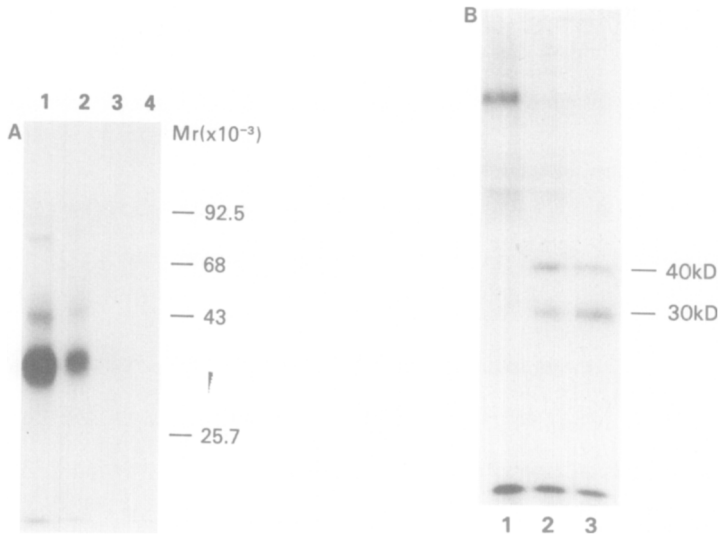

Figure 4. Binding of [^{125}I]GA733 antigen to immobilized MAbs. Panel A. Purified radioiodinated antigen bound to a microtiter plate derivatized with GA733 IgG (lane 1), 17-1A IgG (lane 2), ME75-29 IgG (lane 3), or BR15-6A IgG (lane 4) was analyzed by SDS-PAGE. Panel B. Proteins from extract of surface-labeled SW948 cells bound to microtiter plate derivatized with BR15-6A IgG (lane 1), 17-1A IgG (lane 2), or GA733 IgG (lane 3).

fied antigen. Fig. 5 shows that soluble GA733 MAb completely inhibited binding of [^{125}I]GA733 antigen by GA733 or 17-1A MAb bound to the plate. Soluble 17-1A MAb partially inhibited binding of antigen by GA733 or 17-1A MAb bound to the plate. Unrelated MAb BR15-6A did not significantly inhibit binding.

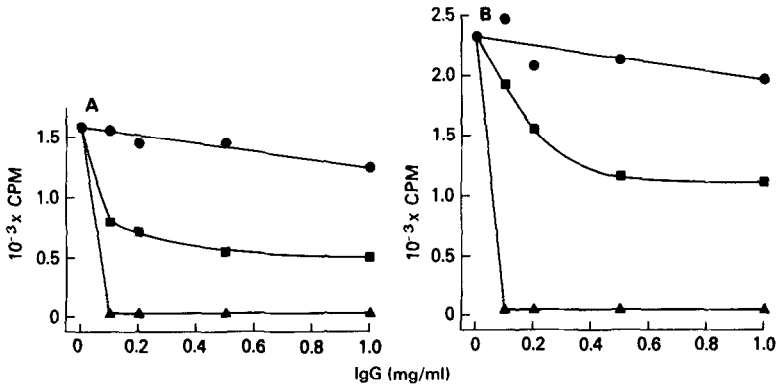

Figure 5. Inhibition by soluble IgG of binding of immobilized MAbs to [^{125}I]GA733 antigen. Antigen bound to an microtiter plate derivatized with either 17-1A IgG (A) or GA733 IgG (B) in the presence of soluble BR15-6A IgG (●), GA733 IgG (▲), or 17-1A IgG (■) was quantitated by gamma counting.

Total cell extract of lactoperoxidase-surface radioiodinated SW948 cells was incubated in MAb-coated microtiter wells, washed, and eluted as above. MAbs GA733 and 17-1A bound both the 30,000- and 40,000-dalton forms of the antigen (Fig. 4B).

Idiotypic analysis of MAbs 17-1A and GA733. By Id analysis (11), no significant cross-reactions between the idiotypes of MAbs GA733 and 17-1A were detected.

Conclusions. We have isolated the GA733 antigen from SW948 tumor cells. The antigenic site is located on a 30,000-dalton protein with a pI of 6.9-7.7 and is sensitive to reduction of disulfide bonds. This purified protein will be useful for more detailed biochemical and sequence analysis as well as for the characterization of anti-Id and anti-anti-Id immune responses in cancer patients treated with MAb GA733 (4, 11, 13). These experiments also raise two questions that require further study. First, the antigen consists of two subunits with apparent molecular weights of 30,000 and 40,000 (5); however, the isolation procedure gave preparations with little or no 40,000-dalton species suggesting that this antigen form is lost during purification of the 30,000-dalton form or that the 30,000-dalton form is a breakdown product that readily forms during purification. Second, both anti-carcinoma MAbs GA733 and 17-1A bind to the GA733 antigen. It is likely that they bind to different but adjacent sites on the protein since they partially compete with each other for binding to the antigen and do not share idiotopes. However, the partial inhibition of GA733 MAb binding by 17-1A MAb may also reflect the 10-fold lower affinity of the 17-1A MAb (12). A detailed mapping of the antigen-antibody binding sites should aid in answering these questions.

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