Tumor-associated Antigen Defined by a Monoclonal Antibody against Neuraminidase-treated Human Cancer Cells

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ABSTRACT

A monoclonal antibody, MH-A6, was produced by immunization with a human gastric cancer cell line, MKN 74, treated with neuraminidase. The antigen defined by the monoclonal antibody was detected on various tumor tissues and a limited number of normal tissues in immunoperoxidase assay, and the expression of MH-A6 antigen was not influenced by neuraminidase treatment except for some cases of tumor tissues. Interestingly, neuraminidase treatment enhanced binding of the antibody to some adenocarcinomas, but diminished binding of the antibody to squamous cell carcinomas. Treatment of the immunizing tissues with trypsin and periodic acid diminished binding of the antibody. In isolation of MH-A6 antigen from MKN 74 cells by the monoclonal antibody coupled-affinity column, the epitope exists on molecules with molecular weights of 30,000 and 72,000, and with an acidic pH range in two-dimensional electrophoresis. CEA and CA 19-9 activities were not detected in purified MH-A6 antigen by solid-phase radioimmunoassay, and the reactivity of the MH-A6 antibody with CEA and CA 19-9 was not detected in enzyme-linked immunosorbent assay. Hemagglutination observed between erythrocytes (Lewis', Lewis', or NE-treated) and anti-Lewis' sera, or anti-T-agglutinin (peanut lectin), respectively, was not inhibited by MH-A6 antigen. The results suggest that MH-A6 antigen is a tumor-associated antigen, probably glycoprotein, and different from CEA, CA 19-9, Lewis', Lewis', and Thomsen-Friedrich (T) antigen.

INTRODUCTION

Many of the monoclonal antibodies prepared by immunization with the nontreated human cancer cells react with terminal carbohydrate structures such as sialyl-Lewis' (1), sialyl-Lewis' (2), and Lewis' (3, 4). On the other hand, neuraminidase treatment of tumor cells removes the sialic acid from cell surfaces, increases the immunogenicity, and reveals the T-antigenic determinant (5, 6). Thus, it was interesting to us whether a new monoclonal antibody (MH-A6), different from many previously reported monoclonal antibodies reacting with tumor-associated antigen, would be produced by immunization with NE'-treated human cancer cells.

In this study, we reported the distribution of the antigen defined by MH-A6 antibody in normal and tumor tissues, and the characterization of the antigen isolated from MKN 74 cells and ascites of a patient with pancreatic carcinoma.

MATERIALS AND METHODS

Preparation of Monoclonal Antibody

The cell line MKN 74, established from a well-differentiated adenocarcinoma of the human stomach, was treated with neuraminidase (from Vibrio cholerae, GIBCO) at a ratio of 20 IU/1 × 10⁷ cells/ml in Dulbecco's minimum essential medium (pH 6.0) for 2 h at 37°C in a 5% CO₂ incubator and then centrifuged. Pellets were suspended in Dulbecco's minimum essential medium with 10% fetal calf serum and prepared at a concentration of 4 × 10⁶ cells/ml for immunization. Six-week-old male BALB/c mice were immunized i.p. with NE-treated MKN 74 cells emulsified in complete Freund's adjuvant. The animals were given three i.p. booster injections a week apart and final injection 3 days prior to fusion experiment. Spleen cells from these mice were fused with P3-X-63-Ag8, 653 myeloma cells according to a modified method of Köhler and Milstein (7). Two to 3 weeks after fusion, culture supernatants were analyzed by ELISA for antibody production. Positive clones were selected and subcloned twice by semisolid cloning and passed into BALB/c mice to produce ascites.

Monoclonal Antibody Screening by ELISA

Microtiter plates (Samco Junyaku Co.) were coated overnight at 4°C with crude extracts of MKN 74 cells (at about 20 μg protein/ml in bicarbonate buffer, pH 9.6), and then 3% BSA in bicarbonate buffer were sufficiently added to the wells, and coated overnight at 4°C. After washing three times with PBS/0.05% Tween 20, 50 μl of culture supernatants of hybrid cells were added and incubated for 2 h at 37°C.

After washing three times with PBS/0.05% Tween 20, 50 μl of 1:100 diluted biotin-labeled horse anti-mouse IgG (Vector Labs.) were allowed to react for 1 h at 37°C. After washing five times, 1:40 diluted avidin-biotin peroxidase complex was added to the wells and incubated for 45 min at 37°C. After washing five times, 100 μl of peroxidase substrate (2,2-azino-di/3-ethyl-benzothiazoline sulfamate, KPL) containing H₂O₂ were added and left for 45 min at room temperature. The absorbance was measured at 405 nm with aTitertec Multiskan MC.

Indirect Immunofluorescence Assay

Indirect immunofluorescence assay was performed by MKN 74 cells with culture supernatants of cloned hybrid cells for 50 min at 37°C. After washing three times with PBS, the cells were incubated in 20 μl of 1:50 diluted fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (KPL) for 50 min at 37°C, followed by washing three times. The cells examined by fluorescence microscopy.

IP Assay

The IP assay was performed by a modification of ABC (avidin-biotin peroxidase complex) IP technique on normal, fetal, and various tumor tissues. The sections of tissues fixed in 10% buffered formaldehyde were deparaffinized and hydrated via a series of xylene and graded ethanol rinses. The tissue sections were pretreated with 0.3% H₂O₂ in methanol for 15 min to inhibit endogenous peroxidase, followed by treatment with 3% BSA in PBS for 30 min at room temperature. The slides were then successively incubated with approximately 0.2 ml of 1:100 diluted mouse ascites overnight at 4°C, with 15 min rinses in PBS containing 0.1% BSA between all steps. The slides were treated for about 5 min with 0.024% (w/v) 3-amino-9-ethylcarbazole in 0.02 M sodium acetate buffer (pH 5.2) and 0.02% H₂O₂, then counterstained with hematoxylin, dehydrated, and mounted in glycerol/PBS. A brownish color distinctly stronger than that of the background was judged as positive staining.

Enzyme Treatment

The IP assay was begun, but after pretreatment with 3% BSA and before incubation with mouse ascites, the slides were incubated with 2 μl per slide neuraminidase (from Vibrio cholerae, GIBCO) for 2 h at 37°C, with 0.1% trypsin (DIFCO Labs.) for 30 min at 37°C, or with
1% periodic acid for 10 min at 37°C, respectively. After each treatment, the slides were washed, exposed to the monoclonal antibody, and processed as described before.

Isolation of the Antigen Reacting with Monoclonal Antibody

The soluble antigen (3 M KCl extract) from MKN 74 cells (1 × 10⁶ cells/ml) was prepared with 3 M KCl extraction according to a modified method of Reisfeld et al. (8). Ascites of a patient with pancreatic carcinoma was fractionated by adding solid ammonium sulfate to 50% saturation at 4°C. The precipitate was dissolved in PBS and dialyzed against PBS for 3 days at 4°C (ascites extract).

For isolation of the specific antigen reacted with MH-A6 antibody from 3 M KCl extract and ascites extract, the monoclonal antibody-coupled affinity column was prepared as followed: 5 ml of ascites from BALB/c mice injected with cloned hybrid cells were dialyzed against 0.01 M phosphate buffer (0.01 M KH₂PO₄, 0.01 M K₂HPO₄, pH 8.0) overnight at 4°C. IgG fractions were prepared with a linear gradient of 0.02 to 0.05 M phosphate buffer in a DE 52 ion exchanger (Whatman) column, concentrated by a microsorution concentrator (AMICON, type B-15) up to a concentration of 5 mg/ml, and dialyzed against 0.1 M NaHCO₃ containing 0.5 M NaCl (pH 8.5). Dialyzed IgG fractions were coupled to CNBr-activated Sepharose 4B gel (Pharmacia) using 0.1 M NaHCO₃ coupling buffer containing 0.5 M NaCl (pH 8.5) and uncoupled gel was blocked by 0.2 M glycine. The extract samples were applied to the affinity column with irrelevant mouse immunoglobulin fraction as a precolumn. Fractions passed through a precolumn were applied to the monoclonal antibody-affinity column, and incubated overnight at 4°C. The column was washed with PBS containing 0.02% Na₂SO₄. The bound materials were eluted with 0.2 M glycine-HCl containing 0.15 M NaCl (pH 3.4). Fractions eluted from the affinity column were collected and neutralized with Tris powder. The eluted fraction was concentrated by a microsorution concentrator up to 50-fold.

SDS-PAGE

SDS-PAGE was performed according to the procedure described by Laemmli (9). Samples were prepared by heating at 37°C in the presence of 5% 2-mercaptoethanol for 2 h in 2% SDS containing sample buffer, and separated on 12% polyacrylamide gels. Electrophoresis was carried out on the following samples: purified antigens by the monoclonal antibody-affinity column from (a) 3 M KCl extract and (b) ascites extract. After electrophoresis, the gel was fixed in 10% trichloroacetic acid and stained by a silver staining procedure (Silver staining kit; Daiichi Chemical Co., Ltd.).

Two-Dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis was carried out according to a method of O’Farrell (10). Nonequilibrium pH gradient gel electrophoresis was performed in the first dimension. 3 M KCl extract from MKN 74 was dissolved in sample buffer containing 9.5 M urea, 2% w/v NP-40, 2% ampholine (Phamalyte, pH range 3–10), and 5% 2-mercaptoethanol. Urea was added to the samples and 10 mg/20 μl solution was prepared. The disc gel was 10 cm long and 1 mm thick. As electrode buffer, 0.01 M H₂PO₄ was used in the upper reservoir and 0.02 M NaOH in the lower reservoir. The reduced samples were electrophoresed at 500 V for 6 h. SDS-PAGE using 12% polyacrylamide gel was processed as described before in the second dimension. After electrophoresis, the gel was fixed in trichloroacetic acid and stained with silver.

Immunoblotting Procedure

Immunoblotting was performed on the following samples: 3 M KCl extract, ascites extract, and NE-treated ascites extract. Neuraminidase treatment was carried out at a final concentration of 20 IU/ml. These reduced samples were developed by SDS-PAGE or two-dimensional gel electrophoresis, and separated proteins were transferred to nitrocellulose sheets with the passive diffusion technique (11). After transfer, specific antigens reacting with the monoclonal antibody in the sheets were detected by IP technique as described before, except that 0.05%

Cross-reactivity of Monoclonal Antibody-defined Antigen

Solid-Phase Radioimmunoassay. The 3 M KCl extract from MKN 74 cells was applied to the monoclonal antibody-coupled affinity column. Fractions eluted from the antibody-affinity column were neutralized, and adjusted to 10 μg protein/ml. Commercially available solid-phase radioimmunoassay kits of CEA (DAINABOT) and CA 19-9 (CIS) were used to determine the activity of CEA and CA 19-9 in both bound and unbound fractions eluted from the antibody-affinity column.

ELISA. Microtiter plates were coated in triplicate overnight at 4°C with the affinity-purified antigen (10 μg protein) from 3 M KCl extract, CEA (100 ng/ml), CA 19-9 (120 U/ml), and 3% BSA (negative control) in bicarbonate buffer (pH 9.5), respectively. In the following procedure, ELISA was performed by the same fashion of the monoclonal antibody screening procedure as described before. The absorbance was measured at 405 nm.

Hemagglutination Inhibition Assay. Hemagglutination inhibition assay was performed with anti-Lewis' (ORTHO, LA340A2; GAMMA, LEA105-3; Hoechst, 01950A), anti-Lewis'' (DADE, 311,1001 la) sera, or anti-T-agglutinin (peanut lectin, Bio-Science Product), respectively. These sera were diluted to four agglutinin titers. One hundred μl of anti-Lewis', anti-Lewis'' sera, or anti-T-agglutinin were mixed with 100 μl of the affinity-purified antigen, respectively. These mixtures were allowed to hold for 2 h at 4°C, and then 2% red cell suspension of known Lewis blood groups (Lewis' and Lewis'') or NE-treated erythrocyte were added to the mixtures. Hemagglutination was observed after 2 h at room temperature.

RESULTS

Production of Monoclonal Antibody. Spleen cells from mice immunized with NE-treated MKN 74 cells were fused with P3 myeloma cells. Four of 48 hybrid cultures secreted antibody reacting with MKN 74 cells in ELISA. In indirect immunofluorescence assay, the culture medium of cloned hybrid cells reacted with membrane and cytoplasm of MKN 74 cells (Fig. 1). The isotype of the monoclonal antibody determined by immunodiffusion was IgG.

Expression of the Antigen Reacting with Monoclonal Antibody in Tissues. The distribution of MH-A6 antigen in the tissue specimens was examined by IP assay. The positive stained specimens in normal tissues were as followed: Limited parts of glands and keratinized layers of squamous epithelium of the esophagus; gastric glands of basal zone; Brunner’s gland of the duodenum; islets, centroacinar cells, and small ducts of the pancreas; small biliary ducts of the liver; columnar epithelium

Fig. 1. Reaction of the monoclonal antibody with MH-A6. Indirect immunofluorescence assay on MKN 74 cells (× 400).
Expression of the monoclonal antibody-defined antigens in tumor tissue specimens determined by IP assay with or without neuraminidase treatment

<table>
<thead>
<tr>
<th>Tumor origin</th>
<th>Frequency of positive antigen staining</th>
<th>Effect of NE treatment¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without NE treatment</td>
<td>With NE treatment</td>
</tr>
<tr>
<td>Esophagus</td>
<td>3/4</td>
<td>0/4</td>
</tr>
<tr>
<td>Stomach</td>
<td>6/6</td>
<td>6/6</td>
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<tr>
<td>Colon</td>
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<td>6/6</td>
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<tr>
<td>Pancreas</td>
<td>5/5</td>
<td>5/5</td>
</tr>
<tr>
<td>CBD*</td>
<td>1/2</td>
<td>2/2</td>
</tr>
<tr>
<td>Liver</td>
<td>1/2</td>
<td>1/2</td>
</tr>
<tr>
<td>Breast</td>
<td>3/3</td>
<td>3/3</td>
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<tr>
<td>Lung</td>
<td>2/3</td>
<td>2/3</td>
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</table>

¹↓, diminished; →, not influenced; ↑, enhanced or newly expressed.
²CBD, common bile duct.

of the gall bladder; paroximal tubules of the kidney; bronchial glands and tissue macrophages.

Erythrocytes, NE-treated erythrocytes, lymphocytes, granulocytes, spleen, pancreatic acinar cells, hepatocytes, goblet cells, brain, veins, arteries, muscles, and connective tissues failed to react with MH-A6 antibody. Fetal tissues showed the same distribution of the antigen as normal tissues.

The result of IP assay in carcinoma tissue specimens are shown in Table 1. The positive staining was observed in six of six gastric carcinomas, six of six colonic carcinomas, five of five pancreatic carcinomas, and two of three lung carcinomas.

The antigen expression in cancerous portions was observed in the cytoplasm of the cancer tubules and in the stroma near cancer tubules, but not observed in the luminal contents.

Effect of Enzyme Treatment on the Expression of the Antigen in Tissues. The binding of the MH-A6 antibody in normal and tumor tissues was not affected by neuraminidase treatment, except that it was enhanced in two of five pancreatic adenocarcinomas and one of two common bile duct adenocarcinomas, and completely diminished in three of three esophageal squamous cell carcinomas (Table 1, Figs. 2 and 3). The binding of MH-A6 antibody in pancreatic and esophageal carcinomas was diminished by periodic acid, and decreased by trypsin treatment.

These results suggest that MH-A6 antigen may be glycoprotein, and that the antigenic determinant recognized by the antibody may be sialic acid free except for cases of esophageal carcinomas.

Isolation and Characterization of the Antigen. The extracted antigens from MKN 74 cells and patient's ascites with pancreatic carcinoma was purified by the monoclonal antibody-affinity column chromatography. For characterization of MH-A6 antigen, the affinity-purified antigen was subjected to SDS-PAGE, and immunoblotting procedure was performed with crude extracts.

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Fig. 2. Sections of pancreatic adenocarcinoma stained by IP assay with the monoclonal antibody. Counterstained with hematoxylin (× 400). A, without NE treatment; B, with NE treatment.

Fig. 3. Sections of squamous cell carcinoma of the esophagus stained by IP assay with the monoclonal antibody. Counterstained with hematoxylin, (× 125). A, without NE treatment; B, with NE treatment.
The results of SDS-PAGE showed that two components were developed as the band with estimated molecular weights of 30,000 and 72,000 from 3 M KCl extract (Fig. 4A) and two components with estimated molecular weights of 67,000 and 72,000 were developed from ascites extract (Fig. 4B). In immunoblotting, two bands with molecular weights of 30,000 and 72,000 of 3 M KCl extract in SDS-PAGE reacted with MH-A6 antibody, and binding of the antibody was prominent on the former (Fig. 5A). Of two bands of ascites extract developed by SDS-PAGE, binding of the antibody exists only in a band with a molecular weight of 72,000 (Fig. 5B).

The results suggest that the band with a molecular weight of 67,000 is an artifact due to presence of albumin, because this component was removed by Cibacron blue F3G-A-Sepharose CL-6B. Neuraminidase treatment did not influence the results of immunoblotting for ascites extract from a patient with pancreatic carcinoma (Fig. 5C).

To find out more about the physicochemical nature of the antigen, 3 M KCl extract was subjected to two-dimensional gel electrophoresis (Fig. 6) and antigens were identified by immunoblotting. The results showed that the spot with an estimated molecular weight of 30,000 is present at the pH 4.8–5.8 region, and the spot with an estimated molecular weight of 72,000 is present at the pH 6.0–6.4 region in two-dimensional electrophoresis (Fig. 7).

Cross-reactivity of the Antigen. When CEA and CA 19-9 activities were measured using solid-phase radioimmunoassay kits, both activities detected in affinity-purified antigen from 3 M KCl extract were the same level as that of negative standard control of assay kits. But CEA activity in the unbound fraction of 3 M KCl extract eluted from the monoclonal antibody-affinity column was found to be higher than that of the negative standard control (Table 2).

The reactivity of MH-A6 antibody with the affinity-purified antigen, CEA, CA 19-9, and 3% BSA (negative control) was examined by ELISA. Table 3 shows that the reactivity of MH-A6 antibody with the affinity-purified antigen was approximately 7-fold as that with CEA, CA 19-9, and 3% BSA.

In order to detect the two Lewis blood group substances and Thomsen-Friedreich antigen in the affinity-purified antigen from 3 M KCl extract, the hemagglutination inhibition assay...
A monoclonal antibody, MH-A6, is described here which defines a new tumor-associated antigen. MH-A6 antigen was found to have somewhat different distribution in normal tissues from that of such tumor-associated antigens as sialyl-Lewis* defined by N-19-9 (1, 13), sialyl-Lewis* defined by CSLEX1 (2), and Lewis* (3, 4, 12). Both MH-A6 antigen and sialyl-Lewis* were detected on the esophagus, proximal tubules of the kidney, and tissue macrophages. In addition to their common distribution, MH-A6 antigen was detected on islets and small ducts of the pancreas, gastric glands, bile ducts, and bronchi, whereas sialyl-Lewis* was detected on acinar cells of the pancreas, hepatocytes, Kupffer's cells, and granulocytes (2). The tissue distribution of Lewis* appears to be almost identical to that of sialyl-Lewis*, except that Lewis* is expressed in stomach and brain tissue (12). The sialyl-Lewis* antigen was found on gastric glands, pancreatic ducts, small biliary ducts, and the gallbladder, and so on (13); such tissue distribution is similar to that of MH-A6 antigen, except that MH-A6 antigen is expressed in pancreatic islets, Brunner's glands, and proximal tubules of the kidney and the esophagus, and sialyl-Lewis* is not (13). In addition, CEA and CA 19-9 activities were not detected in the affinity-purified antigen by solid-phase radioimmunoassay, and in ELISA the reactivity of MH-A6 antibody with CEA and CA 19-9 was similar to that with negative control absorbance.

**DISCUSSION**

Table 2 shows that the reactivity of the monoclonal antibody with affinity-purified antigen from 3 m KCl extract of MKN 74 cells, CEA, CA 19-9, and 3% BSA in ELISA. The results of the affinity-purified antigen were performed with anti-Lewis*, anti-Lewis* sera, and anti-T-agglutinin. The affinity-purified antigen was tested at a dilution range of from 1:1 to 1:100. No inhibition of hemagglutination was observed.

**REFERENCES**


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