Immunochemical Analysis of the Determinant Recognized by a Monoclonal Antibody (MBr1) Which Specifically Binds to Human Mammary Epithelial Cells

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ABSTRACT

A monoclonal antibody (MBr1) raised against a membrane preparation (CM) of a human breast cancer line (MCF-7) and characterized as mammary gland epithelium associated (S. Ménard, E. Tagliabue, S. Canevari, G. Fossati, and M. I. Colnaghi. Generation of monoclonal antibodies reacting with normal and cancer cells of human breast. Cancer Res., 43: 1295–1300, 1983), was used to biochemically define and partially purify its target antigen. The antigenic activity recognized by MBr1 was unaffected by treatment of MCF-7 cells with trypsin, protease K, or Vibrio cholerae neuraminidase and by heating at 100° but was abolished by treatment with methanol. Since this behavior suggested a glycolipid nature of the MBr1-defined antigen, total lipids obtained by chloroform:methanol or tetrahydrofuran:phosphate buffer extractions from crude membrane preparations of MCF-7 cells and of breast cancer surgical specimens. Total absorption of MBr1 activity was found by breast cancer lipid extracts, whereas no absorbing capability was detected with a series of highly purified acid and neutral glycolipids or with normal and neuraminidase-treated red blood cells of human, ox, and sheep species. The same pattern of inhibition of MBr1-binding activity was obtained with total lipid extract and both phases after diethyl ether partition. However, when the three extracts were chromatographed on diethylaminoethyl-Sepharose, the antigenic activity was recovered only in the neutral glycolipid fractions. Periodate oxidation of MCF-7 crude membrane preparation abolished MBr1-binding activity, suggesting that the carbohydrate portion of the molecule may constitute the antigenic determinant.

INTRODUCTION

Monoclonal antibodies recognizing tumor-associated antigens in human tumors have been used to characterize biochemically the target antigens in melanoma, colon cancer, and neuroblastoma (1, 7, 9, 12, 18, 27, 28). The chemical study of these antigens showed that, besides glycoproteins, glycolipids are also involved (7, 9, 18, 29).

In the preceding paper (10), we have reported the generation of 2 monoclonal antibodies which appeared specifically directed to an antigen present on epithelial cells of human normal and cancerous mammary gland. In the case of particular tumors, such as breast or ovarian tumors, normal antigens, provided they are strictly tissue specific, could be operationally useful as tumor-specific antigens with a view towards a clinical use. Here we describe the immunochemical analysis of the antigen defined by one of the 2 monoclonals, called MBr1.

MATERIALS AND METHODS

Chemicals. If not otherwise stated, chemicals were purchased from Merck, Darmstadt, West Germany. Neuraminidase (1 IU/ml) was from Behringwerke, Marburg, West Germany. Trypsin (1:250) was from Difco Laboratories, Detroit, Mich. DEAE-Sepharose Cl 6B was from Pharmacia, Uppsala, Sweden. Nonidet P-40 was from BDH Chemicals, Ltd., Poole, United Kingdom. Radioisotopes were from New England Nuclear, Boston, Mass.

Cells and Tissues. The characteristics, culture maintenance, and methods of CM preparations from the MCF-7 cell line or from surgical specimens are reported in the accompanying paper (10).

Antisera. The production and characterization of monoclonal antibody MBr1 was described in the accompanying paper (10). Antibodies were used as ascitic fluids obtained from hybridoma-bearing mice.

In the absorption experiments, a rabbit antiserum specific for human β-microglobulin (kindly provided by Dr. R. Tosi) and a human typing serum for N blood group antigen (Merz-Dade AG, Dudinghen, Switzerland) were also used.

Immunoprecipitation Procedures. MCF-7 cells were labeled as follows: (a) surface labeling with 125I by the lactoperoxidase procedure (25); (b) metabolic incorporation of [35S]methionine by using 250 μCi in 10 ml of methionine-free medium for 16 hr. Radiolabeled cells were dissolved in Nonidet P-40 buffer, and immunocomplexes were formed and precipitated as described by Dippold et al. (1).

Chemical and Enzyme Treatments. Methanol treatment of CM was carried out at 0° for 30 min directly on CM seeded on EIA plates (Dynatech Laboratories, Inc., Alexandria, Va.) immediately before testing. Heat treatment at 100° for 30 min was performed on CM suspensions before seeding on plates.

Periodate oxidation of CM was carried out at 4° for 2 hr directly on CM seeded on EIA plates, immediately before testing, adding to each well 10 μl phosphate buffer (pH 7.2) which contained different concentrations of sodium metaperiodate ranging from 0 to 50 mM.

Enzyme treatments of cells were performed as follows. MCF-7 cells (4 × 10⁶/ml) in medium were incubated with trypsin (0.25%), protease K (2 mg/ml), or neuraminidase (0.04 unit/ml) for 60 min at 37° with occasional shaking. For neuraminidase treatment, the pH of the medium was adjusted to 6.0 with 1 N HCl. Following incubation, the cells were washed twice with culture medium and then used in absorption experiments. The viability was always more than 85% and was similar to that of the untreated cells.

Preparation of Lipid Fractions. The extraction procedure is depicted in Chart 1. Briefly, 1 to 2 × 10⁹ cells or 10 to 20 mg CM were lyophilized, extracted with chloroform:methanol:water according to the

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method of Momoi et al. (13) or with THF, and partitioned with diethyl ether according to the method of Tettamanzi et al. (24).

The total lipid extracts and the aqueous and organic phases obtained by diethyl ether partition were evaporated to dryness, resuspended by ultrasonication (Biosonik III; Bronwill Scientific, Rochester, N. Y.) in 5 ml distilled water, and dialyzed according to the method of Ghiboni et al. (3). After dialysis, the contents of each bag were rinsed from the columns with methanol and acid fractions were eluted with 100 ml 1 M sodium acetate in methanol. All fractions were then evaporated to dryness, resuspended in distilled water, dialyzed, lyophilized, and dissolved in chloroform:methanol (2:1, by volume).

Brain total gangliosides, GM₃ (II°NeuAc-LacCer), GM₁ (II°NeuAc-GgOse₅Cer), Fuc-G₂₀, (II°NeuAc, IV°-α,FucGgOse₅Cer), and G₀₁₂ (II°NeuAc, IV°NeuAc-GgOse₅Cer) gangliosides were prepared according to the same methodology and were characterized according to the criteria of Sonnino et al. (20). Neutral glycolipids were prepared, starting from the corresponding gangliosides, by acid hydrolysis (20).

Assay System and Absorptions. The binding of monoclonal antibody MBr1 was evaluated by ELISA as described by Poli et al. (17). Unless otherwise stated, CM from MCF-7 cells was used as target antigen and were seeded at a concentration of 2 ug/well in phosphate-buffered saline (136 mM NaCl;2.7 mM KCl; 8.0 mM Na₂HPO₄; 1.5 mM KH₂PO₄; 0.9 mM CaCl₂, 0.5 mM MgCl₂, pH 7.4) in EIA plates. The plates were air dried at 37° overnight and stored at 4° for a maximum of 2 months until use.

The following antisera were used: goat anti-mouse IgG peroxidase conjugate (1:500); goat anti-rabbit IgG (1:20); peroxidase anti-peroxidase rabbit serum (1:100); and rabbit anti-human IgG peroxidase conjugate (1:500) (Miles Yeda, Ltd., Rehovot, Israel). Absorbance at 492 nm was determined by Titertek Multiskan (Flow Laboratories, Ltd., Milan, Italy).

Antibody absorptions were performed to test the absorbing capability of cells or CM after different treatments. The dilutions of reagents were 1:1000 for monoclonal antibody MBr1, 1:100 for anti-β₂m antisera, and 1:30 for anti-N blood group antigen antisera. Diluted antisera (150 µl) were mixed with different cell numbers ranging from 1 x 10⁴ to 8 x 10⁶ or different CM quantities ranging from 0.5 to 400 µg.

Lipid fractions at various concentrations were dried under nitrogen immediately before testing and resuspended by ultrasonication in 150 µl of monoclonal antibody MBr1 diluted 1:1000. The absorptions were performed at room temperature with occasional shaking; after 60 min, particulate materials were removed by centrifugation; and the remaining binding activity of the sera was evaluated by ELISA.

RESULTS

No specific immunoprecipitation was obtained with monoclonal antibody MBr1 from 125I-surface- or internally 35S)methionine labeled MCF-7 material, suggesting a nonproteic nature of the membrane structure recognized by MBr1.

In further experiments, cell suspensions or CM from MCF-7 cell line were exposed to different chemical or enzymatic treatments and then tested for residual antigenic activity with MBr1 monoclonal antibody by direct binding or quantitative absorption in ELISA. As control, 2 other antisera were absorbed which were directed against 2 molecules of known chemical nature also expressed on MCF-7 cells, i.e., the protein antigen β₂m and the N blood group carbohydrate antigen.

The results are reported in Table 1. MBr1-defined antigen was

![Chart 1](chart.png)

**Chart 1. Procedures for extraction and fractionation of glycolipids from MCF-7 materials.** Total lipid extract and the aqueous and organic phases obtained by diethyl ether partition were evaporated to dryness, resuspended by ultrasonication in 5 ml distilled water, and dialyzed according to the method of Ghiboni et al. (3). After dialysis, the contents of each bag were rinsed from the columns with methanol and acid fractions were eluted with 100 ml 1 M sodium acetate in methanol. All fractions were then evaporated to dryness, resuspended in distilled water, dialyzed, lyophilized, and dissolved in chloroform:methanol (2:1, by volume).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MBr1 (1:1000)</th>
<th>Anti-β₂m (1:100)</th>
<th>Anti-N (1:30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>6.5</td>
<td>10 x 10⁶</td>
<td>Binding ratio</td>
</tr>
<tr>
<td>Heat (100°C)</td>
<td>6.0</td>
<td>1.8</td>
<td>3.5</td>
</tr>
<tr>
<td>Methanol</td>
<td>1.3</td>
<td>4.3</td>
<td>3.4</td>
</tr>
<tr>
<td>Trypsin</td>
<td>5 x 10⁶</td>
<td>2 x 10⁵</td>
<td>4 x 10⁶</td>
</tr>
<tr>
<td>Protease K</td>
<td>5 x 10⁵</td>
<td>10 x 10⁶</td>
<td>ND</td>
</tr>
<tr>
<td>Vibrio cholerae neuraminidase</td>
<td>5 x 10⁵</td>
<td>ND</td>
<td>10 x 10⁶</td>
</tr>
</tbody>
</table>

* ND, not done.

Table 1: MBr1-binding activity on MCF-7 CM tested by ELISA: direct and absorption experiments with MCF-7 cells or CM treated with various chemicals and enzymes

Enzyme treatments of cells were performed as follows: 4 x 10⁶ MCF-7 cells/ml in medium were incubated with trypsin (0.25%), protease K (2 mg/ml), or neuraminidase (0.04 IU/ml) for 60 min at 37° with occasional shaking. Diluted antisera (150 µl) were mixed with different cell numbers ranging from 1 x 10⁴ to 8 x 10⁶. The absorptions were performed at room temperature.

The following antisera were used: goat anti-mouse IgG peroxidase conjugate (1:500); goat anti-rabbit IgG (1:20); peroxidase anti-peroxidase rabbit serum (1:100); and rabbit anti-human IgG peroxidase conjugate (1:500) (Miles Yeda, Ltd., Rehovot, Israel). Absorbance at 492 nm was determined by Titertek Multiskan (Flow Laboratories, Ltd., Milan, Italy).

Antibody absorptions were performed to test the absorbing capability of cells or CM after different treatments. The dilutions of reagents were 1:1000 for monoclonal antibody MBr1, 1:100 for anti-β₂m antisera, and 1:30 for anti-N blood group antigen antisera. Diluted antisera (150 µl) were mixed with different cell numbers ranging from 1 x 10⁴ to 8 x 10⁶ or different CM quantities ranging from 0.5 to 400 µg.

Lipid fractions at various concentrations were dried under nitrogen immediately before testing and resuspended by ultrasonication in 150 µl of monoclonal antibody MBr1 diluted 1:1000. The absorptions were performed at room temperature with occasional shaking; after 60 min, particulate materials were removed by centrifugation; and the remaining binding activity of the sera was evaluated by ELISA.
stable after treatment of CM for 30 min at 100°C, whereas it was completely removed by treatment with methanol. On the contrary, βm was denatured by 5 min heating and was unaffected by methanol; neither treatment affected N blood group antigen. Treatment of MCF-7 cells with proteolytic enzymes and neuraminidase did not modify their absorbing capability, except for protease K, which seemed to better expose antigenic determinants on the surface of MCF-7 cells. This was confirmed also in immunofluorescence experiments (data not shown) in which after protease K treatment the relative fluorescence intensity was greater than in untreated cells. As regards the control antigens, proteolytic enzymes removed βm, and neuraminidase removed N blood group antigen, as indicated by suppression of absorption of antibody activity.

Since these results suggested a glycolipid nature of the MBr1-defined antigen, total lipids were extracted from CM of MCF-7 cells and 2 surgical specimens of breast carcinoma. After lipid extraction, either by chloroform:methanol:water or THF, all the antigenic activity was found within the organic solvent phase. Chart 2 illustrates the inhibition of MBr1-binding activity to CM of a surgical specimen (MaCa 4354) by the lipid extracts. The same pattern of inhibition was obtained either with CM or with their lipid extracts with 50% inhibition at 7 μg of diluted monoclonal antibody per ml. No inhibition was detected with CM defatted with either method or with a purified neutral glycolipid [asialo-GM1 (GgOse,Cer)] and a purified ganglioside [Gm1] included as controls.

In order to verify whether the glycolipid involved was a known one, absorption experiments were carried out using a mixture of brain gangliosides and a series of purified neutral and acid glycolipids. As shown in Table 2, none of the listed glycolipids gave inhibition, whereas lipids obtained with various treatments from CM of 2 breast carcinoma surgical specimens (MaCa 4354 and MaCa 4717) and MCF-7 cells inhibited the reaction. We checked by absorption experiments with RBC of different species the possibility that the MBr1-defined antigen could be a blood group-type antigen or a Forssman-like antigen. No inhibition of MBr1 reactivity was obtained after absorption with sheep or ox RBC or with normal or neuraminidase-treated ABO blood group human RBC (data not shown).

<table>
<thead>
<tr>
<th>Antigen source</th>
<th>No. of cells × 10^6</th>
<th>Relative absorption capability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>CM</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>THF lipids</td>
<td>5.4</td>
<td>0.92</td>
</tr>
<tr>
<td>THF lipids after dialysis</td>
<td>5.2</td>
<td>0.95</td>
</tr>
<tr>
<td>Diethyl ether partition of THF lipids after dialysis</td>
<td>Organic phase</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>Aqueous phase</td>
<td>5.5</td>
</tr>
<tr>
<td>DEAE-Sepharose Cl 6B of THF lipids after dialysis</td>
<td>Neutral fraction</td>
<td>5.3</td>
</tr>
<tr>
<td></td>
<td>Acid fraction</td>
<td>100</td>
</tr>
<tr>
<td>DEAE-Sepharose Cl 6B of aqueous phase of THF lipids after dialysis</td>
<td>Neutral fraction</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>Acid fraction</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 3.

Absorption capability of different purification steps during lipid extraction (see Chart 1) from MCF-7 cells

MBr1 (150 μl; 1:1000) was mixed with different cell numbers ranging from 1 x 10^4 to 10 x 10^6 or different CM quantities ranging from 0.5 to 400 μg. Lipid fractions at various concentrations were dried under nitrogen immediately before testing and resuspended by ultrasonication in 150 μl of diluted MBr1. The remaining MBr1-binding activity on MCF-7 CM was evaluated by ELISA.

Table 2.

Analysis in ELISA of binding reactivity of MBr1 on MCF-7 CM: summary of absorption tests

The ganglioside nomenclature is that of Svennerholm (22) and is in keeping with the recommendations of the IUPAC-IUB (6).

<table>
<thead>
<tr>
<th>Complete absorption</th>
<th>No absorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7 cell line</td>
<td>MCF-7-defatted CM</td>
</tr>
<tr>
<td>CM</td>
<td>4354-defatted CM</td>
</tr>
<tr>
<td>CM 30 min 100°C</td>
<td>Cholesterol</td>
</tr>
<tr>
<td>THF lipids</td>
<td>Egg lecin</td>
</tr>
<tr>
<td>Fresh specimens</td>
<td>Brain total gangliosides</td>
</tr>
<tr>
<td>MaCa 4717</td>
<td>Asialo-GM1 (LacCer)</td>
</tr>
<tr>
<td>CM</td>
<td>Gm1</td>
</tr>
<tr>
<td>Chloroform:methanol lipid extract</td>
<td>Gm1</td>
</tr>
<tr>
<td>MaCa 4354</td>
<td>Fuc-Gm1</td>
</tr>
<tr>
<td>CM</td>
<td>0.3</td>
</tr>
<tr>
<td>CM, 30 min, 100°C</td>
<td>Gm1</td>
</tr>
<tr>
<td>Chloroform:methanol lipid extract</td>
<td>THF lipids</td>
</tr>
</tbody>
</table>
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To partially define the nature of the MBr1-recognized antigen, the extraction procedure shown in Chart 1 was applied to a surgical specimen of breast carcinoma (MaCa 4354) and to MCF-7 cells. The quantitative absorption analysis of the different purification steps of MCF-7 material is reported in Table 3 as the number of cells or glycolipid extracts derived from cells necessary to absorb 50% of antibody-binding activity. The same pattern of inhibition was obtained with cells, CM, total lipid extracts, also after dialysis, and with both phases after diethyl ether partition. However, after chromatography on DEAE-Sepharose, the antigenic activity was found only within the neutral lipid fractions with a 90% recovery. Superimposable results were obtained with the surgical specimen of breast carcinoma.

To further characterize the MBr1-recognized determinant, MCF-7 CM were treated with various concentrations of sodium metaperiodate and tested for MBr1-binding activity. As control, the anti-β2m rabbit serum was assayed on the same treated CM. As shown in Chart 3, increasing periodate concentrations denatured the MBr1-recognized determinant since the MBr1 activity was abolished after 25 mw treatment, whereas the β2m was unaffected even at higher concentrations.

DISCUSSION

Recently, glycolipid molecules present on plasma membranes have acquired increasing attention as markers of certain normal and neoplastic cells (2, 7, 9, 13, 14, 16, 18, 29, 30). Some studies have suggested a specific role of membrane glycolipids in the regulation of cell growth and cellular interactions (4). In this paper, we present evidence indicating that the antigen defined by the monoclonal antibody MBr1 which specifically binds to duct epithelial cells of normal human breast as well as to primary and metastatic breast carcinomas belongs to the neutral glycolipids. Due to its chemical nature, this antigen seems to be different from those already described on normal or neoplastic breast tissues (5, 8, 11, 21, 23).

In addition, it seems also different from the other glycolipids that have been reported recently to be tumor associated (7, 9, 18, 29), since all of them belong to gangliosides with different degrees of sialylation.

It was unlikely that the MBr1-recognized antigen was a Forssman-like antigen since the latter is not immunogenetic in the mouse, being present on murine normal tissues as a differentiation antigen. Nevertheless, absorption experiments with RBC of human, sheep, and ox species excluded any cross-reactivity between Forssman antigen and the MBr1-defined determinant. Carbohydrate blood group determinants, which are part of both membrane glycoproteins and glycolipids (4), were also considered as possible antigens cross-reacting with the MBr1-defined determinant, keeping in mind that the Thomsen-Friedenreich antigen, a precursor of the human MN blood group antigens, has been reported to be associated with human breast cancer (21) and to be present on normal breast tissues also (15). The possibility that the Thomsen-Friedenreich antigen carries the MBr1-defined determinant could be ruled out since no inhibition of MBr1-binding activity was obtained after absorption of MBr1 with normal and neuraminidase-treated A, B, and O blood group human RBC. In addition, the MBr1-defined antigen is distinct from some known gangliosides.

Our study was aimed mostly at isolation of the MBr1-recog-


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