Human-Human Hybridomas and Human Monoclonal Antibodies Obtained by Fusion of Lymph Node Lymphocytes from Breast Cancer Patients

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ABSTRACT

Lymphocytes from lymph node cells obtained from breast cancer patients undergoing mastectomy were fused with the 0467.3, UC729HF2, or KR-12 human cell lines, totaling 42 fusions with lymphocytes from 23 patients. A total of 1696 human-human hybridomas were generated, 675 (39.8%) of which produced human IgG and/or IgM.

Seventy-three human hybridomas produced antibodies binding to autologous malignant breast tissue and/or MCF-7 cells, as assayed by immunohistology or by cell-binding enzyme-linked immunosorbent assay. Twelve of these hybridomas, all reacting with malignant breast tissue, were subcloned to stabilize the production of human immunoglobulin. The reaction patterns of these 12 human monoclonal antibodies were investigated further by immunohistology on formalin-fixed, paraffin-embedded tissues. The reaction patterns of the various antibodies showed substantial variation and the antibodies reacted with a varying frequency with antigens expressed by different malignant breast tumors. One of these antibodies, MAC 40/43 (IgM), reacted with malignant breast and colon carcinomas and other epithelial derived neoplasms but did not react with normal breast tissue or with other normal and malignant tissues tested, except for a weak reaction with certain normal epithelial tissues. The antigen defined by MAC 40/43 was identified as a M, = 47,000 glycoprotein.

INTRODUCTION

The monoclonal antibody technique has had a major impact on the ability to define cell components characteristic of various types of normal and malignant cells. Monoclonal antibodies have aided in identification and characterization of a number of epitopes which are expressed in a highly restricted fashion on cancer cells. Most of these monoclonal antibodies have thus far been of murine origin. Antigens recognized by these antibodies have therefore generally been defined by their immunogenicity in mice and are not necessarily equivalent to tumor-associated antigens that are capable of eliciting an immune response in humans. Certain tumor-associated antigens have been shown to be able to elicit an immune response by the host and consequently in the generation of antibodies toward these antigens (1–3).

To investigate the autologous humoral immune response to tumor-associated antigens and to define the biochemical nature of tumor-associated antigens capable of eliciting antibody formation, human monoclonal antibodies were here generated by fusion of different human fusion partners with lymphocytes from axillary lymph nodes draining the primary breast tumor of breast cancer patients.

The human hybridomas produced monoclonal antibodies, some of which showed selective binding to human malignant breast tissue in immunohistological staining. One of these monoclonal antibodies (MAC 40/43) reacted specifically with a M, = 47,000 glycoprotein associated with breast carcinomas, colon carcinomas, and other neoplasms of epithelial origin.

MATERIALS AND METHODS

Media and Cell Lines. Human fusion partners and human hybridomas were grown in RPMI 1640 (Gibco, United Kingdom), supplemented with 2 mM glutamine, 50 µg streptomycin/ml, 50 units penicillin/ml, 3.5 × 10−3 M 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO), and 10–15% heat-inactivated fetal calf serum (all from Gibco). In the following designated complete RPMI. HAT medium consisted of complete RPMI supplemented with 13.6 µg hypoxanthine/ml, 0.18 µg aminopterin/ml, and 3.9 µg thymidine/ml (all from Gibco). HT medium consisted of complete RPMI supplemented with 13.6 µg hypoxanthine/ml and 3.9 µg thymidine/ml. The human breast cancer cell line MCF-7 was grown in complete RPMI medium containing 5% fetal calf serum. The human fusion partner UC729HF2 (4) is a human B-lymphoblastoid cell line resistant to 6-thioguanine. UC729HF2 is a nonsecreting variant of UC729-6 (5), kindly provided by Dr. T. Plesner, Rigshospitalet, Denmark. The human fusion partner KR-12 (6) is a hybrid between the human myeloma RPMI-8226 (a producer of α chains) and the human lymphoblastoid cell line KR-4 (a producer of γ and κ chains), which is an ouabain-resistant mutant of the human B-lymphoblastoid cell line GM 1500. The KR-12 cell line is phenotypically similar to human myeloma cells and proliferates like the lymphoblastoid cell line KR-4. KR-12 secretes γ, α, and κ chains. KR-12 was kindly provided by Dr. D. Kozbor, The Wistar Institute, Philadelphia, PA. The human fusion partner 0467.3 (7) was obtained from Dr. A. Rosén, Karolinska Institute, Stockholm, Sweden, and is a hypoxanthine-guanine phosphoribosyltransferase-deficient subclone of GM0467.

Production of Hybridomas. Segments of sterile human axillary lymph nodes were obtained from breast cancer patients undergoing mastectomy. Fat was removed from the nodes and a single cell suspension was prepared by homogenization. The suspended lymph node cells were washed 3 times in RPMI 1640. Human lymph node cells and one of the 3 different human fusion partners in the log phase of growth were mixed in a ratio of 2:1 and fused with polyethylene glycol 4000 (50% w/v in RPMI 1640) (Merck, West Germany) as described by Köhler and Milstein (8) and modified by Gefter et al. (9). One ml of polyethylene glycol 4000 heated to 37°C was slowly added to the cell pellet over a period of 1 min, the cells were mixed gently for 1.5 min, and 10 ml of RPMI 1640 were added dropwise over a period of 5 min. The cells were allowed to stand for 10 min.) After fusion the cell suspension was washed 3 times with RPMI 1640, resuspended (5 × 10⁶ cells/ml), and seeded in wells on 3 to 5 microtiter plates (Costar, Cambridge, Mass.), depending on the total number of cells. Cells were seeded directly to 200 µl HAT medium. Two days later 100 µl medium/well were removed and replaced with 100 µl fresh medium. Cells were maintained in HAT medium for 3 weeks and the medium was gradually changed to HT medium. When hybridomas grew vigorously, the medium was changed to complete RPMI without supplements. Hybridomas usually appeared between weeks 3 and 4 after fusion. Hybridoma culture supernatants were subsequently tested for the presence of human immunoglobulins. Hybridoma cultures producing human immunoglobulins (>1 mg/ml) were transferred to 1.5-ml wells (Multidish 24 plates; NUNC, Denmark). Hybridoma culture supernatants containing immunoglobulins were assayed for reactivity with the human breast cancer cell line MCF-7 by cell-binding ELISA and with autologous breast tumor tissue by immunohistology. Hybridomas producing antibodies reacting with either MCF-7, or autologous breast tumor, or both were cloned under standard conditions by limiting dilution at densities of 10

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1 To whom requests for reprints should be addressed.

2 The abbreviations used are: ELISA, enzyme linked immunosorbent assay; PBS, 0.05 M sodium phosphate buffer/0.15 M NaCl, pH 7.2.

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cells/well and 1 cell/well. Peritoneal macrophages from one BALB/c mouse were distributed in each microtiter plate as feeder cells. Eight to 10 days after cloning, the wells were examined by microscopy for growth of single clones. Hybridomas were cloned at least twice. Human immunoglobulins in hybridoma culture supernatants were measured by ELISA. The ELISA used was a modification of the method described by Douillard and Hoffman (10), using rabbit anti-human IgG, IgM, IgA, and IgG antibodies (all from DAKO, Denmark).

Cell-binding ELISA. The reactivity of human monoclonal antibodies with MCF-7 fixed with glutaraldehyde was determined by cell-binding ELISA. Cells were suspended in PBS at 10⁶ cells/ml and distributed in immunoplates pretreated with 0.001% poly-L-lysine (Sigma) in PBS. Plates were centrifuged at 100 x g for 5 min and the cells were fixed with 0.025% glutaraldehyde in PBS for 10 min. After 3 washes with PBS containing 0.05% Tween 20, 200 µL PBS containing 1% bovine serum albumin containing 0.1 M glycine were added to each well and the plates were incubated for 1 h at 22°C. Hybridoma supernatant (50 µL) was added to each well and allowed to react for 1 h at 22°C.

After 3 washes, 50 µL of peroxidase-conjugated goat anti-human IgG antibody (Tago, Burlingame, CA) diluted 1:3000 in PBS containing 1% bovine serum albumin were added to each well and the plates were incubated for 1 h at 22°C. After 3 washes, 100 µL enzyme substrate were added to each well and the enzyme reaction was terminated with H₂SO₄ after 5–15 min.

Biotinylation. Antibodies were biotinylated according to the method of Kendall et al. (11). After purification, the antibodies (1 mg/ml) were dialyzed for 24 h at 4°C against several changes of 0.1 M Na₂CO₃ buffer. Biotinyl-N-succinimide (Sigma) (120 µL; 5 mmol in dimethylformamide per mg of antibody) was added; the reaction mixture was incubated for 4 h at 22°C and dialyzed against several changes of PBS at 4°C.

Immunohistology. The initial screening for reactivity was performed on 5-µm sections of freshly frozen, otherwise untreated autologous tumor tissue sliced on a cryostat. Specimens were quick-frozen in cold liquid isopentane and subsequently stored at −80°C. The sections were preincubated for 30 min with normal goat serum, washed in Tris buffer-0.15 M NaCl, and incubated overnight at 4°C with undiluted supernatant from antibody-producing hybridomas. The sections were then rinsed in Tris buffer-0.15 M NaCl and incubated for 45 min with goat anti-human IgG or IgM (depending on the subclass of the primary antibody) diluted 1:300. After another rinse, the sections were incubated for 30 min with 1:1000 diluted peroxidase-conjugated swine anti-goat IgG. The reaction was developed with AEC, and the sections were counterstained with hematoxylin and mounted in Aquamount.

In the case of a positive reaction, the antibodies were applied to sections of formalin-fixed, paraffin-embedded tissue, and only such antibodies where the corresponding epitope was preserved despite fixation were included in further screening.

To reduce the background staining and to enhance the specific reaction, the above three-layer method was substituted with an avidin-biotin system. Biotinylated primary antibody was applied to the deparaffinized sections for 30 min, followed by washing in PBS and application of an avidin-biotin system (ABC kit; Vector Laboratories). The positive reaction was developed with AEC; the sections were counterstained with hematoxylin and mounted in Aquamount.

The specificities of the antibodies were tested on a wide range of different tissue types of epithelial, mesenchymal, and lymphoid origin. The reaction in breast tissue was investigated in 150 primary breast carcinomas, 19 benign breast tumors, and 9 samples of normal breast tissue. The further testing of selected monoclonal antibodies was in all cases performed on formalin-fixed, paraffin-embedded tissue sections.

Antigen Characterization. MCF-7 cells were washed twice in PBS and solubilized in 15 mM deoxycholate in 20 mM Tris-HCl, pH 8.0, containing 1 mM phenylmethylsulfonyl fluoride, for 30 min at 4°C. The suspension was centrifuged at 50,000 x g for 30 min and mixed 1:1 with sample buffer containing 5% β-mercaptoethanol, boiled for 5 min, and applied to a 3.5–12.5% sodium dodecyl sulfate-polyacrylamide gel system.

Electrophoretic transfer of proteins to nitrocellulose paper (0.2 µm; Sartorius, West Germany) was performed using a JC semidry electroblotter (Jancos, Denmark) according to the manufacturer’s instructions. After blotting, additional protein binding sites on the nitrocellulose paper were saturated by exposure to 2% Tween 20 in 50 mM Tris-HCl-0.15 M NaCl-0.05% Tween 20 for 5 min.

Carbohydrates were cleaved by mild periodate oxidation at an acid pH, according to the method of Woodward et al. (12). Briefly, strips were incubated with 10 mM sodium periodate in 50 mM sodium acetate, pH 4.5, for 1 h at 22°C, and aldehyde groups were reduced by exposure to 50 mM sodium borohydride in PBS for 30 min at 22°C. Control strips were incubated with 50 mM sodium acetate, pH 4.5, and 50 mM sodium borohydride. The strips were incubated with MAC 40/43 supernatant for 2 h at 22°C. After 4 washes at 5-min intervals, the strips were incubated with peroxidase-conjugated goat anti-human IgM (Tago) diluted 1:3000 in 50 mM Tris-HCl-0.15 M NaCl-0.05% Tween 20 for 1 h at 22°C.

Finally, strips were washed 4 times with 50 mM sodium acetate and the peroxidase activity was demonstrated using AEC as substrate.

RESULTS

Forty-two human-human fusions were performed with lymph node cells from a total of 23 patients. A total of 1696 human hybridomas were obtained (Table 1), 675 (39.7%) of which produced human immunoglobulin. The percentage of seeded wells yielding viable hybridomas showed significant patient-to-patient variation. The explanation for this variability is unknown at present. Seventy-three human hybridomas initially produced human immunoglobulin reactive either with MCF-7 cells, with autologous tumor tissue, or with both. These monoclonal antibodies were derived from 5 of the total of 23 patients.

The distribution of the tumor-reactive antibodies between hybridomas from different patients may not be random since hybridomas from certain patients produced a higher incidence of monoclonal antibodies reacting with autologous breast tumor cells.

The 3 different human fusion partners utilized in this study were: 0467.3 (which resulted in 0.38 hybridoma per 10⁶ lymph node cells); UC726HF₂ (1.65 hybridomas/10⁶ lymph node cells); and KR-12 (1.0 hybridoma/10⁶ lymph node cells). The fusion frequencies of KR-12 and UC729HF₂ found in this study correspond to those found by others (6, 13). The frequency of fusion obtained with the 0467.3 cell line was low, and this fusion partner was therefore abandoned after the initial experiments. Once established, the human hybridomas showed rapid growth, with doubling times of 24–36 h.

Quantitation of Human Immunoglobulin. Hybridoma culture supernatants were screened 3–5 weeks after the fusion for production of human IgG and IgM. Of the 1696 wells containing human hybridomas, 675 wells contained hybridomas producing >1 µg human immunoglobulin per ml supernatant (Table 1). 303 wells contained hybridomas producing IgM, 275 wells contained hybridomas producing IgG, and 97 wells contained hybridomas producing both IgM and IgG.

Cloning of Human Hybridomas. Thirty human hybridomas producing antibodies with strong reactivity with either MCF-7 or autologous tumor tissue were selected for stabilization of immunoglobulin production. Twelve of these hybridomas were cloned to stability. The number of clones appearing after cloning was generally low; 8 of 18 hybridomas were lost due to unsuccessful recloning and 10 were lost due to unstable immunoglobulin production.

Once stabilized, the human hybridomas were found to remain stable, producing human immunoglobulin in the range of 1.5 to 17.0 µg/ml supernatant (Table 2).

Immunohistology. Of the total of 73 monoclonal antibodies...
initially found to react with frozen tissue, the antigenic determinants were fully preserved in spite of fixation for 12 (as listed in Table 2) of these antibodies (17%; formalin-fixed, paraffin embedded tissue sections).

The specificity of the reaction of 6 of these antibodies studied in further detail is illustrated in Table 3, which shows that none of these antibodies were absolutely specific for either breast tissue or malignant tissue. Most of these antibodies also were found to react with mesenchymal tumors, yet apparently not with normal mesenchymal tissue. None of the antibodies reacted with lymphoid tissue or with lymphocytes. An example of immunohistological staining of formalin-fixed paraffin-embedded tissue sections by one of these monoclonal antibodies (MAC 40/43) is shown in Fig. 1. A positive antigen-antibody reaction was characteristically observed as a reddish-brown, intracytoplasmic staining (Fig. 1). There was no tendency towards a preferential apical staining in tubular structures. The nuclei were always negative.

In general, reactions with breast carcinomas were characterized by marked heterogeneity. Thus, some of the antigens detected were present only in approximately 20% of the cases investigated, whereas the antigen detected by the monoclonal antibody MAC 14/39 was detected in 80% of the carcinoma samples.

Table 4 shows the reaction of the monoclonal antibody MAC 40/43 in breast tissue. It appears from these data that we have been unable to detect the corresponding antigen in normal, resting breast tissue. The reaction in benign breast tumors and in carcinomas is heterogeneous with both negatively and positively reacting tumors. In the carcinomas, there was no relation between the presence of the antigen and the type of carcinoma, e.g., whether the carcinoma was of the ductal or lobular type or whether it represented some of the more rarely occurring types such as mucinous or medullary carcinomas. Similarly, areas of intraductal carcinoma reacted in the same manner as the infiltrating carcinoma. Furthermore, there was no relationship between the presence of the antigen and well known prognostic factors such as lymph node status, estrogen receptor content, and the grade of anaplasia (results not shown). Likewise, the recurrence-free survival of the patients appeared to be independent of the expression of the MAC 40/43 antigen.

Table 5 summarizes the reaction of the monoclonal antibody MAC 40/43 with a range of different epithelial tissues and the corresponding carcinomas. As summarized here, the antigen can be detected in epithelium other than cylindrical epithelium. However, it is noteworthy that the reaction in colon and rectum tissue is identical to the one detected in breast tissue with a positive reaction in adenocarcinomas of the colon and rectum but no detectable reaction in the normal epithelium of the large intestine. Fig. 1 illustrates the reaction of the MAC 40/43

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**Table 1. Generation of human hybridomas with lymph node lymphocytes from breast cancer patients and different human fusion partners**

<table>
<thead>
<tr>
<th>Fusion partner (ref.)</th>
<th>No. of patients</th>
<th>No. of fusions</th>
<th>No. of hybridomas</th>
<th>% hybridomas producing immunoglobulin</th>
<th>MCF-7/tumor reactive antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.467.3 (7)</td>
<td>4</td>
<td>7</td>
<td>67</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>UC729.HF2 (4)</td>
<td>21</td>
<td>24</td>
<td>1281</td>
<td>18.1</td>
<td>11.2</td>
</tr>
<tr>
<td>KR-12 (6)</td>
<td>11</td>
<td>11</td>
<td>348</td>
<td>20.9</td>
<td>28.7</td>
</tr>
</tbody>
</table>

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**Table 2. Characterization of 12 human monoclonal antibodies reacting with breast tumors**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>µg immunoglobulin/ml&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Reaction with MCF-7 cells&lt;sup&gt;b&lt;/sup&gt;</th>
<th>ELISA for HC&lt;sup&gt;c&lt;/sup&gt;</th>
<th>ELISA for LC&lt;sup&gt;d&lt;/sup&gt;</th>
<th>PAGE</th>
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</thead>
<tbody>
<tr>
<td>MAC 37/14&lt;sup&gt;e&lt;/sup&gt;</td>
<td>7.5</td>
<td>++</td>
<td>γ</td>
<td>γ, LC</td>
<td></td>
</tr>
<tr>
<td>MAC 40/43&lt;sup&gt;f&lt;/sup&gt;</td>
<td>17.0</td>
<td>++</td>
<td>γ + μ</td>
<td>γ, LC</td>
<td></td>
</tr>
<tr>
<td>MAC 37/8&lt;sup&gt;g&lt;/sup&gt;</td>
<td>7.5</td>
<td>−</td>
<td>γ</td>
<td>γ, LC</td>
<td></td>
</tr>
<tr>
<td>MAC 37/135&lt;sup&gt;h&lt;/sup&gt;</td>
<td>6.0</td>
<td>++</td>
<td>γ + μ</td>
<td>γ, LC</td>
<td></td>
</tr>
<tr>
<td>MAC 36/124&lt;sup&gt;i&lt;/sup&gt;</td>
<td>1.5</td>
<td>−</td>
<td>γ</td>
<td>γ, LC</td>
<td></td>
</tr>
<tr>
<td>MAC 34/19&lt;sup&gt;j&lt;/sup&gt;</td>
<td>4.0</td>
<td>++</td>
<td>γ</td>
<td>γ, LC</td>
<td></td>
</tr>
<tr>
<td>MAC 37/121&lt;sup&gt;k&lt;/sup&gt;</td>
<td>5.0</td>
<td>+</td>
<td>γ</td>
<td>γ, LC</td>
<td></td>
</tr>
<tr>
<td>MAC 37/102&lt;sup&gt;l&lt;/sup&gt;</td>
<td>8.0</td>
<td>−</td>
<td>γ</td>
<td>γ, LC</td>
<td></td>
</tr>
<tr>
<td>MAC 14/39&lt;sup&gt;m&lt;/sup&gt;</td>
<td>16.0</td>
<td>++</td>
<td>γ</td>
<td>γ, LC</td>
<td></td>
</tr>
<tr>
<td>MAC 36/44&lt;sup&gt;n&lt;/sup&gt;</td>
<td>2.6</td>
<td>−</td>
<td>γ + (μ)</td>
<td>γ (a)</td>
<td></td>
</tr>
<tr>
<td>MAC 37/142&lt;sup,o&lt;/sup&gt;</td>
<td>4.0</td>
<td>−</td>
<td>γ</td>
<td>γ, LC</td>
<td></td>
</tr>
<tr>
<td>MAC 37/69&lt;sup&gt;p&lt;/sup&gt;</td>
<td>4.0</td>
<td>−</td>
<td>γ</td>
<td>γ, LC</td>
<td></td>
</tr>
</tbody>
</table>

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* Determined by ELISA, Ig production per 48 h.
<sup>a</sup> Performed with hybridoma culture supernatant.
<sup>b</sup> Hybridoma produced with parent cell line UC729HF2.
<sup>c</sup> Hybridoma produced with parent cell line KR-12.
<sup>d</sup> Hybridoma produced with parent cell line UC729HF.
<sup>e</sup> Determined by ELISA, Ig production per 48 h.
<sup>f</sup> Performed with hybridoma culture supernatant.
<sup>g</sup> Hybridoma produced with parent cell line UC729HF.
<sup>h</sup> Hybridoma produced with parent cell line UC729HF.
<sup>i</sup> Hybridoma produced with parent cell line UC729HF.
<sup>j</sup> Hybridoma produced with parent cell line UC729HF.
<sup>k</sup> Hybridoma produced with parent cell line UC729HF.
<sup>l</sup> Hybridoma produced with parent cell line UC729HF.
<sup>m</sup> Hybridoma produced with parent cell line UC729HF.
<sup>n</sup> Hybridoma produced with parent cell line UC729HF.
<sup)o</sup> Hybridoma produced with parent cell line UC729HF.
<sup>p</sup> Hybridoma produced with parent cell line UC729HF.
<sup>q</sup> Hybridoma produced with parent cell line UC729HF.
<sup>r</sup> Hybridoma produced with parent cell line UC729HF.
<sup>s</sup> Hybridoma produced with parent cell line UC729HF.
<sup>t</sup> Hybridoma produced with parent cell line UC729HF.
<sup>u</sup> Hybridoma produced with parent cell line UC729HF.
<sup>v</sup> Hybridoma produced with parent cell line UC729HF.
<sup>w</sup> Hybridoma produced with parent cell line UC729HF.
<sup>x</sup> Hybridoma produced with parent cell line UC729HF.
<sup>y</sup> Hybridoma produced with parent cell line UC729HF.
<sup>z</sup> Hybridoma produced with parent cell line UC729HF.
<sup>A</sup> Hybridoma produced with parent cell line UC729HF.
<sup>B</sup> Hybridoma produced with parent cell line UC729HF.
<sup>C</sup> Hybridoma produced with parent cell line UC729HF.
<sup>D</sup> Hybridoma produced with parent cell line UC729HF.
<sup>E</sup> Hybridoma produced with parent cell line UC729HF.
<sup>F</sup> Hybridoma produced with parent cell line UC729HF.
<sup>G</sup> Hybridoma produced with parent cell line UC729HF.
<sup>H</sup> Hybridoma produced with parent cell line UC729HF.
<sup>I</sup> Hybridoma produced with parent cell line UC729HF.
<sup>J</sup> Hybridoma produced with parent cell line UC729HF.
<sup>K</sup> Hybridoma produced with parent cell line UC729HF.
<sup>L</sup> Hybridoma produced with parent cell line UC729HF.
<sup>M</sup> Hybridoma produced with parent cell line UC729HF.
<sup>N</sup> Hybridoma produced with parent cell line UC729HF.
<sup>O</sup> Hybridoma produced with parent cell line UC729HF.
<sup>P</sup> Hybridoma produced with parent cell line UC729HF.
<sup>Q</sup> Hybridoma produced with parent cell line UC729HF.
<sup>R</sup> Hybridoma produced with parent cell line UC729HF.
<sup>S</sup> Hybridoma produced with parent cell line UC729HF.
<sup>T</sup> Hybridoma produced with parent cell line UC729HF.
<sup>U</sup> Hybridoma produced with parent cell line UC729HF.
<sup>V</sup> Hybridoma produced with parent cell line UC729HF.
<sup>W</sup> Hybridoma produced with parent cell line UC729HF.
<sup>X</sup> Hybridoma produced with parent cell line UC729HF.
<sup>Y</sup> Hybridoma produced with parent cell line UC729HF.
<sup>Z</sup> Hybridoma produced with parent cell line UC729HF.
Fig. 1. Immunohistochemical staining patterns obtained with the MOC-6/43 monoclonal antibody when the adenocarcinoma and different specimens of malignant tissues: (d) breast carcinoma, (f) fibroadenomas, and (c) colon carcinoma. × 250. Positive staining in the immunoperoxidase reaction presents itself as a diffuse brown coloration in the cytoplasm of epithelial cells, whereas nuclear and stromal components appear negative.
antibody in breast carcinoma (Fig. 1a), fibroadenomatosis (Fig. 1b), and colon carcinoma (Fig. 1c).

Characterization of the MAC 40/43 Antigen. Deoxycholate extracts of MCF-7 cells were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose paper. After incubation with MAC 40/43 antibody followed by peroxidase-conjugated goat anti-human IgM, a band with an approximate molecular weight of 47,000 could be visualized (Fig. 2). The reactivity was diminished after treatment of MCF-7 cell components with 10 mM periodate, suggesting that the MAC 40/43 antigen is a glycoprotein. Parallel experiments using deoxycholate extracts of human lymphoma cells (Daudi) were negative in immunoblotting experiments using the MAC 40/43 antibody. Furthermore, the monoclonal antibody MAC 40/43 was found not to react with glycolipids extracted from MCF-7 breast carcinoma cells (results not shown).

DISCUSSION

This study demonstrates that stable human-human hybridomas secreting complete human immunoglobulin can be established by fusion of human axillary lymph node lymphocytes with suitable human fusion partners and that some of these human monoclonal antibodies react with autologous breast cancer cell-cell lines or with extracts from breast carcinoma cells. In the present series of experiments, a total of 1696 human-human hybridomas have been generated using lymph node cells from a total of 23 breast cancer patients. The study was undertaken with the assumption that B-lymphocytes obtained from lymph nodes draining primary breast tumors or themselves containing metastatic breast tumor cells were already primed to antigens shed by the tumor cells (1). The results of the present study confirm that breast cancer patients may produce antibodies that recognize antigens expressed by breast carcinoma cells. It should be pointed out, however, that lymph nodes from normal donors (i.e., donors other than mastectomy patients) have not been analyzed in this series of experiments. Therefore the question remains whether the production of antibodies reactive with breast carcinomas is restricted to breast cancer patients or whether antibodies of a similar specificity can be generated from normal donors.

It was surprising that only 189 of 348 human hybridomas constructed with the KR-12 cell line as fusion partner produced more than 1 µg of immunoglobulin per ml of supernatant, when the KR-12 cell line itself secretes IgG (3 µg/ml supernatant). The decrease in, or loss of, immunoglobulin production of certain hybridomas constructed with the KR-12 cell line may be a result either of postfusion chromosome loss or of the donor cell phenotype in some way dominating the secretion of endogenous KR-12 immunoglobulin.

All stabilized human hybridomas secreted IgG and 3 of these hybridomas also secreted IgM. The 2 human hybridomas MAC 40/43 and MAC 37/135 were found to secrete both IgG and IgM. In both cases, IgM was the immunoglobulin type which contained the specificity for MCF-7 cells. MAC 40/43 and MAC 37/135 were constructed with the KR-12 cell line as the fusion partner and it is therefore possible that the irrelevant
IgG could be of KR-12 origin. The hybridoma line MAC 36/44 secretes IgG as well as a small amount of IgM. MAC 36/44 was constructed with the UC729HF2 cell line as the fusion partner. It has not as yet been analyzed which immunoglobulin class of MAC 36/44 immunoglobulins that react with breast cancer cells. It is, however, plausible that a small amount of UC729HF2-derived IgM-secreted immunoglobulin has been induced in this hybridoma. Therefore, it is most likely that the IgG fraction of the MAC 36/44 immunoglobulins are capable of binding to human breast cancer cells. At this point not all the hybridoma lines have been verified as being true hybridomas.

The MAC 40/43 antigen is presumably different, since we have been unable to detect MAC 40/43 in normal breast epithelium. Edwards et al. (16) report a $M_\ell \approx 43,000$ membrane glycoprotein associated with human breast cancer cells defined by the murine monoclonal antibody 323/A3. MAC 40/43 both reacted with approximately 59% of the primary breast tumors tested and recognized an antigen of a very similar molecular weight. However, since the 323/A3 antigen is sensitive to $\beta$-mercaptoethanol while the MAC 40/43 antigen is not, it is likely that either 323/A3 and MAC 40/43 recognize different antigens or that they at least recognize two different epitopes on the same antigen.

The MAC 40/43 antigen is also different from the Tn (N-acetylgalactosamine-O-serine/threonine) and the T (galactose $\beta$-3 $N$-acyetylgalactosamine-O-serine/threonine) antigens since MAC 40/43 does not react with neuraminidase-treated glycoporin A or ovine submaxillary mucin. The Tn- and T-antigens are well known carcinoma-associated antigens which are able to elicit an immune response towards autologous carcinoma cells (17).

The expression of tumor-associated antigens as defined by murine monoclonal antibodies is now well established, (for a review see Ref. 18). In the present study, we have been able to produce human monoclonal antibodies by fusion of lymph node lymphocytes from breast cancer patients in order to investigate the humoral immune response of these patients towards their autologous cancer cells. A number of monoclonal antibodies reacting with autologous tumor cells were produced in the course of this work, and from these hybridomas, 12 hybridomas were selected and cloned in order to yield stable hybridomas. One of these hybridomas, MAC 40/43, and its monoclonal antibody (IgM isotype) have been investigated demonstrating an interesting tissue distribution of the cognate antigen which itself has been identified as a $M_\ell \approx 47,000$ glycoprotein. The diagnostic and therapeutic applications of the monoclonal antibody MAC 40/43 are currently under further evaluation.

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