ABSTRACT

A monoclonal antibody (7B10) which displays differential reactivity with breast carcinomas compared to benign lesions or normal breast tissue was selected by fusion of spleen cells from BALB/c mice immunized with the T47D human mammary carcinoma cell line. The antigen, recognized by 7B10 on T47D cells, appeared to be both surface and cytoplasmic localized, as demonstrated by indirect immunofluorescence, immunoperoxidase, and electron microscopy studies. This antibody (IgG₁) bound with four human breast cancer cell lines (T47D, MCF7, ZR-75-1, and HSL53) which express estrogens receptors. No binding was observed with cancer cell lines of other origin or with normal cells.

In vivo, by immunoperoxidase staining of frozen sections of normal breast, the antigen recognized by 7B10 appeared to be located on epithelial cell membranes, whereas in benign and malignant mammary disorders, staining also involved the cytoplasm, as confirmed by electron microscopy on fresh cancer tissue. On formalin-fixed, paraffin-embedded sections, cytoplasmic staining was detected in breast cancer, but no immunostaining was observed with benign lesions or normal breast.

In paraffin sections, most normal tissues investigated did not react with 7B10 antibody. However, ducts in the parotid gland, tubules in the kidney and some biliary ducts, and apocrine glands in the skin showed irregular, diffuse weak staining. 7B10 was unreactive with adenocarcinomas of origin other than breast, except for some cells in ovarian clear cell carcinoma. No reactivity was observed with squamous carcinomas, lymphomas, or melanomas.

The antigen recognized by 7B10 appeared to be a M, 32,000 protein, as identified by immunoprecipitation from extracts of T47D after labeling with [35S]methionine.

Since the antigen was present only on the membrane of differentiated normal mammary epithelial cells, and was also expressed in the cytoplasm of tumor cells, it may be of interest in immunological studies of mammary epithelial cell differentiation. Moreover, since in formalin-fixed tissues immunostaining is virtually confined to mammary carcinomas, monoclonal antibody 7B10 may have diagnostic applications in breast cancer.

INTRODUCTION

Breast cancer is the most frequently occurring malignant tumor and the principal cause of cancer death in women in Europe and in the United States. For this reason, numerous investigators have attempted to produce specific markers for identification of mammary tumors which may have clinical application in diagnosis and therapy. Several mAbs of human and mouse origin which are reactive with breast tumors (1–10) have been developed for this purpose.

Many mouse monoclonal antibodies, in spite of a wide range of tissue reactivity, are already being used in the differential diagnosis of epithelial tumors, in the detection of metastatic tumor cells in bone marrow and in lymph nodes, in immunoradio-localization of tumors, and in immunoradiotherapy.

This is the case for several monoclonal antibodies, including HMFG1 and HMFG2 (11, 12), M8 and M18 (13, 14), NCRC11 (15), F36/22 (16), and 1BE12 (17).

Other monoclonal antibodies are more specifically reactive with tumor antigens, such as B72-3 (18), 323/A3 (19), or 3B18 (20), or with antigens differentially expressed in normal and benign versus malignant mammary tissue, such as DF3 (21) or 3E1 (22). They may provide more information about the antigenicity of tumor cells compared to their normal counterparts and to differentiation processes.

Monoclonal antibody 7B10, which is described in this paper, belongs to this latter group. The reactivity of this monoclonal antibody has been characterized by immunocytochemical, immunohistochemical, and electron microscopy studies, and the antigen detected by 7B10 mAb has been preliminarily characterized by biochemical methods.

MATERIALS AND METHODS

Cell Lines

A human breast carcinoma cell line, T47D, was used for immunization. This cell line was derived from a metastatic pleural effusion from a breast adenocarcinoma. These cells exhibit epithelial morphology, form monolayers in culture, contain intracellular casein and receptors for estradiol, progesterone, glucocorticoids, and androgens (23). T47D cells were cultured in Dulbecco’s modified Eagle’s Medium supplemented with 10% FCS, 2 mM L-glutamine, 100 IU/ml penicillin, and 50 µg/ml streptomycin.

The other human breast cancer cell lines used in these studies were MCF-7, ZR-75.1, HSL-53, MDA-MB-231, BT-20, and 466-B. Culture conditions and properties of these cell lines have been previously described (24, 25).

The other human cell lines used were PC3 and DU 145, which are prostatic carcinoma cell lines, 385 F₂, an ovarian carcinoma cell line, HeLa, derived from a carcinoma of the cervix, Raji, a B-lymphoblastoid cell line, and MRC5, a cell line derived from human fetal lung fibroblasts.

The murine myeloma cell line SP₂/O, was cultured in RPMI 1640 supplemented with 20% FCS, 20 µmol/ml glucose, 1 mmol/ml sodium pyruvate, 2 mM L-glutamine, 100 IU/ml penicillin, and 50 µg/ml streptomycin.

All cell lines were free of Mycoplasma contamination.

Immunization

BALB/c mice (female, age 4 weeks) were immunized by i.p. injection of 2 × 10⁴ live T47D cells (detached from culture flasks with a rubber policeman) in PBS, four times at 2-week intervals, and boosted i.v. and i.p. with the same cell numbers 3 days before fusion.

Cell Fusion and Hybridoma Production

Fusions were performed as previously described (26). Cell suspensions of immunized mouse spleens were mixed with mouse SP₂/O myeloma cells at a 5:1 ratio and fused with 50% (w/v) polyethylene glycol (M, 4000) in serum-free RPMI 1640. Fused cells were plated in...
Linbro 24-well plates and grown in selective medium (hygromycin) in RPMI 1640 supplemented with 20% FCS subcloned and grown as ascitic tumors in pristane-primed BALB/c mice. Clonal lines were cryopreserved in 90% FCS and 10% dimethyl sulfoxide.

**Analysis of Antibody Class**

The heavy chain subclass of the secreted antibody was determined by Ouchterlony gel immunodiffusion and by ELISA. Specific antisera (purified fraction 75%) to immunoglobulin subclasses were obtained from Nordic Laboratories.

**Purification of Monoclonal Antibody**

Immunoglobulins contained in culture supernatants were precipitated with 40% ammonium sulfate and dialyzed against PBS before use.

Ascitic fluids were centrifuged at 600 x g for 10 min to remove cells and were further clarified by centrifugation at 10,000 x g for 15 min. The supernatant was precipitated with 40% ammonium sulfate, resuspended, dialyzed against 10 mM phosphate buffer, pH 8, and applied on a DEAE-acrylamide column. The IgG fraction was eluted with a 50-75 mM NaCl gradient. The degree of purification was determined by polyacrylamide gel electrophoresis. Protein concentration was determined with Coomassie blue (27).

**Immunofluorescence Staining**

The indirect immunofluorescence method on cell suspensions was used. Briefly, 100 μl of live cell suspension (10⁷/ml) in PBS with 1% BSA and 0.1% NaN₃ was incubated with hybridoma supernatant for 60 min at 4°C. After two washes in PBS/BSA, cells were incubated with 1:50 fluorescein-labeled sheep anti-mouse antibody (Institut Pasteur, France) for 1 h at 4°C. After two washes in PBS/BSA, the cell pellet was resuspended in 30 μl of buffer with 10% glycerol and 100 μg/ml p-phenylenediamine, mounted on glass slides, and examined under a Zeiss fluorescence microscope.

An IgG₁ monoclonal antibody (160C19) (26) directed against rotavirus was used as negative control.

**ELISA Assays**

Cells growing as monolayers were plated in 96-well culture dishes (Nunc) at a density of 5 x 10⁴ cells/well and allowed to attach to plastic for 24 h. Dishes were immersed in PBS with 0.25% glutaraldehyde for 5 min at 4°C. After three washes in PBS, the cells were incubated 1 h at room temperature with antibody-containing culture supernatant and ELISA was processed by using the two-step staining technique with biotin and avidin-peroxidase (Vector, Vector Laboratories). Peroxidase activity was revealed with 2,2'-Azinodi(3-ethylbenzthiazolin-sulfonic acid) (Sigma), 0.2 mg/ml in citrate/phosphate buffer, pH 5, 0.001% H₂O₂, 100 μl/well. The colorimetric reaction was detected by using a Dynatech ELISA reader at 405 nm, after 15-20 min. A monoclonal antibody directed against rotavirus (160C19) was used as negative control.

Cells growing in suspension (peripheral blood lymphocytes) were fixed to plastic by poly-L-lysine (28). Fifty μl of 0.05% poly-L-lysine (Sigma) in PBS were added in 96-well dishes (Nunc) for 90 min at 37°C, then removed. Cells 1.5 x 10⁶ in 50 μl PBS were then plated in each well and incubated 1 h at 4°C. The plates were immersed in 0.25% glutaraldehyde in PBS for 5 min at 4°C and processed as above for ELISA.

**Table 1 Reactivity of monoclonal antibody 7B10 with breast cancer cell lines and other cell types using ELISA and indirect immunofluorescence**

<table>
<thead>
<tr>
<th>Monoclonal Antibody</th>
<th>Reactivity</th>
<th>ELISA</th>
<th>Immunofluorescence</th>
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<tbody>
<tr>
<td>T47D</td>
<td>++</td>
<td>1280</td>
<td>+</td>
</tr>
<tr>
<td>MCF7</td>
<td>++</td>
<td>320</td>
<td>±</td>
</tr>
<tr>
<td>ZR-75-1</td>
<td>+</td>
<td>80</td>
<td>±</td>
</tr>
<tr>
<td>HSL53</td>
<td>+</td>
<td>ND</td>
<td>±</td>
</tr>
<tr>
<td>BT20</td>
<td></td>
<td></td>
<td>±</td>
</tr>
<tr>
<td>466B</td>
<td>±</td>
<td>5</td>
<td>±</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>±</td>
<td>5</td>
<td>±</td>
</tr>
<tr>
<td>PC3</td>
<td></td>
<td></td>
<td>±</td>
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<tr>
<td>DU145</td>
<td></td>
<td></td>
<td>±</td>
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<tr>
<td>385F5</td>
<td></td>
<td></td>
<td>±</td>
</tr>
<tr>
<td>HeLa</td>
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<td>±</td>
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<tr>
<td>Raji</td>
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<td>±</td>
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<tr>
<td>MRC5</td>
<td></td>
<td></td>
<td>±</td>
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<tr>
<td>PBL</td>
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</tr>
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- * Result of ELISA; ++, absorbance >4 x mean of negative control antibody; +, absorbance >3 x mean of negative control antibody; ±, absorbance >2 x mean of negative control antibody; --, negative.
- ** End dilution of monoclonal antibody which gave positive absorbance.
- ** ND, not done.
- ** Tested only in indirect immunofluorescence.

**Electron Microscope Studies**

**Materials.** Cell suspensions (T47D and MCF7) in growth culture medium were plated on Terasaki plates (10⁴ cells/well) and allowed to adhere to plastic. After 24 h they were fixed with 0.25% glutaraldehyde in PBS for 5 min at 4°C. For membrane permeabilization, cells were fixed with 0.5% glutaraldehyde, 1% Triton X-100, in PBS for 10 min at 4°C. After 3 washes in PBS, plates were incubated with 1 mg/ml NaBH₄ for 10 min to reduce free glutaraldehyde radicals.

Cells were then incubated with antibody-containing culture supernatants for 3 h at room temperature. After 3 washes in PBS, cells were incubated for 2 h with 20 μl of 15 nm colloidal gold-labeled anti-mouse IgG (Jansen Pharmaceuticals) in PBS. Cells were then fixed with 1% glutaraldehyde in PBS for 1 h at 4°C. Plates were incubated with 1% osmium tetroxide for 1 h at 4°C, washed with distilled water, and dehydrated in 25 to 100% ethanol. Inclusion in Epon (for 24 h at 60°C) was performed as described, and the grids were contrasted by using standard methods. Sections were analyzed by using a Philips EM 301 electron microscope.

**Tissue Preparations.** Tumor tissues removed in the operating room were immediately cut into 5-5 x 2-mm slices, fixed for 75 min at 4°C in 4% paraformaldehyde in PBS, and washed for 60 min in 0.01 M sucrose-PBS. Sections (100 μm) were cut with a vibratome (Lancero 1000).

**Immunostaining Procedure.** Free-floating sections were incubated in Petri dishes at room temperature unless otherwise stated: 30 min with normal goat serum; 3 min at room temperature. After 3 min washes in PBS; overnight at 4°C under constant shaking with 7B10 diluted in a 0.05% saponin solution; three 10-min washes in PBS; 2 h in biotin-labeled goat anti-rabbit IgG antiserum; three 10-min washes in PBS; 45 min in 0.3% hydrogen peroxide solution; three 10-min washes in PBS; 2 h in the avidin-peroxidase complex solution; three 10-min washes in PBS; 20 min in 2% glutaraldehyde solution; three 10-min washes in PBS; 30 min in 0.05% 3,3'-diaminobenzidine-tetra-HCl (Serva Laboratories) in phosphate buffer, pH 7.6; another 30 min with the same solution containing 0.03% hydrogen peroxide; three 10-min washes in PBS; 45 min in 2% osmium tetroxide solution.

**The sections were then dehydrated in graded ethanol solutions, embedded in Araldite, and ultrathin sections were cut. Sections were analyzed by using a JEOL 1200 electron microscope.**

**Cell Radiolabeling and Immunoprecipitation**

[35S]methionine (60 μCi/ml; Nag-009A; L-[35S]methionine, 1154 Ci/mmol) was added to the culture medium. Culture medium was removed 11 to 14 days later for antibody screening with the use of indirect immunofluorescence and an ELISA test, with T47D cells.

The positive hybridomas were then cloned directly by limited dilution into 96-well dishes (Nunc) in which a feeder layer of 30-γ-irradiated mink fibroblast cell line CCL64 had been previously plated at a density of 10⁶ cells/well. Wells containing hybridoma colonies were assayed 12 to 15 days later by using ELISA. T47D cells were the target cells and the MRC5 cell line was used as the negative control.

Positive cultures were expanded in RPMI 1640 with 20% FCS, subcloned, and grown as ascitic tumors in pristane-primed BALB/c mice. Clonal lines were cryopreserved in 90% FCS and 10% dimethyl sulfoxide.

**Table 1 Reactivity of monoclonal antibody 7B10 with breast cancer cell lines and other cell types using ELISA and indirect immunofluorescence**

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- ** End dilution of monoclonal antibody which gave positive absorbance.
- ** ND, not done.
- ** Tested only in indirect immunofluorescence.
Autoradiography was then performed at −80°C for 24 h.

The insoluble material was removed by centrifugation (1,000 x g for 10 min, followed by centrifugation at 100,000 x g for 60 min) at 4°C. The supernatant was incubated with the monoclonal antibody overnight at 4°C. Bound antigen-antibody complexes were eluted by resuspending the pellets in 100 μl Laemmli buffer and boiling for 5 min; 30 μl of eluates were submitted to electrophoretic analysis in a continuous 5—15% gradient sodium dodecyl sulfate-polyacrylamide gel by using a stacking gel of 3% (w/v) acrylamide. An IgG-negative control mAb and two positive controls [OKT9 mAb and 1BE12 mAb (17)] were run concomitantly with 7B10.

Autoradiography was then performed at −80°C for 24 h.

Radioactivity, washed twice in PBS, harvested by scraping, and pelleted with a Dounce homogenizer.

Buffer (10 mM Tris-HCl, pH 7.4, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 1 mmol; DuPont, NEN Products) or [14C]glucosamine (10 μCi/ml; d-[1-14C]glu d-[1-14C]glucosamine chlorohydrate CB70, C.E.A., France, 45-60 mCi/milliatom), 2% dialyzed PCS, glutamine, and sodium pyruvate.

Cells were incubated for 15 min at 4°C in a hypotonic lysis buffer (10 mM Tris-HCl, pH 7.4, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, and 1 mM EDTA) and disrupted with a Dounce homogenizer.

Homogenates were mixed with the same volume of lysis buffer containing 0.4 mM NaCl, 1% Nonidet P-40 and incubated for 30 min at 4°C. Bound antigen-antibody complexes were eluted by resuspending the pellets in 100 μl Laemmli buffer and boiling for 5 min; 30 μl of eluates were submitted to electrophoretic analysis in a continuous 5—15% gradient sodium dodecyl sulfate-polyacrylamide gel by using a stacking gel of 3% (w/v) acrylamide. An IgG-negative control mAb and two positive controls [OKT9 mAb and 1BE12 mAb (17)] were run concomitantly with 7B10.

Autoradiography was then performed at −80°C for 24 h.

### Immunohistochemistry

#### Materials.

Normal tissues (n = 41) were obtained during necropsy or surgery and corresponded to normal tissue adjacent to various tumorous or nontumorous disorders.

Nonmalignant disorders (n = 11), mainly of breast origin, were also investigated. These included fibrocystic disease, apocrine metaplasia, papilloma, and adenofibroma. Malignant tumors included squamous carcinomas (n = 7) and adenocarcinomas of various types and grades (n = 30) (Table 2 and 4). Lymphomas (2) and a melanoma were also tested.

#### Tissue Preparations.

Variations of 7B10 immunoreactivity in frozen and paraffin sections were compared in breast tissue (normal, benign, and malignant tumors) and endometrial and ovarian tumors. Samples of normal tissues and tumors were embedded in paraffin after fixation in formalin or Bouin’s liquid. Paraffin sections were mounted on coated slides and dried at 37°C for 12 h.

Frozen sections were obtained from blocks embedded in OCT (Tissue-Tec, Miles) and stored at −80°C for 1 to 3 years. Tissue sections 4- to 5-μm thick were cut immediately prior to the immunostaining procedure and mounted on coated glass slides.

#### Immunostaining Procedure.

Immunoperoxidase staining was performed by using avidin-biotin-peroxidase complex kits (Vector Laboratories) as previously described (29).

Briefly, the sections were incubated first with normal horse serum, then with 7B10 (40 μg/ml protein) for 1 h at room temperature. Tissue sections were subsequently incubated with biotin-labeled horse antimouse immunoglobulins. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide solution for 20 min before incubation in avidin-biotin-peroxidase complex. The slides were stained with 3-amino-9-ethylcarbazole (Sigma), and then counterstained with Mayer’s

### Table 2 Comparison of 7B10 immunoreactivity in frozen and paraffin sections of human breast, endometrium, and ovarian tissues (avidin-biotin-peroxidase)

<table>
<thead>
<tr>
<th>Tissues n = 31</th>
<th>Frozen sections</th>
<th>Paraffin sections</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of cells + to +++</td>
<td>Intensity + to +++</td>
<td>Distribution Membrane Cytoplasm</td>
</tr>
<tr>
<td>Breast (n = 26)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal breast (7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epithelial cells</td>
<td>+++ (7/7)</td>
<td>+++</td>
</tr>
<tr>
<td>Myoepithelial cells</td>
<td>+ (7/7)</td>
<td>+++</td>
</tr>
<tr>
<td>Stromal cells</td>
<td>− (7/7)</td>
<td>+++</td>
</tr>
<tr>
<td>Cyst (n = 3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epithelial cells</td>
<td>++++/+++ (3/3)</td>
<td>+++</td>
</tr>
<tr>
<td>Myoepithelial cells</td>
<td>++/++ (3/3)</td>
<td>+++</td>
</tr>
<tr>
<td>Stromal cells</td>
<td>− (3/3)</td>
<td>+++</td>
</tr>
<tr>
<td>Apocrine metaplasia</td>
<td>NT</td>
<td>++ (1/1)</td>
</tr>
<tr>
<td>Adenosis (n = 3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epithelial cells</td>
<td>+++ (2/3)</td>
<td>+++</td>
</tr>
<tr>
<td>Myoepithelial cells</td>
<td>++ (2/3)</td>
<td>++</td>
</tr>
<tr>
<td>Stromal cells</td>
<td>− (3/3)</td>
<td>+++</td>
</tr>
<tr>
<td>Papilloma (n = 1)</td>
<td>NT</td>
<td>−</td>
</tr>
<tr>
<td>Adenofibroma (n = 1)</td>
<td>NT</td>
<td>−</td>
</tr>
<tr>
<td>Tubular carcinoma Grade I (n = 1)</td>
<td>+++ IV</td>
<td>+++</td>
</tr>
<tr>
<td>Grade II (n = 2)</td>
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<tr>
<td>Intra ductal invasive Grade II carcinoma (n = 2)</td>
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<td></td>
</tr>
<tr>
<td>Invasive lob</td>
<td>+++ (2/2)</td>
<td>+++</td>
</tr>
<tr>
<td>Invasive lob</td>
<td>++ (2/2)</td>
<td>+++</td>
</tr>
<tr>
<td>Grade II (n = 1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mucinous (n = 1)</td>
<td>+++ (1/1)</td>
<td>+++</td>
</tr>
<tr>
<td>Medullary (n = 1)</td>
<td>++ (1/1)</td>
<td>+</td>
</tr>
<tr>
<td>Lymph node metastases (n = 2)</td>
<td>+++ (1/1)</td>
<td>+++</td>
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<td>Endometrium (n = 2)</td>
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<tr>
<td>Adenocarcinoma (n = 2)</td>
<td>+ (2/2)</td>
<td>+++</td>
</tr>
<tr>
<td>Ovaries (n = 3)</td>
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<td></td>
</tr>
<tr>
<td>Clear cells</td>
<td>NT</td>
<td>−</td>
</tr>
<tr>
<td>Papillary serous carcinoma grade I (n = 1)</td>
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<td></td>
</tr>
<tr>
<td>Germinoma</td>
<td>NT</td>
<td>−</td>
</tr>
</tbody>
</table>

* NT, Not treated.

Grade index from + to ++++. % of cells: +, <30% positive cells; ++, <60% positive cells; ++++, >60% positive cells.

mmol; DuPont, NEN Products) or [14C]glucosamine (10 μCi/ml; d-[1-14C]glu d-[1-14C]glucosamine chlorohydrate CB70, C.E.A., France, 45-60 mCi/milliatom), 2% dialyzed FCS, glutamine, and sodium pyruvate.

Cells were incubated for 15 min at 4°C in a hypotonic lysis buffer (10 mM Tris-HCl, pH 7.4, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, and 1 mM EDTA) and disrupted with a Dounce homogenizer.

Homogenates were mixed with the same volume of lysis buffer containing 0.4 mM NaCl, 1% Nonidet P-40 and incubated for 30 min at 4°C. The insoluble material was removed by centrifugation (1,000 x g for 10 min, followed by centrifugation at 100,000 x g for 60 min) at 4°C.

One hundred μl of radiolabeled cell extract (5-6 × 10⁴ cpm) were incubated for 1 h at 4°C with 5 μl of normal mouse serum, then mixed with 50 μl of a suspension of protein A-Sepharose (Pharmacia) for another 1 h. After centrifugation (12,000 x g for 1 min) the supernatant was incubated with the monoclonal antibody overnight at 4°C.

One hundred μl protein A-Sepharose coated with rabbit anti-mouse serum were added to antigen-antibody mixture. After incubation for 3 h at 4°C, beads were washed 3 times with 0.1 M Tris (pH 8.0)—0.5 mM NaCl buffer. Bound antigen-antibody complexes were eluted by resuspending the pellets in 100 μl Laemmli buffer and boiling for 5 min; 30 μl of eluates were submitted to electrophoretic analysis in a continuous 5—15% gradient sodium dodecyl sulfate-polyacrylamide gel by using a stacking gel of 3% (w/v) acrylamide. An IgG-negative control mAb and two positive controls [OKT9 mAb and 1BE12 mAb (17)] were run concomitantly with 7B10.

Autoradiography was then performed at −80°C for 24 h.
mAbs were selected and were cloned at least twice by end point dilution. One of these mAbs, 7B10 (IgG) was selected for further analysis and is described in this paper. The other mAbs (12BB22, 1BE12, and 1BD6) were IgM mAb (17).

**RESULTS**

**Generation of Monoclonal Antibodies and Screening**

BALB/c mice were immunized with live T47D cells four times and boosted 3 days before fusion. The first screening was performed with T47D cells by using indirect immunofluorescence with live cells and ELISA with weakly fixed cells (0.25% glutaraldehyde) to preserve cell membrane integrity. The MRC5 cell line derived from fetal human lung fibroblast was used as a control. Antibodies reactive with both lines were discarded.

Twenty hybridomas were subsequently cloned. Fourteen persistently positive culture supernatants were tested for reactivity with other breast cancer lines, cancer cell lines of other origin, and peripheral blood lymphocytes. These 14 hybridomas were also screened on a limited number of breast tumors, normal breast tissues, and lymphomas by using immunoperoxidase staining of frozen and paraffin-embedded tissue sections. Four mAbs were selected and were cloned at least twice by end point dilution. One of these mAbs, 7B10 (IgG), was selected for further analysis and is described in this paper. The other mAbs (12BB22, 1BE12, and 1BD6) were IgM mAb (17).

**7B10 Binding to Normal and Malignant Cells**

Culture supernatants were tested by ELISA and IIF for binding to a panel of cell lines (Table 1). 7B10 strongly bound to four breast cancer cell lines (T47D, MCF7, ZR-75.1, and HSL-53) known to be estrogen steroid receptor-positive cells and bound weakly to MDA and 466B; it did not react with BT20.

No binding was observed with cancer cell lines of other origin (PC3, DU145, 385F5, HeLa, and Raji) or with normal cells (MRC5 and peripheral blood lymphocytes).

**Immunohistopathological Results with 7B10**

**Frozen Sections (Table 2)**

**Normal Tissues.** In normal breast, positive staining was observed in the seven cases tested. It involved epithelial cells as well as myoepithelial cells, whereas stromal cells and vessels were always negative (Fig. 1a). In epithelial cells, positivity was found mainly on cell membrane, whereas the cytoplasm and the nucleus were negative. On cell membrane, positive immunostaining consisted of a thick continuous line around individual cells, or irregular dots distributed on cell membranes. In myoepithelial cells, positive immunostaining was more difficult to define, due to cell shape and size, and was observed in cell membrane and cytoplasm.

Most epithelial cells in normal breast were positive, with a similar pattern of distribution in the 7 cases examined: 60 to 90% (+++) with the semiquantitative grading system. In con-
In contrast, fewer myoepithelial cells were stained: 10 to 30% (+). The intensity of positivity was always strong in both epithelial and myoepithelial cells.

SAMBA analysis of 7B10-positive immunostaining showed that the total (epithelial and myoepithelial) positive surface to total cell surface ratio evaluated by the nuclear counterstain varied from 45 to 55%. The histogram of the IOD showed only slight variations in staining intensity in individual cases. MOD was almost similar in the different cases investigated, showing that the intensity of positive staining did not vary from case to case.

Nonmalignant Disorders. In breast fibrocystic disease, epithelial and myoepithelial cells showed the same positive staining as in normal tissues. However, some epithelial cells displayed increased positive staining on the apex of the cells. In adenosis (Fig. 1b) (microcystic, sclerosing, and nodular), myoepithelial cells were clearly stained in cell membrane and in the cytoplasm. Also, positive staining was visible in the cytoplasm of epithelial cells, in contrast to normal breast. No stromal positive staining was observed. SAMBA analysis showed the same homogeneity of staining intensity as in normal breast, with nonsignificant variations in MOD and IOD histograms.

Malignant Disorders. The nine primary breast carcinomas and lymph node metastases were all positive with 7B10. Staining involved the cell membrane and cytoplasm of epithelial cells, never the stromal cells (Fig. 1c; Fig. 2, a and c). Most cells were positive, but in ductal grade III and medullary carcinomas, fewer cells were positive (Table 2). The intensity of staining was similar to that of normal breast and nonmalignant disorders, as reflected by nonsignificant variations in MOD. However, variations in IOD histograms revealed some heterogeneity of positive immunostaining in tumors (Fig. 2, b and d). Also, in contrast to normal breast, both cell membrane and cytoplasm were positive.

In ovarian papillary serous carcinoma grade I, the epithelial cells were positive and stromal cells were negative. Positive cells were less frequent (30%) than in breast carcinomas; however, staining intensity was similar. The positive reaction involved both the cell membrane and cytoplasm. One case of seminoma (a dysgerminoma) was completely negative. Endometrial carcinomas (n = 2) were 7B10 positive, with 30% positive cells. Positive staining was mainly located on cell membrane and focally within the cytoplasm. Staining intensity was homogeneous and similar to that observed in breast cancer.

No staining was observed in two malignant lymphomas and two reactive lymph node frozen sections (not shown).

Paraffin Sections (Tables 2–4)

Normal Tissues (Tables 2 and 3). None of the 7 normal breast tissues (Table 2) tested were 7B10 reactive in paraffin section.
Most of the normal human tissue investigated (Table 3) did not react with 7B10 antibody. However, ducts in the parotid gland, tubules in the kidney, and apocrine glands in the skin showed some irregular, diffuse weak (+) cytoplasmic 7B10 positivity. Also, in some biliary ducts in the liver, the apex of the epithelial cells was immunostained.

Nonmalignant Disorders (Tables 2 and 4). Fibrocystic disease, adenosis, fibroadenoma, and microcystoma of the breast were negative (Table 2). Only in apocrine metaplastic epithelial cells did breast cysts show some diffuse cytoplasmic-positive staining.

Pleomorphic adenoma of the parotid and prostatic adenoma were 7B10 negative (Table 4).

Malignant Disorders (Tables 2 and 4). In breast carcinomas, positive staining was observed in all cases tested (Fig. 2e). However, the pattern of 7B10 reactivity on paraffin sections differed from that observed on frozen sections: (a) fewer cells were immunoreactive, since 30 to 60% were positive (+ to ++++) on semiquantitative analysis. However, SAMBA analysis of the positive surface was, in some cases, higher than in frozen sections because of the diffuse cytoplasmic distribution of positive staining; (b) positive staining was observed only within the cell cytoplasm, diffusely distributed, or gathered in intracytoplasmic clumps; (c) staining intensity was lower than on frozen sections, as reflected by lower MOD and IOD (Fig. 2f). Also, staining intensity varied from one case to another.

All adenocarcinomas from other sites tested, except some cells in ovarian clear cell carcinomas, were 7B10 negative.
formed to determine the proteic or carbohydrate nature of the antigen. No immunoreactive band was detected with the d-[¹⁴C]-glucosamine-labeled NonidetP-40 extract of T47D; in contrast, the mAb precipitated a $M_r$ 32,000 protein from the [³⁵S]-methionine-labeled extract (Fig. 4).

**DISCUSSION**

We have reported on a mAb, 7B10, which reacts with a human mammary gland antigen. This antibody was generated by immunization of BALB/c mice with a human breast cancer cell line, T47D. 7B10 is an IgG$_1$ antibody which reacts strongly with estrogen receptor-positive breast cancer cell lines T47D, MCF7, ZR75-1, and HSL53, and binds poorly or not at all with estrogen receptor-negative breast cancer cell lines, tumor cell lines of other origin, and normal cells tested. These results show a marked specificity for breast cancer cell lines. In *vitro* results were confirmed with the use of histological material. Indeed, immunohistochemical assays showed different distribution of the antigen between malignant and normal breast tissues.

Using frozen sections of histological specimens, the reactivity of 7B10 was shown to be positive with cancer tissue as well as with benign and normal breast tissue. Although all the specimen types showed cell surface staining, cytoplasmic reactivity was shown only in benign and malignant tumors. These observations suggest that antigenic expression is not regulated in the same way in normal and tumor cells. This may be related to an increased expression of the antigen or to its accumulation in cytosol, and may be of great interest in the study of differentiation of breast cells. Similar patterns of antigenic heterogeneity between membrane and cytoplasmic staining were described with other mAbs, (Refs. 16, 21, 22, 32, and 33; reviewed in Refs. 34 and 35).

An interesting difference in staining was observed with 7B10 in paraffin sections. No reactivity was shown in the seven normal mammary glands and the six nonmalignant lesions tested, the only exception being the case containing apocrine metaplasia. Conversely, breast cancers were stained with an intracytoplasmic localization. Although this study should be considered preliminary, it is highly probable that the alteration in membrane antigenic determinants caused by fixation and
embossing procedures was responsible for these differences in reactivity. 7B10 thus appears to discriminate between cancer cells and nonmalignant breast cells in paraffin sections. The recognition of a breast cancer-associated antigen seems to be relatively specific, since the only other cell specimens giving positive results were from an ovarian clear cell carcinoma and a negative control and OKI 9 (Lane d, antitransferrin receptor used as positive control), were analyzed by 5-15% continuous gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Lane e, migration of molecular weight standards). Further in-depth analysis with a large number of paraffin-embedded human tissues is in progress.

Fig. 4. Characterization of antigen recognized by mAb 7B10. T47D cells were labeled metabolically with [35S]methionine. Cell lysates, following immunoprecipitation with 7B10 (Lane a), 1BE12 (Lane b, mAb raised against T47D cells used as a positive control (17)), 160C19 (Lane c, antirotavirus IgG, mAb (26) used as a negative control), and OKT 9 (Lane d, antitransferrin receptor used as positive control), were analyzed by 5-15% continuous gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Lane e, migration of molecular weight standards).

In an attempt to further determine the localization of the detected antigen on cancer samples, electron microscopy studies of membrane and cytoplasm binding were undertaken. Breast cancer cell lines and clinical specimens showed marked staining involving membrane, which seemed to be polarized to the apical border of the cells, with no staining of membranes at the intercellular junctions. Specific cytoplasmic staining of breast cancer cell lines T47D and MCF7 was diffuse, without nuclear reactivity, while, in fresh tumors, the antigen appeared concentrated in ergastoplasm saccules.

Further biochemical analysis using immunoprecipitation of cells labeled with [35S]methionine revealed a band of M, 32,000. Further studies are required to know the nature of the antigen detected by 7B10, since no immunoreactivity of breast cancer tumors was observed with D5 after formalin fixation, and no membrane staining was observed with glutaraldehyde in either normal or malignant tissue.

A comparative epitope analysis of antigens defined by monoclonal antibodies of similar molecular weight or immunological origin, as exemplified by Price for mAb NCRC-11-defined antigen (37), would be of interest.

Further studies using receptor-characterized clinical specimens are in progress by using 7B10 to determine whether this cross-reacting antigen might be associated with the hormonal receptor status of breast tumor. Other works in progress are focused on the use of 7B10 for the histological detection of occult tumor cells in lymph nodes and bone marrow.

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has a molecular weight of 290,000. Like antibody 7B10, DF3 shows a cytoplasmic antigen location for mammary carcinoma, while benign lesions of the breast show only an apical location of antigen, suggesting a relationship with the functional activity of mammary cells as well as with the degree of differentiation of tumor cells.

mAb H59, described by Yuan et al. (2), has been shown to recognize an estrogen-regulated cell surface antigen. The antigen appears to consist of two peptides with molecular weights of 28,000. This mAb reacts with the majority of breast cancer cell lines tested, except with T47D, which was the immunizing cell line in our study. Moreover, normal frozen tissue sections of breast gland did not show any reactivity with H59, and no cytoplasmic staining could be detected with this antibody on tumor samples. Although close in molecular weight to the antigen described in this paper, the antigen recognized by H59 is presumably different. Another estrogen receptor-related antigen has been described (D5) with a molecular weight of 29,000. This antigen appears to be distinct from the M, 32,000 antigen detected by 7B10, since no immunoreactivity of breast tumors was observed with D5 after formalin fixation, and no membrane staining was observed with glutaraldehyde in either normal or malignant tissue.


A Novel Monoclonal Antibody (7B10) with Differential Reactivity between Human Mammary Carcinoma and Normal Breast

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