Monoclonal Antibody Identification and Characterization of a M, 43,000 Membrane Glycoprotein Associated with Human Breast Cancer

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ABSTRACT

A monoclonal antibody (323/A3) with a high degree of selectivity for binding to breast cancer cells was produced by immunization of mice with MCF-7 human breast cancer cells. The antigen recognized by 323/A3 on MCF-7 appears to be surface localized, and by enzyme-linked immunosorbent assay, the antibody was found to bind strongly with four of six breast cancer cell lines examined while no binding was detectable with non-breast cancer cell lines. In vivo distribution of the 323/A3 antigen was screened by immunoperoxidase staining of formalin-fixed paraffin sections of normal human tissues and tumors. Among breast tissues, positive staining was detected with 75% (6 of 8) of metastatic lymph nodes, 59% (76 of 128) of primary breast tumors, 20% (13 of 63) of benign breast lesions, and 0% (0 of 10) of normal breast. No immunostaining was detected with a large variety and number of other normal human tissues with the exception of staining observed with epithelium of normal colon. Antigen distribution appears not to be disease specific, since positivity was also observed with adenocarcinomas other than breast.

The antigen recognized by the 323/A3 antibody was identified by Western blot analysis as a M, 43,000 protein. The glycoprotein nature of the antigen was demonstrated by its binding to concanavalin A, specific elution with sugar, and immunoprecipitation of a M, 43,000 radiolabeled protein from extracts of MCF-7 cells after pulse-labeling with [3H]glucosamine. The 323/A3 antigen appears to be the same M, 43,000 protein in cell lines as in breast tumours in vivo. Based on a comparison with the molecular weights of other known tumor-associated antigens and with their immunocytochemical tissue distribution, the M, 43,000 glycoprotein described here represents a tumor-associated antigen previously undescribed in breast cancer or in other tumors.

Since the M, 43,000 glycoprotein is present on the surface of most breast cancer cells and is either absent or expressed at very low levels in most normal tissues including normal breast, the monoclonal antibody described here may have potential applications in diagnosis and management of breast cancer.

INTRODUCTION

MAbs reactive with breast cancer-associated antigens have been developed recently by several investigators. These antibodies fall into three general categories. There are those produced through immunization with breast tumor cells or cell extracts which display highly selective binding to breast cancer and little or no binding with normal tissues (1-7). These antibodies have the capability of distinguishing between breast tumor cells and their normal cell counterparts and are potentially useful, therefore, in diagnosis and therapy of breast cancer. Another group of MAbs have been raised also against breast cancer cells but react with epithelial antigens of both normal and neoplastic mammary tissues (8-12). A third group of antibodies has been produced to tissue differentiation antigens of normal breast epithelium by immunization with membranes of the human milk fat globule (13-18). These normal breast epithelial antigens are also expressed, although in reduced amounts, on a majority of breast carcinomas and are potentially useful as well in diagnosis of breast cancer (17, 18). Antigens of the human milk fat globule, for example, have been detected in elevated levels in sera of breast cancer patients (19), and monoclonal antibodies against these normal differentiation antigens have been used to localize breast carcinomas in patients by radioimaging techniques (20, 21).

Our studies were designed to produce monoclonal antibodies to breast cancer determinants of the first category. Antibodies were produced by immunization of mice with human breast cancer cell lines. We report here characterization of one such MAb, 323/A3, which displays highly selective binding to breast carcinomas and benign breast lesions and a very narrow distribution of binding with normal tissues. Immunohistochemical studies were performed to evaluate the in vivo tissue distribution of antibody binding, and we also present preliminary biochemical characterization of the antigen recognized by this monoclonal antibody.

MATERIALS AND METHODS

Cell Cultures

Human Breast Cancer Cells. The following human breast cancer cell lines were used in these studies: MCF-7; T47D; ZR-75.1; MDA-231; 1

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The abbreviations used are: MAb, monoclonal antibody; MEM, minimal essential medium; PBS, phosphate-buffered saline (8 mM sodium phosphate:2 mM potassium phosphate:0.137 M sodium chloride:0.003 M potassium chloride, pH 7.4); HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; SDS, sodium dodecyl sulfate; ABC, avidin:biotin:horseradish peroxidase complex; NP-40, Nonidet P-40 nonionic detergent; CEA, carcinoembryonic antigen; Con A, concanavalin A; WMCP, 15 mM Tris (pH 7.4):1 mM MgCl2:2 mM phenylmethylsulfonyl fluoride: + /- M, with /- mercaptoethanol; - /- M, without /- mercaptoethanol.
MDA-330; and BT-20. Culture conditions and properties of these cell lines have been previously described (22, 23). MCF-7 cells, which are estrogen receptor and progesterone receptor positive, were used as the source of antigen. Growth medium for MCF-7 consisted of Eagle's MEM supplemented with insulin (6 ng/ml), 2 mM L-glutamine (Gibco), 1% nonessential amino acids, 10 mM HEPES buffer, pH 7.3, 0.2% sodium bicarbonate, gentamicin (25 µg/mL) (Irvin Scientific), and 5% calf serum (K. C. Biologicals, Inc.), and cells were grown as previously described (24).

Other Cell Lines. HBL-100 are spontaneously transformed receptor-negative cells derived from human milk epithelium and grown as previously described (22). KB human epidermoid cells, WI-38 (VA 13. subline 2RA) virally transformed human lung fibroblasts, and 3T3 (clone A31) mouse embryoid fibroblasts were obtained from the American Type Culture Collection and grown according to cell bank protocols. Chinese hamster ovary cells were obtained as a gift from Dr. Victor Ling (Ontario Cancer Institute) and were grown as previously described (25).

For large scale production of cultures and isolation of plasma membrane vesicles, cells (MCF-7, MDA-231, and HBL-100) were plated in Corning plastic roller bottles (850 cm²) at a density of 40 x 10⁶ cells/bottle and grown at 37°C in a warm room without CO₂. For ELISA wells, cells were plated into 96-well plastic microtiter culture dishes (Costar) in appropriate growth medium at a density of 5 x 10⁵ cells/well. Cells were allowed to attach as a monolayer and then grown for an additional 24 h in a 5% CO₂ incubator at 37°C.

Isolation of Plasma Membranes

Plasma membranes were isolated from cultured cells by sedimentation on sucrose density gradients as described by Riodin and Ling (26). Cells from 10 roller bottles (10 g of cells) were homogenized at 4°C in 20 ml of TMCP buffer in a Duvaal glass homogenizer fitted with a Teflon pestle. Homogenates at 4°C were centrifuged at 800 x g to pellet nuclei and unbroken cells, and the supernatant was saved. The pellet was resuspended in 10 ml of TMCP buffer, rehomogenized, and centrifuged at 800 x g. The resulting supernatant was added to the first and layered onto a 30-ml discontinuous sucrose gradient consisting of 45, 31, and 23% sucrose layers prepared immediately before use in TMCP buffer. Centrifugation was for 2 h at 4°C at 27,000 rpm (=100,000 x g average) in a Beckman SW27 swinging bucket rotor. Sedimented membrane vesicles at the 23:31% interface were drawn off with a syringe and pelleted by centrifugation for 1 h at 4°C at 100,000 x g. The supernatant containing solubilized membrane proteins was collected and stored at −70°C.

Protein in the soluble detergent extract was measured by the method of Lowry et al. (27). Membrane proteins were also extracted by continuous mixing overnight at 4°C with 3 M KCl. Criteria for determining purity of membrane preparations were electron microscopy and measurement of the membrane enzyme marker adenylate cyclase. The 23:31% interface of the sucrose gradients contained the highest enrichment in membrane vesicles. This fraction on electron microscopy was composed almost entirely of membrane vesicles and showed a 7-fold increase in adenylate cyclase activity over that of cell homogenates.

Immunizations

BALB/c mice (female, age 8 wk) were immunized by injection of 1 x 10⁷ live MCF-7 cells i.p. once a week for 3 wk. Animals were boosted daily beginning 3 days before fusion with 100 µg of either a 3 M KCl extract or an NP-40 detergent extract of isolated membrane vesicles from MCF-7. Booster injections 3 days before fusion were given i.p. in PBS.

Some mice were immunized with 100 µg of MCF-7 detergent membrane extracts emulsified in complete Freund's adjuvant and given in multiple s.c. sites. Second and third injections of 100 µg of membrane extracts were given 1 wk apart in incomplete Freund's adjuvant. Beginning 3 days before fusion, these animals were also boosted daily by i.p. injection of 100 µg of MCF-7 membrane extracts diluted in PBS.

Cell Fusion and Hybridoma Production

Fusions were performed as previously described (28). Single cell suspensions of mouse spleens were prepared and mixed at a 4:1 ratio (spleen:NS-1) with mouse NS-1 myeloma cells and fused with 50% (w/v) polyethylene glycol (MW, 1540 obtained from Koch light) in serum-free RPMI-1640 medium. Mouse NS-1 cells were cultured and expanded for fusion in RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum (Hyclone Sterile Systems), 2 mM L-glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids, penicillin/streptomycin (50 units/ml), 10 mM HEPES, pH 7.4, and 0.2% sodium bicarbonate. Fused cells were plated and incubated for 24 h in 100-mm Petri dishes (Falcon) in the NS-1 growth medium, except supplemented with 20% fetal bovine serum. Cells were then seeded into 96-well microtiter dishes (Costar) in selection medium (same medium described above with the addition of 0.1 mM hypoxanthine, 0.4 µM aminopterin, and 16 µM thymidine) at a density of 5 x 10⁵ cells/well. Culture medium was removed 1 to 2 wk later for screening. After the first screening, positive cultures producing antibodies against MCF-7 antigens were transferred to larger 1 ml culture wells (Coster; 24-well plates), allowed to grow for 2 to 3 days, and then reassayed. Selected hybridoma cultures were then cloned directly from 1-ml culture wells by limited dilution into 96-well dishes seeded in advance with a feeder layer of BALB/c thymus cells plated at a density of 5 x 10⁵ cells/well. Wells containing single hybridoma colonies (1 to 2 wk after cloning) were assayed, and positive cultures were expanded and grown up in 100-mm Petri dishes. Hybridoma lines were subcloned twice by limited dilution to ensure clonality of the line. Clonal lines were cryopreserved in 95% fetal bovine serum and 5% dimethyl sulfoxide (American Type Culture Collection) in liquid nitrogen.

Purification of Monoclonal Antibody

Hybridomas were grown as ascites tumors in pristane-primed BALB/c mice. Pristane (tetramethylpentadecane) was injected in volumes of 0.5 ml i.p., 1 wk prior to i.p. injection of 2 x 10⁶ live hybridoma cells per animal. Ascites fluid was drained 1 to 2 wk later by abdominal puncture and was used as a source for antibody purification. The IgG fraction of ascites fluids was purified by two successive precipitations of immunoglobulins at 40% saturation of ammonium sulfate followed by DEAE-cellulose chromatography (Whatman; DE-52). The IgG fraction eluted with 50 mM sodium chloride and was approximately 90 to 95% pure based on SDS-gel electrophoresis and staining with Coomassie blue. Antibody typing for mouse immunoglobulin class and subclass was by ELISA, using the Boehringer-Mannheim mouse immunoglobulin subtype identification kit.

ELISA Assays

Both fixed cells and solubilized membrane protein extracts were used as the target antigen in ELISA assays. For fixed-cell assays, the method of Layton and Smithyman (29) was followed with some modifications. Cells were plated in 96-well culture dishes (Costar) at a density of 5 x 10⁵ cells/well and allowed to grow for 24 to 48 h to form an attached monolayer. Growth medium was flicked out, and cells were washed by a brief 30-s rinse (with shaking) in PBS containing 0.1% Tween-20. Supernatant was removed by inverting the dish, and cells were dried by incubation of the plates without covers for 30 min in a dry oven at 37°C. Cells were then fixed by a 5-min incubation at room temperature with 70% methanol:3% hydrogen peroxide (H₂O₂). Fixative was removed, and cells were rehydrated by incubation for 20 min at room temperature with PBS (200 µl/well). Plates were washed 2 times with PBS and incubated for 1 h at 4°C with 1% BSA (200 µl/well) as a block step. Each well was...
incubated overnight at 4°C with either 50 μl of hybridoma supernatant or purified antibody diluted in 1% BSA:PBS. Plates were then washed 3 times with PBS:PBS, incubated for 3 h at room temperature with 150 μl of goat anti-mouse IgG:peroxidase (Cappel) diluted 1:5000 with 1% BSA:PBS. Following incubation with anti-mouse peroxidase, plates were washed 5 times with PBS and incubated for 10 min at room temperature with 150 μl of enzyme substrate (2.5 mg/ml of O-phenylenediamine:0.05% H₂O₂ prepared in citric acid buffer (50 mM citric acid monohydrate; 0.2 m sodium phosphate dibasic, pH 4.9). Reaction was stopped by addition of 50 μl of 1 M sulfuric acid to each well. Plates were read in a Dynatech MR-600 ELISA reader at a wavelength of 490 nm.

For ELISA with solubilized membrane proteins, detergent extracts were diluted to a protein concentration of 6 μg/ml in borate buffer (0.1 M boric acid:0.025 M sodium tetraborate, pH 8.2), and 150 μl aliquots of this antigen solution were added per well to Immulon II microtiter dishes (Dynatech) and allowed to incubate overnight at 4°C. Plates were washed twice in PBS and incubated for 1 h at 4°C with 150 μl of 1% BSA:PBS per well to block remaining binding sites. The remaining steps of the assay were the same as described above for ELISA of fixed cells.

SDS-Polyacrylamide Gel Electrophoresis and Immunoelectroblotting Technique (Western Blot)

Proteins from membranes or cell extracts were separated by SDS-polyacrylamide gel electrophoresis on a vertical slab gel apparatus as originally described by Laemmli (30) and modified by Wykoff et al. (31), of goat anti-mouse IgG:peroxidase (Cappel) diluted 1:5000 with 1% methanol. Following incubation with anti-mouse peroxidase, plates were washed twice in PBS and incubated for 1 h at 4°C with 150 μl of 1% BSA:PBS for 24 h at room temperature with 5 μl of purified monoclonal antibody (1 mg/ml). The incubation mixture was then diluted 1:3 with TMCP containing 1% NP-40, 1% sodium deoxycholate, and 0.2 M sodium chloride and incubated overnight at 4°C. A 10-μl aliquot of rabbit anti-mouse IgG (4 mg/ml) was added and incubated for 3 h at 4°C, followed by addition of a 100-μl suspension of Protein A-Sepharose (Pharmacia) and incubation for another 1 h at 4°C. [Protein A-Sepharose beads were washed prior to use in PBS and suspended in a 1:1 (w/v) ratio of beads to buffer.] Beads were washed 3 times by resuspension and centrifugation in a microfuge tube using 0.1 M Tris (pH 8.0):0.5 M sodium chloride as wash buffer. Washed Protein A-Sepharose beads were boiled in SDS-sample buffer, and extracted proteins were analyzed by SDS-electrophoresis and liquid scintillation counting of gel slices. Gel slices were processed for counting by incubation for 24 h at 37°C in 10.0 ml of scintillation cocktail containing 5% Protosol (New England Nuclear), 4 g of PPO, and 0.05 g of POPP per liter of toluene. Samples were counted in a Beckman LS-233 scintillation counter with a counting efficiency of 42% for tritium.

Radioiodination of Antibodies

Radioiodination of goat anti-mouse IgG second antibody (Cappel) was performed by a solid-phase lactoperoxidase:glucose oxidase method as originally described by Morrison et al. (33). Briefly, 50 μg of antibody (in a volume of 1 to 15 μl) were incubated at room temperature for 40 min with 50 μl of 0.2 M sodium phosphate (pH 7.2):50 μl of Enzymobeads (Bio-Rad):1 mCi of Na₁²⁵I(Amersham):25 μl of 1% O-D-glucose. Enzyme reaction was stopped by centrifugation of the Enzymobeads, and free radiolabeled antibody was diluted in 1% BSA:PBS and 0.2% sodium azide at a concentration of 500,000 cpm/50 μl for storage at 4°C.

Immunofluorescence

Indirect immunofluorescence assays were performed on live and fixed MCF-7 cells. For fixed cell assays, cells were plated in growth medium at 5 x 10⁴ cells/well into an 8-well chamber slide and allowed to grow as a monolayer for 24 to 48 h at 37°C. Chambers were washed twice in PBS, fixed in ether:ethanol for 10 min at room temperature, and then rehydrated and washed in PBS. Cells in the chambers were incubated with monoclonal antibody for 2 h at room temperature. Control incubations included myeloma spent culture medium as a medium blank, an unrelated mouse monoclonal antibody (anti-human IgE), and a positive control (anti-24K; Ref. 34). Chambers were washed with PBS and incubated with goat anti-mouse fluorescein isothiocyanate (Cappel; anti-mouse IgG, IgA, and IgM) for an additional 2 h at room temperature. Slides were washed in PBS and mounted in immersionfluorescence mounting medium (90% glycerol:0.1% p-phenylenediamine in PBS) with a 0.05 μl of galactose for R. communis. Following centrifugation and sugar-elicited fractions were each analyzed for specific antigens by Western blot assays.

Internal Radiolabeling and Analysis of Immunoprecipitated Proteins

MCF-7 cells grown in 75-cm² flasks were pulsed labeled in culture with [³H]glucosamine (10 μCi/ml) (43.2 Ci/mmol glucosamine hydrochloride; [¹⁻⁻³H]; New England Nuclear Corp.). Cells were grown to near confluence and pulsed overnight in MCF-7 culture medium at 37°C. Flasks were washed twice with MEM to remove free radioactive sugar, harvested by scraping into MEM, and pelleted by low speed centrifugation. Cells were then homogenized in TMCP buffer, and monomeric proteins were adjusted to 1% NP-40, 1% sodium deoxycholate, and 0.2 M sodium chloride and incubated with slow stirring at room temperature for 20 min. Solubilized proteins were collected as the supernatant fraction after centrifugation at 100,000 x g for 60 min at 4°C. Aliquots (100 μl) of the radiolabeled cell extracts were incubated for 30 min at room temperature with 5 μl of purified monoclonal antibody (1 mg/ml). The incubation mixture was then diluted 1:3 with TMCP containing 1% NP-40, 1% sodium deoxycholate, and 0.2 M sodium chloride and incubated overnight at 4°C. A 10-μl aliquot of rabbit anti-mouse IgG (4 mg/ml) was added and incubated for 3 h at 4°C, followed by addition of a 100-μl suspension of Protein A-Sepharose (Pharmacia) and incubation for another 1 h at 4°C. [Protein A-Sepharose beads were washed prior to use in PBS and suspended in a 1:1 (w/v) ratio of beads to buffer.] Beads were washed 3 times by resuspension and centrifugation in a microfuge tube using 0.1 M Tris (pH 8.0):0.5 M sodium chloride as wash buffer. Washed Protein A-Sepharose beads were boiled in SDS-sample buffer, and extracted proteins were analyzed by SDS-electrophoresis and liquid scintillation counting of gel slices. Gel slices were processed for counting by incubation for 24 h at 37°C in 10.0 ml of scintillation cocktail containing 5% Protosol (New England Nuclear), 4 g of PPO, and 0.05 g of POPP per liter of toluene. Samples were counted in a Beckman LS-233 scintillation counter with a counting efficiency of 42% for tritium.
coverslip and viewed on a Leitz Dialux-20 fluorescent microscope equipped with a short arc lamp and a K-480 filter to eliminate autofluorescence. Identical fields of view were photographed under visible and fluorescent light using Kodak ektachrome film.

For assay with unfixed cells, MCF-7 cells harvested from T-flasks were incubated in suspension (PBS) with MAb for 40 min at room temperature. Cells were then washed in PBS, incubated for another 40 min at room temperature with goat anti-mouse fluorescein isothiocyanate, suspended in PBS, mounted, and viewed as above.

**Avidin:Biotin:Immunoperoxidase Staining**

Cell lines to be stained with peroxidase were fixed in Bouin's solution while tissues were fixed in 10% buffered formalin. Fixed cells and tissues were then processed and embedded in paraffin. Normal human tissues were obtained at autopsy from the Pathology Department at Audie Murphy Veterans Administration Hospital, San Antonio, TX, or as surgical specimens from pathology at Medical Center Hospital, San Antonio, TX. Normal breast tissues were obtained from reduction mammoplasty, and specimens of human benign breast disease were from three sources: University of Wales Hospital, Cardiff, Wales; Seton Medical Center, Austin, TX; and Medical Center Hospital, San Antonio, TX. Human tumors were obtained as formalin-fixed paraffin-embedded sections from the Department of Anatomical Surgical Pathology at Medical Center Hospital, San Antonio, TX. Five-μm-thick tissue sections were immunostained by the avidin:biotin:peroxidase method (Vector Laboratories) according to the manufacturer’s instructions and as previously described by Ciocca et al. (34). Control slides included incubation with NS-1 culture medium as a medium blank, an unrelated antibody (anti-human IgE), and a positive control antibody (anti-24K). Diaminobenzidine:hydrogen peroxide was used as the chromagen, and sections were counterstained with hematoxylin and mounted with Permount.

**RESULTS**

**Screening Strategy and Production of MAbs.** Mice were immunized with soluble extracts of purified membranes from MCF-7 human breast cancer cells or with live MCF-7 cells. In each case, animals were boosted 3 days before fusion with solubilized MCF-7 membrane proteins, and spleen cells were fused with mouse NS-1 myeloma to produce hybridoma cultures as described in "Materials and Methods."

Since our goal was to define antigens that might serve as tumor markers in breast cancer, a screening strategy was designed to select monoclonal antibodies reactive with surface membrane antigens highly restricted to breast cancer cells and not expressed on normal cell counterparts.

Screening of hybridomas was performed in several stages, each stage designed to eliminate unwanted MAbs. (a) The first level of screening was by ELISA against a panel of methanol-fixed tissue culture cell lines. This panel included six breast cancer cell lines, HBL-100, and some non-breast cell lines, WI-38, KB, 3T3, and CHO. Those antibodies showing high binding to breast cancer lines and low or no binding to other cells were retained and others discarded. (b) Since we were interested in MAbs against surface membrane antigens, cultures selected by the above ELISA were screened secondarily by an indirect immunofluorescence assay with viable and fixed MCF-7 cells to assess whether antigens were localized on the surface of cells. Antibodies reactive with intracellular structures were eliminated, and only those giving surface fluorescence staining with viable unfixed cells were taken for further analysis. (c) Further selection was based on ability of MAbs to recognize antigen in paraffin-embedded sections. To accomplish this, MCF-7 cells were fixed in Bouin’s solution and embedded in paraffin, and thin sections were prepared and stained by the avidin:biotin:immunoperoxidase method. (d) Finally, antibodies meeting all the above criteria were screened for immunoperoxidase staining with a limited number of formalin-fixed paraffin sections of breast carcinomas and normal human tissues, including normal breast. Those reactive with breast carcinomas but failing to react with normal breast were taken for more extensive immunohistochemical and biochemical studies. Others were dropped from the study.

Approximately 3000 hybridomas have been screened by the above strategy using either MCF-7 cells or MCF-7 membranes as immunogen. A number of MAbs were selected which displayed various degrees of restrictive binding to breast carcinomas. One particular MAB, designated 323/A3, has proven to be the most specific and will be the subject of this study. Properties and characterization of other MAbs will be presented elsewhere. The 323/A3 MAB is a mouse IgG1 and was produced from a mouse immunized with live MCF-7 cells and boosted 3 days prior to cell fusion with a 3 M KCl extract of MCF-7 plasma membranes. This hybridoma cell line has been subcloned repeatedly and found to be a stable antibody-producing line. 323/A3 binds to the surface of MCF-7 cells as evidenced by the immunofluorescence pattern observed with both lightly fixed MCF-7 cells grown on chamber slides (Fig. 1A) and with live unfixed MCF-7 cells in suspension (Fig. 1B). A fairly even fluorescent staining of cell membranes and a diffuse weaker cytoplasmic staining were observed with fixed cells. On the other hand, a patchy immunofluorescent staining was observed with viable unfixed cells, which is indicative of surface localization of the antigen. 323/A3 also binds strongly on ELISA to detergent and 3 M KCl extracts of purified MCF-7 plasma membranes (not shown). We observed that immunoperoxidase staining of fixed, paraffin-embedded sections of MCF-7 cells taken from culture is also predominantly membrane as shown in Fig. 1C. Thus, antigen appears to be localized, at least in part, on the surface of MCF-7 cells and is stable to fixation and embedding in paraffin. Also observed in Fig. 1C is that 323/A3 immunostaining of MCF-7 cells in culture appears homogeneous since nearly all cells show a uniform staining. By contrast, when MCF-7 cells are grown as solid tumors in nude mice, the immunoperoxidase staining pattern with 323/A3 becomes more heterogeneous, and staining is both membrane and cytoplasmic (primarily cytoplasmic) (Fig. 1D). The mechanism of this acquired heterogeneous expression of antigen in vivo may have important implications with regards to heterogeneity often seen in many human tumors. We have also observed that expression of the 323/A3 antigen is stable with prolonged passage of MCF-7 cells in culture.

For these initial screenings and characterization of the 323/A3 MAb, hybridoma culture supernatants were used as the antibody source. Subsequent studies were performed with highly purified IgG preparations isolated from mouse ascites fluids.

**Binding of 323/A3 to Cultured Cell Lines.** Purified 323/A3 was examined by ELISA for binding to a panel of methanol-fixed cell lines (Fig. 2). Strong binding was obtained with four (MCF-7, T47-D, ZR-75.1, and BT-20) of the six human breast cancer cell lines assayed when using a single concentration of MAB. MCF-7, T47-D, and ZR-75.1 are steroid receptor-positive cells, while BT-20, MDA-231, and MDA-330 are receptor negative.
Fig. 1. 323/A3 immunostaining of MCF-7 breast cancer cells. A, 323/A3 immunofluorescence of fixed MCF-7 cells. MCF-7 cells were briefly fixed in ether-ethanol and stained by indirect immunofluorescence using DEAE-purified 323/A3 MAb. Arrows point to areas of membrane staining. × 312.5. B, 323/A3 immunofluorescence of unfixed MCF-7 cells. Live MCF-7 cells were stained by indirect immunofluorescence using the 323/A3 antibody. Granular membrane staining (single-headed arrow) and punctate staining (double-headed arrow) illustrate the surface membrane localization of the antigen. Occasional capping (C) was also observed. × 312.5. C, 323/A3 immunoperoxidase staining of paraffin-embedded MCF-7 cells. Bouin’s fixed, paraffin-embedded MCF-7 cells show predominantly membrane staining (arrows) using the 323/A3 antibody. Sections were stained by the ABC:immunoperoxidase technique and counterstained with hematoxylin as described in “Materials and Methods.” × 500. D, 323/A3 immunoperoxidase staining of solid MCF-7 tumor. MCF-7 cells grown as a solid s.c. tumor in nude mice were fixed in Bouin’s, embedded in paraffin, and stained by the ABC method. Sections were counterstained in hematoxylin. Intense membrane stain (single-headed arrow) as well as diffuse cytoplasmic staining (double-headed arrow) was observed. Connective tissue (CT) is negative. × 312.5.

23). The ELISA data therefore suggest a lack of relationship between steroid receptors and expression of 323/A3 antigen in breast cancer. Little or no binding of the 323/A3 MAb was obtained with the other cell lines examined, including HBL-100 which is a spontaneously transformed cell derived originally from normal breast epithelium.

Fig. 3 shows titration of 323/A3 binding, by fixed cell ELISA, to MCF-7 cells compared with other negative cell lines. At the highest concentrations of purified 323/A3, no binding above background was obtained with any of these other cell lines. By contrast, half-maximal binding of 323/A3 with MCF-7 cells occurs at about 10 ng of MAb per ml with the end point dilution occurring at about 750 pg/ml. Antigen densities, therefore, on MCF-7 are several orders of magnitude higher than on other negative cell lines.

Fig. 4 shows titration of 323/A3 binding to the four positive breast (MCF-7, T47D, ZR-75-1, and BT-20) cancer cell lines and for comparison one of the negative breast cancer cell lines, MDA-330. Also shown in Fig. 4 is the background binding to MCF-7 cells obtained with negative control mouse NS-1 ascites fluid. We occasionally see positive ELISA reaction with MCF-7 cells at the highest concentration of NS-1 ascites fluid, but we attribute this to nonspecific binding since the binding quickly drops off with dilution. Based on these ELISA titration curves (Figs. 3 and 4), antigen densities are highest and roughly equivalent on MCF-7 and ZR-75 cells and slightly less dense on BT-20 and T47-D cells. Antigen is either absent or below level of ELISA detection on MDA-231 and MDA-330 breast cancer cells and all the other cells examined.

Immunohistochemical Analysis of Human Tumors and Normal Tissues. The results of 323/A3 immunoperoxidase staining with formalin-fixed paraffin sections of human breast tissues are
summarized in Table 1. Normal breast, benign tumors, and malignant lesions of the breast were examined. No staining was observed with normal breast, which included two cases of lactating breast and eight of resting breast. Of the benign tumors examined, 20% showed binding with 323/A3. We found that immunostaining of benign tissue was heterogeneous and was localized primarily to surface membranes in areas of epithelial cell hyperplasia and apocrine glands. Histologically normal lobules and stroma did not stain. In further examination of benign lesions by histological categories, we observed that the frequency of 323/A3 staining of fibroadenomas without hyperplasia was low, whereas an intermediate frequency of staining was observed in fibroadenomas with hyperplasia, and all sections of hyperplasia with morphological dysplasia stained strongly positively. This pattern of reactivity suggests that expression of the 323/A3 antigen may increase with progression of breast disease. A more detailed analysis of 323/A3 reactivity with various histological categories of benign breast lesions and its possible identification of patients at high risk to develop breast cancer will be presented elsewhere. Of the primary breast carcinomas examined, 59% reacted with 323/A3 and, although the number of cases is low, a slightly higher percentage (75%) of metastatic lymph nodes stained positively (Table 1). Thus, the incidence of immunocytochemical staining with 323/A3 was observed to increase in a progressive manner from normal, to benign disease, to carcinoma.

The pattern of 323/A3 immunoperoxidase staining was documented for all 76 of the positive breast carcinomas examined in Table 1, and those results are given in Table 2. We observed that staining of breast tumors was heterogeneous not only among tumors (since 76 of 128 tumors were positive) but also within given tumors. For example, the percentage of immunocytochemical staining with 323/A3 was observed to increase...
stained tumor cells within a given tumor ranged from 1 to 100% (mean, 58%), and the intensity of staining ranged on an arbitrary scale from +1 to +4 (Table 2). Intracellular location of staining was also highly heterogeneous. Some tumors showed predominantly cytoplasmic staining, others predominantly membrane staining, while the majority (54%) gave both cytoplasmic and membrane staining. In many tumors (i.e., 31%), 323/A3 bound to every tumor cell, but the majority of tumors (45%) displayed regional heterogeneity in staining, meaning that most tumor cells in an area of involvement stained either uniformly positive or uniformly negative. A smaller percentage of tumors displayed cell-to-cell heterogeneity in staining within the same region of tumor cell involvement, while some tumors displayed both regional and cell-to-cell heterogeneity.

Representative immunoperoxidase-stained sections of human breast tissues are shown in Fig. 5. Negative staining of a normal breast is shown in Fig. 5A. Fig. 5B is a section of primary breast cancer which displays homogeneous cytoplasmic staining with the rest of tumor cells contained in a lymphatic vessel. Fig. 5C is another primary breast cancer which gives predominantly membrane staining of tumor cells. In this particular tumor, heterogeneity in the intensity of membrane staining was observed from cell to cell. Staining of a metastatic lymph node is shown in Fig. 5D. In lymph nodes, we observed staining only with tumor cells (in this case staining of tumor cells is membrane), and no staining was observed with normal lymphocytes or surrounding connective tissue. In no case did we observe 323/A3 staining in sections of breast carcinoma with blood vessels, surrounding connective tissues, or with morphologically normal breast epithelium. Antibody reactivity therefore appears to be confined to neoplastic epithelial cells, with the exception that staining was associated in some tumor sections with hyperplastic epithelial

Table 2

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<th>Parameter examined</th>
<th>Result</th>
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<td>Range of the percentage of positive cells</td>
<td>1–100% (58%)</td>
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<tr>
<td>Range staining intensity</td>
<td>+1→+4 (+2)</td>
</tr>
<tr>
<td>Staining location</td>
<td></td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>45%</td>
</tr>
<tr>
<td>Membrane</td>
<td>11%</td>
</tr>
<tr>
<td>Membrane &amp; cytoplasm</td>
<td>54%</td>
</tr>
<tr>
<td>Staining heterogeneity</td>
<td></td>
</tr>
<tr>
<td>(within same tumor)</td>
<td></td>
</tr>
<tr>
<td>Homogeneous</td>
<td>31%</td>
</tr>
<tr>
<td>Regional</td>
<td>45%</td>
</tr>
<tr>
<td>Cell-to-cell</td>
<td>19%</td>
</tr>
<tr>
<td>Regional and cell-to-cell</td>
<td>5%</td>
</tr>
</tbody>
</table>

*Numbers in parentheses, mean.

Fig. 5. 323/A3 immunoperoxidase staining of human breast tissues. All sections were cut from formalin-fixed paraffin-embedded blocks. Sections were stained by the ABC technique and counterstained with hematoxylin as described in "Materials and Methods." A, normal breast tissue. Normal breast obtained from reduction mammoplasty shows no immunostaining using the 323/A3 antibody. Myoepithelial cells (ME) x 200. B, primary breast cancer. Homogeneous cytoplasmic staining of a cluster of breast cancer cells is indicated by arrows in a lymphatic vessel (L). x 200. C, primary breast cancer. Heterogeneous membrane staining (arrows) of breast cancer cells. x 200. D, metastatic breast cancer to lymph node. Intense membrane immunostaining of breast cancer cells (arrows) found in axillary lymph node. Lymphocytes (Ly) are negative. x 200. E, benign breast lesion. Membrane immunostaining of hyperplastic breast epithelium (arrows) lining a duct. This is a different area of the section of breast cancer pictured in Fig. 2B. x 500. F, apocrine gland. Membrane immunostaining (arrows) associated with cells in apocrine glands of a section of primary breast cancer. Tumor cells in this section also stained positively with 323/A3 (not shown). Connective tissue elements (CT) are negative. Apocrine cell budding (A). x 200.

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cells and apocrine glands in areas adjacent to tumor cell involvement as shown in Fig. 5, E and F, respectively.

We have also examined immunocytochemical reactivity of 323/A3 with some nonbreast human tumors, the results of which are summarized in Table 3. Binding was observed with several other adenocarcinomas, and in each case, it was confined to involved epithelial cells. As with breast carcinoma, immunoperoxidase staining was localized in both membranes and cytoplasm. Although we have examined only a few cases, 323/A3 has not shown reactivity with tumors of nonepithelial origin. The antibody therefore does not react exclusively with breast carcinomas but binds also with other cancers of epithelial origin.

To further evaluate in vivo tissue distribution of the 323/A3 antigen, immunocytochemistry was performed with a large variety and number of normal human tissue taken as formalin-fixed paraffin sections. No staining was observed with the majority of tissues examined, which included multiple cases of normal breast (Table 1), major organs, lymph nodes, skin, bone marrow, and blood vessels (Table 4). A commonly observed cross-reaction with MAbs is binding with sweat glands and salivary glands. Sections of skin examined in Table 4 contained sweat glands that did not stain with 323/A3. We were not able, however, to obtain sections of normal salivary gland, so we have not evaluated 323/A3 for this possible cross-reaction. The only positive reactions observed with normal tissues were two of eight kidneys and five of five colons. Staining in kidney was weak and localized to collecting tubules and was not observed in glomerulus or capillary systems. All five cases of colon examined gave a positive heterogeneous staining localized to surface epithelium and intestinal glands. Goblet cells do not stain, and binding appears to be localized to the basal region of epithelial cells.

Based on these immunocytochemical analyses, the 323/A3 MAb binds with a high degree of selectivity to mammary and other carcinomas but not with the majority of normal human tissues examined, including normal breast. Since 323/A3 reacts solely with epithelial elements (whether tumor or normal cells), it may be recognizing a normal epithelial antigen expressed in abnormally high concentrations in tumors of epithelial origin.

The immunocytochemical staining pattern obtained with 323/A3 was compared in sections of MCF-7 cells with that of antibodies to some other known tumor-associated antigens. Paraffin sections of MCF-7 cells were immunostained with a MAb to Thomsen Friedenrich T-antigen and with rabbit antibodies to CEA, α-fetoprotein, and β-microglobulin. Three antibodies, Thomsen Friedenrich antigen, CEA, and β-microglobulin, gave positive staining of MCF-7 cells. Anti-α-fetoprotein was negative. The positive stainings observed, however, were cytoplasmic in each case and very different from the pattern observed with 323/A3 on serial sections of the same block of MCF-7 cells. We were also unable to block 323/A3 staining of MCF-7 using a highly purified preparation of CEA.

Biochemical Characterization of the 323/A3 Antigen. Western blot analysis was used to define the structure of the antigen recognized by 323/A3. In initial experiments, NP-40 extracts of purified MCF-7 membranes were resolved by SDS:electrophoresis gels, transferred to nitrocellulose filters, and reacted with 323/A3 and a 125I-labeled second antibody as described in "Materials and Methods." Under these conditions, no immunoreactive bands were detected. Subsequently we examined the conditions for preservation of antigenicity by a dot blot assay in which MCF-7 membrane extracts were spotted onto nitrocellulose discs and incubated with MAbs, followed by a 125I-labeled second antibody and counting of the filter disc for radioactivity. Heating to 100°C or denaturation with various detergents, including 1% SDS, did not effect 323/A3 binding to antigen. However, treatment with reducing agents, such as β-mercaptoethanol, destroyed antigenicity. Western blots were repeated using a SDS:sample buffer both with and without β-mercaptoethanol, and these results are shown in Fig. 6. Also included in Fig. 6 is a 323/A3 Western blot of membrane extracts from MDA-231 cells which by ELISA are negative for the 323/A3 antigen (Figs. 2 and 3). In the presence of reducing agent (+βME), no immunoreactive bands were detected in either MDA or MCF-7 cell membranes. In the absence of reducing agent (−βME), a single radioactive band was detected in MCF-7 membranes at M, 43,000 which was absent in MDA cells. In this experiment, decreasing amounts of MCF-7 membrane extracts were applied to gels ranging from 95 to 12 μg of total protein.

### Table 4

<table>
<thead>
<tr>
<th>Tissue</th>
<th>No. of positives/no. of tissues tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen</td>
<td>0/9</td>
</tr>
<tr>
<td>Pancreas</td>
<td>0/5</td>
</tr>
<tr>
<td>Heart</td>
<td>0/8</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>0/6</td>
</tr>
<tr>
<td>Lung</td>
<td>0/5</td>
</tr>
<tr>
<td>Liver</td>
<td>0/8</td>
</tr>
<tr>
<td>Small intestine</td>
<td>0/2</td>
</tr>
<tr>
<td>Prostate</td>
<td>0/3</td>
</tr>
<tr>
<td>Stomach</td>
<td>0/2</td>
</tr>
<tr>
<td>Lymph node</td>
<td>0/3</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>0/3</td>
</tr>
<tr>
<td>Endometrium</td>
<td>0/2</td>
</tr>
<tr>
<td>Thyroid</td>
<td>0/5</td>
</tr>
<tr>
<td>Skin</td>
<td>0/4</td>
</tr>
<tr>
<td>Ovary</td>
<td>0/1</td>
</tr>
<tr>
<td>Fallopian tube</td>
<td>0/2</td>
</tr>
<tr>
<td>Umbilical cord</td>
<td>0/4</td>
</tr>
<tr>
<td>Placenta/fetus</td>
<td>0/4</td>
</tr>
<tr>
<td>Tongue</td>
<td>0/2</td>
</tr>
<tr>
<td>Kidney</td>
<td>2/8</td>
</tr>
<tr>
<td>Colon</td>
<td>5/6</td>
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</tbody>
</table>

All tissue sections were cut from formalin-fixed paraffin-embedded blocks and were stained by the avidin:biotin:peroxidase method as described in "Materials and Methods." A section was considered positive if greater than 1% of cells stained.
Immunoprecipitation studies were next performed with MCF-7 cells metabolically labeled in culture with [3H]glucosamine. Radiolabeled cell lysates were incubated with 323/A3 and then immunoprecipitated with a rabbit anti-mouse second antibody and Protein A-Sepharose beads. After washing, the Protein A beads were extracted with SDS:sample buffer, and the extracts were analyzed by SDS:gel electrophoresis and scintillation counting of gel slices. As illustrated in Fig. 7, a single radioactive peak at \( M, 43,000 \) was detected on SDS: gels. By contrast, no radioactivity was observed for membrane extracts without \( \beta \)-mercaptoethanol (\(-\beta ME\)) and the other without \( \beta \)-mercaptoethanol (\(+\beta ME\)). As an internal molecular weight standard, approximately 25,000 cpm of \( ^{125}\text{I}-\text{IgG} \) were also run under reducing and nonreducing conditions.

Structural properties of the antigen were further characterized by determining lectin-binding specificities. NP-40 membrane extracts of MCF-7 cells were passed over various lectin:agarose affinity columns. The columns were washed extensively and then applied to a 0.5-ml Con A:agarose affinity resin packed into a 0.7-cm inner-diameter column and recirculated over the column for 2 h at 4°C at a flow rate of 6 ml/h. The column was then washed extensively with equilibrating buffer \([10 \text{mM HEPES (pH 7.2)}; 0.15 \text{M NaCl}; 0.1\% \text{NP-40}]\) and eluted at room temperature into 0.5-ml fractions with 0.1 M \( \alpha \)-methylmannoside diluted in the wash buffer. The starting membrane material (MemB), the column flow through, and eluted fractions were analyzed by Western blot under nonreducing conditions as described in Fig. 6. The 323/A3 antigen was bound to the resin. Release of the 323/A3 antigen was obtained by elution with \( \alpha \)-methylmannoside as indicated by the immunoreactive bands in Fractions 4 and 5. The 323/A3 antigen
plasma membranes (of either MCF-7 cells or tumors) suggests that the higher molecular weight band detected in cell lysates may be an intracellular precursor of the M, 43,000 antigen.

**DISCUSSION**

We have described an antigen which is expressed predominantly on breast cancer and is not found by the methods used in this study, on most normal tissues examined including normal breast. The 323/A3 MAb used to detect the antigen was generated by immunization of mice with MCF-7 human breast cancer cells. 323/A3 is directed against a M, 43,000 protein which appears to be the same protein in breast tumors in vivo as in cell lines. The antigen was demonstrated to be a glycoprotein by its ability to bind to and elute from Con A columns with specific sugars and by MAb immunoprecipitation of a radiolabeled M, 43,000 protein from MCF-7 cells after pulse labeling with [3H]-glucosamine. As yet, we do not know if the determinants recognized by the MAb are carried on the carbohydrate or the polypeptide portion of the antigen. Preliminary tunicamycin inhibition experiments indicate that the MAb binds with protein and not with carbohydrate residues (not shown). We know that the M, 43,000 antigen is stable to denaturation with detergents (ionic and nonionic) and heating to 100°C, suggesting that antigenic determinants are contained in a linear sequence of amino acids. Antigenic sites, however, are sensitive to reducing agents such as β-mercaptoethanol. This mechanism of sensitivity to reduction is not known. After immunoprecipitation of radiolabeled antigen in solution, we observe only a single M, 43,000 band on either nonreducing or nonreducing SDS: gels. Thus, epitopes for 323/A3 would appear not to span different polypeptide chains of a subunit protein but are likely to be localized within (or next to) a linear sequence containing an intrachain disulfide bond.

In MCF-7 cell cultures, the 323/A3 antigen appears, at least in part, to be surface membrane localized and to be a minor component of the cell surface. Surface binding of the 323/A3 MAb was demonstrated in this study by live-cell immunofluorescence binding assays with MCF-7. Also supportive of surface membrane localization is the subcellular distribution of antigen. On subfractionation of MCF-7 cells, we find some M, 43,000 antigen in the soluble cytoplasm, but the bulk of antigenic material is contained in isolated plasma membrane fractions. Evidence that the 323/A3 antigen is present in MCF-7 in trace amounts is provided by Con A affinity chromatography of membrane extracts. Silver-stained SDS: gels of the proteins eluted from Con A columns revealed a minor stained band at M, 43,000 corresponding to the immunoreactive band which, by semiquantitative Western blot analysis, contained most of the starting 323/A3 antigen. In most of our experiments, isolated plasma membrane fractions were used in Western blot assays. Later experiments were repeated using detergent cell lysates of MCF-7, and these analyses revealed an additional immunoreactive protein of M, 70,000. Since we invariably detect a single M, 43,000 antigen in isolated plasma membranes, this suggests the M, 70,000 band may represent another form of the antigen or possibly an intracellular precursor. Further studies will be required to determine the possible precursor-product relationship between the M, 70,000 and 43,000 antigens.

Only a handful of MAbs have been produced by other investi-
TUMOR-ASSOCIATED BREAST CANCER ANTIGEN

The 323/A3 MAb is able to detect antigen in formalin-fixed paraffin sections. This has allowed us to obtain a greater variety and number of tissues for immunocytochemical screening (as routine pathology paraffin blocks) that might otherwise have been inaccessible in other forms (i.e., fresh frozen). We found after a rather extensive screening of tissue sections by immunohistochemistry that the 323/A3 MAb is not tumor specific but reacts with a very limited range of normal tissues and in tissue distribution based on immunocytochemistry studies with antibodies to CEA (38). Moreover, we were unable to block binding of 323/A3 MAb to MCF-7 cells with purified CEA. The 323/A3 MAb therefore appears to recognize a previously undescribed tumor-associated antigen and thus adds to the small repertoire of MAbs available for use in human breast disease in identifying those benign lesions which, based on morphological criterion, are believed to be at high risk to development of cancer. We observed that the highest frequency of 323/A3 staining of benign breast lesions was with areas of greatest epithelial cell hyperplasia and morphological dysplasia. Both prospective and retrospective studies are now being done to examine the relationship between 323/A3 antigen in benign breast disease and risk to develop cancer. The 323/A3 MAb may also prove useful in measurement of circulating antigen in patient serum as an early screening for breast cancer or in monitoring disease recurrence. Since the 323/A3 antigen is not detected in most normal tissues and appears to be, in part, surface membrane in location, it may be a useful marker in human serum of early stages of breast cancer. As yet, we do not know if antigen present in normal colon will complicate or preclude the development of a serum screening assay. Finally, the 323/A3 MAb may have some therapeutic usefulness. Since the antibody does not react with normal bone marrow, it may be effective as an antibody:toxin conjugate in eliminating breast cancer cells in vitro from bone marrow of breast cancer patients to be used for autologous marrow transplantation. As recently reported by LeMaistre et al. (39), 323/A3 MAb conjugated with the A-chain of ricin was found to be a very potent and highly selective cytotoxic agent for breast cancer cells in vitro.

Potential applications, however, must be considered in the context that the 323/A3 antigen is expressed in about 60% of breast tumors in vivo. Since this MAb by itself will not detect all breast tumors in vivo, it will be necessary to either develop additional MAbs with characteristics similar to 323/A3, but directed toward antigens on a different subset of tumors, or to combine the 323/A3 MAb with other currently available MAbs in order to achieve detection of 100% of breast tumors.

ACKNOWLEDGMENTS

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TUMOR-ASSOCIATED BREAST CANCER ANTIGEN

Note Added in Proof
Immunofluorescent staining of frozen sections of human tissues showed a somewhat wider range of 323/A3 MAb distribution on normal tissues than was observed in our studies using immunoperoxidase staining of formalin-fixed paraffin-embedded sections. The authors acknowledge Dr. Art Frankel and Dr. David Ring of Cetus Corporation for these unpublished observations with frozen sections.

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Monoclonal Antibody Identification and Characterization of a $M_r$ 43,000 Membrane Glycoprotein Associated with Human Breast Cancer

Dean P. Edwards, Kathleen T. Grzyb, Lynn G. Dressler, et al.


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