

Generation and Characterization of a High-affinity Monoclonal Antibody for MUC1 Measurement in Breast Cancer

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Breast mucin is secreted by breast tumor cells and serves as a marker for breast cancer. Thus, antibodies against breast mucin will be valuable in the development of immunotherapy and laboratory diagnostic tests. Monoclonal antibodies (MAbs) against breast cancer-associated antigen were generated and characterized. Balb/c mice were immunized with breast cancer-associated antigen CA15-3, and subsequently splenocytes from immunized mice were fused with myeloma cells. After fusion, culture supernatants from hybridomas surviving HAT medium were screened by enzyme-linked immunosorbent assay (ELISA). A total of eight hybridomas producing MAbs against breast cancer showed significant levels of antibody activity against CA15-3. Two selected stable hybridomas were adapted into CELLline CL 350 bioreactors, and the MAbs produced were characterized for their subclass, specificity, and affinity. The MAbs were of high specificity and affinity as shown by ELISA. The MAbs produced may represent a powerful tool and are considered promising reagents for use in diagnosis and detection of early stage of the disease.

Introduction

BREAST CANCER IS ONE of the most common cancers in women worldwide, with 3738 new cases reported in Malaysia in 2003.⁽¹⁾ Therefore, specific and sensitive methods for early diagnosis and therapy are necessary to detect, target, and treat breast cancer.

MUC1, a highly glycosylated transmembrane protein, also known as polymorphic epithelial mucin, episialin, DF3 antigen, epithelial membrane antigen, or CA15-3, is a member of the mucin family. It is normally present on the apical surface of most glandular epithelial-type cells, including cells in breasts, pancreas, salivary glands, and lungs.⁽²⁾ In contrast to healthy tissue, many adenocarcinomas, especially malignant breast tumors, overexpress MUC1 in the form of the underglycosylated molecule. It can be found on the entire cell surface and substantial amounts are shed into the circulation.^(3,4) This underglycosylated form of MUC1 makes MUC1 a unique candidate for therapy⁽⁵⁻⁷⁾ and for use as a tumor marker.⁽⁸⁻¹⁰⁾

A number of assays based on competitive or sandwich-type immunoassay have been employed to measure circulating levels of MUC1 protein. Being a product of MUC1, breast-associated antigen CA15-3 plays an important role as a serum tumor marker in the management of breast cancer patients.

Although multiple serum-based tumor markers have been described for breast cancer, not much work has been done in

Malaysia to develop locally produced MAbs for use in development of diagnostic assays for cancer management and therapy. In this study, we report the generation of murine MAbs against breast cancer antigen with the aim of producing MAbs for local diagnosis and therapy. We describe the generation, purification, and characterization of stable hybridomas secreting specific MAbs against CA15-3 and the scale-up production of these MAbs to laboratory level via an *in vitro* culture method. Due to their characteristics, these MAbs are promising reagents for use in the diagnosis and therapy of breast cancer.

Materials and Methods

Hybridoma cells secreting specific MAb against CA15-3 protein were generated by fusion of myeloma cells with splenocytes from mice immunized with CA15-3. The use of animals and immunization protocol were approved by the Animal Care and Use Committee of the Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia (FIS/06/04/2009/CSH[R]).

Immunization

Immunization was carried out based on standard protocols.⁽¹¹⁾ CA15-3 (Sigma-Aldrich, St Louis, MO) was dissolved in normal saline and emulsified with an equal volume of

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complete Freund's adjuvant. Five 6-week-old female Balb/c mice were each immunized by subcutaneous injection of 10 µg of CA15-3 in a volume of 200 µL. Booster injections with 10 µg/200 µL of CA15-3 similarly emulsified with incomplete Freund's adjuvant were given at 1-month intervals for at least 4 months. Blood was collected at intervals from the tail vein by cutting the tip of the tail for determination of serum antibody titers by indirect ELISA. When serum antibody titers had reached appropriate levels, mice were prepared for harvest of splenocytes. Three days prior to fusion, a final intraperitoneal injection comprising 10 µg CA 15-3 in 200 µL normal saline was given.

Hybridoma methodology

On the day of fusion, the selected mouse was sacrificed by cervical dislocation under aseptic conditions, the spleen was isolated, and splenocytes were harvested. Splenocytes were fused with P3X63.Ag8.653 myeloma cell line from American Type Collection Center (ATCC, Rockville, MD) at a ratio of 5:1 (splenocytes–myelomas) using the polyethylene glycol (Sigma) method.⁽¹¹⁾ Cells were then plated into four 96-well plates (NUNC, Rochester, NY) in Dulbecco's modified Eagle's medium (DMEM, Sigma) containing 15% heat-inactivated fetal bovine serum (FBS; Gibco, Carlsbad, CA), 1% penicillin-streptomycin (Gibco), and hypoxanthin-aminopterin-thymidine (HAT) (Gibco) for at least 7 days. When the cells had grown to 2/3 confluence in the wells (between days 8 and 10), culture supernatants were screened for levels of antibodies against CA15-3 using ELISA. Once a well tested strongly positive for CA15-3 (OD at least two times that of control wells), the hybridomas in that well were recloned and made monoclonal by the limiting dilution technique in medium containing hypoxanthin-thymidine (HT). The resulting secreting hybridomas were recloned three times to ensure monoclonality and to obtain stable cells.⁽¹²⁾ The hybridomas were stored in liquid nitrogen and expanded in culture flasks when needed.

Laboratory-scale production

The hybridoma cells designated D2 and F11 were expanded *in vitro* using CELLLine CL 350 bioreactors (Integra Biosciences, Wallisellen, Switzerland). Cells were first cultured in culture flasks in DMEM with 10% FBS, antibiotics, and L-glutamine. Hybridoma cells from culture flasks in log growth phase were pooled into a centrifuge tube and centrifuged at 180 g. Cells were resuspended in growth medium (DMEM with 15% FBS, antibiotics, and L-glutamine) to arrive at a concentration of 1.5×10^6 viable cells/mL. Five mL of this cell suspension (totaling about 8×10^6 cells) were inoculated into each cell compartment. DMEM without serum was supplied to the nutrient medium compartment and the devices were placed in a CO₂ tissue culture incubator under 37°C and 5% CO₂. The cultures were harvested every 5 days and maintained for up to 45 days. At each harvest, a small volume of cells was taken and total, viable, and dead cells were counted using the Trypan Blue exclusion method.⁽¹³⁾ The cells were separated from the medium by centrifugation at 180 g. Cells were split appropriately based on the results of the total and viable cell counts. Fresh medium was added to the cells to achieve $0.8\text{--}1 \times 10^7$ viable cells/mL, and 5 mL of this cell suspension were reinoculated into the cell compart-

ment of the bioreactor. The harvested supernatants were centrifuged for 5 min at 180 g to separate cell debris from medium. Antibody concentration in the medium was measured by ELISA and production rate calculated. The medium was stored at –20°C until purification.

Antibody purification

A crude antibody preparation was obtained by precipitation of protein from the culture supernatant through the slow addition of an equal volume of saturated ammonium sulphate solution. The mixture was centrifuged at 1620 g for 30 min, the supernatant discarded, and the resulting pellet dissolved with 1–2 mL of 0.02 M phosphate buffer (pH 7.4). The dissolved pellet was dialyzed against 0.02 M phosphate buffer (pH 7.4) with three changes of buffer. IgG was then purified using HiTrap Protein-G HP column (GE Healthcare, Bucks, United Kingdom). Briefly, an equal volume of 0.02 M sodium phosphate buffer (pH 7.4) was added to the dialysate, loaded onto the equilibrated protein G column, and washed with 0.02 M sodium phosphate buffer. The bound IgG was then eluted with 0.1 M glycine-HCl buffer (pH 2.7) and collected as fractions in test tubes (1 mL eluate per tube) each containing 50 µL of neutralizing buffer, 1 M Tris-HCl (pH 9.0). The purified MAb was then desalted by dialysis against 0.02 M phosphate buffer (pH 7.4) with three changes of buffer and later stored at 4°C or –20°C. The concentration of purified MAb (protein) was determined by Protein Bradford Assay (Bio-Rad, Hercules, CA) using bovine serum albumin (BSA, Bio-Rad) as the protein standard.

Isotyping of monoclonal antibodies

The MAbs against CA15-3 that were produced were isotyped using monoAb IDKit (HRP) from Zymed Laboratories (South San Francisco, CA), according to the manufacturer's instructions.

Selection of MAb positive wells by non-competitive ELISA

During culture, the presence of MAbs against CA15-3 in culture supernatant was detected by a non-competitive ELISA method. ELISAs were carried out in 96-well microtiter Maxisorb plates (NUNC, Rochester, NY) coated with CA15-3 antigen. Coating was done by the addition of 100 µL of CA15-3 antigen (0.5 µg/mL) in coating buffer (0.01 M sodium carbonate buffer, pH 9.6) into wells incubating overnight at 4°C. After washing three times with washing buffer (0.02 M phosphate buffer containing 0.05 % Tween-20 [pH 7.4]), wells were blocked by incubation with 100 µL of 0.2% BSA in 0.02 M phosphate buffer for 1 h at 37°C. The wells were then washed three times with washing buffer. To perform the assay, 100 µL of supernatant from hybridoma cell cultures were added to each well and incubated at 37°C for 2 h. The wells were washed with washing buffer. Then, 100 µL of HRP-conjugated goat anti-mouse IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD) at 1:2000 dilution were added. After incubation at 37°C for 1 h, the wells were washed five times with washing buffer. One hundred µL of 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate system (Sigma) were added and incubated for another 5 min at room temperature. The reaction was stopped with 50 µL 1 M H₂SO₄,

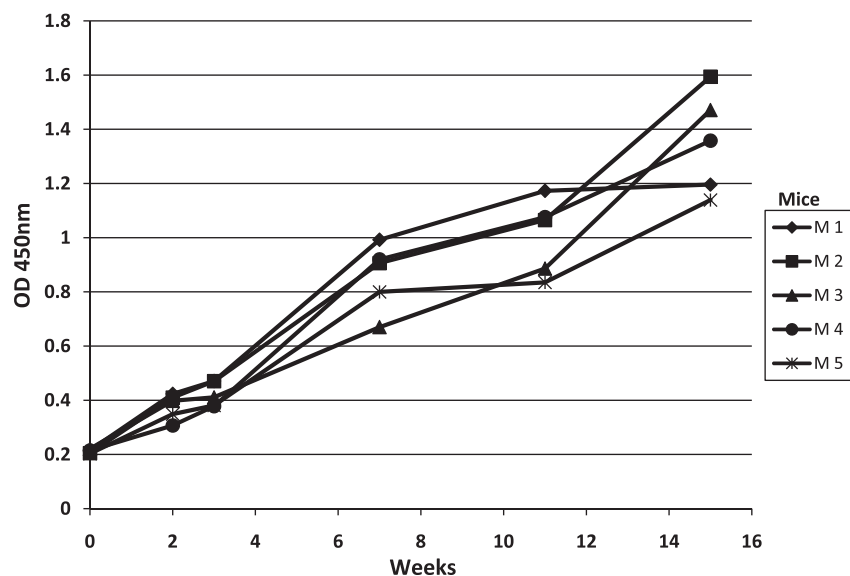


FIG. 1. Levels of serum IgG in mice immunized with CA15-3 by direct immunoassay. Serum was prepared from blood collected from tail vein. The antibody titers increased over time, indicating good immune response.

and the optical density was measured at 450 nm using a micro plate reader (Anthos, Eugendorf, Austria).

Affinity and cross-reactivity studies using competitive ELISAS

The affinity and specificity studies were conducted using competitive ELISA procedures. Supernatant containing MAbs D2 and F11 were first diluted with 0.02 M phosphate buffer (pH 7.4) to concentrations yielding 50% of maximum binding as determined by the non-competitive ELISA procedure described above.

Affinity determination

One hundred μ L of the appropriately diluted supernatants of MAbs D2 and F11 were mixed with 100 μ L of a serial di-

lution of soluble CA15-3 antigen in the range of 1–12 μ g/mL in marked 1.5 mL eppendorf tubes and incubated overnight at 4°C. Next, 50 μ L of each mixture were applied to wells of CA15-3-coated ELISA plates and incubated at 37°C for 2 h. The wells were then washed five times with washing buffer, and the bound antibodies in the wells were quantified using HRP-conjugated goat anti-mouse IgG and TMB. Measurement of the resulting OD was done as described above. The affinities were calculated using the method proposed by Freguet and colleagues.⁽¹⁴⁾

Cross-reactivity analysis

The specificities of MAbs D2 and F11 were determined by competitive ELISA using different tumor markers, PSA, Cyfra, CA 72-4, CA 19-9, CA 125, and CA 15-3, as binding

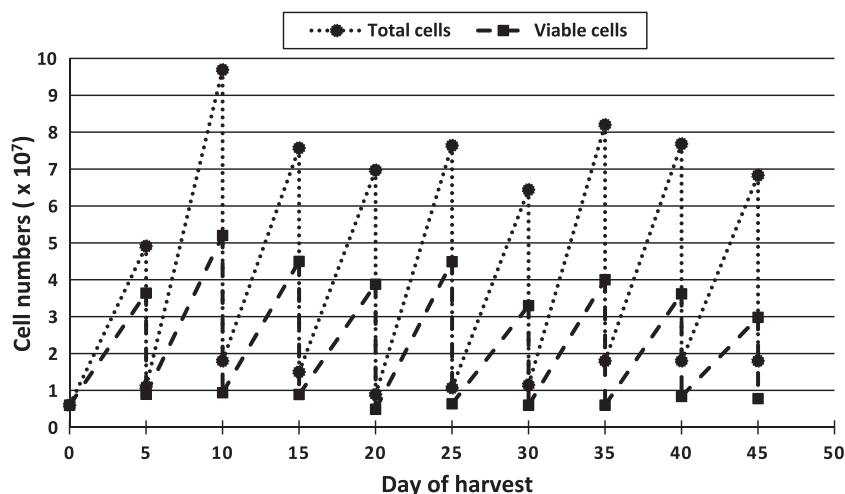


FIG. 2. Growth curve of D2 hybridoma cells in CELLLine CL 350 showing concentration of viable and total cell during culture. The dotted line shows total cells at harvest (higher number) and total cells reinoculated (lower number) while the dashed line shows viable cells at harvest (higher number) and viable cells reinoculated (lower number).

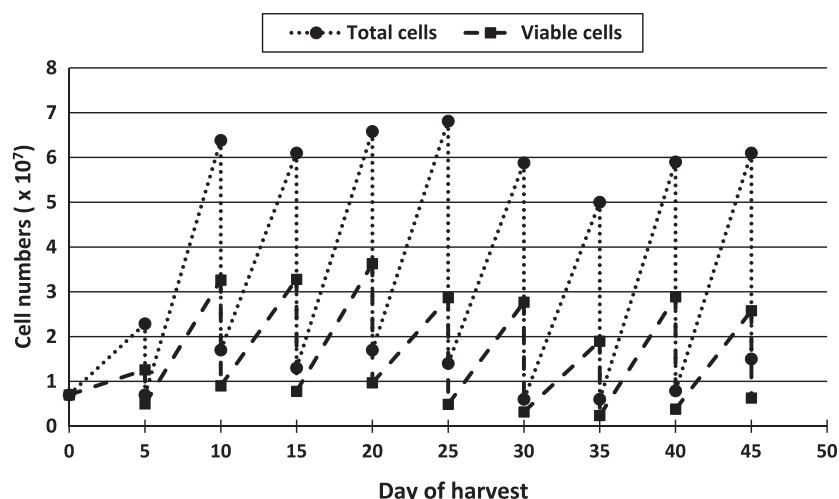


FIG. 3. Growth curve of F11 hybridoma cells in CELLLine CL 350 showing concentrations of viable and total cells during culture. Similar to Figure 2, the dotted line shows total cells at harvest (higher number) and total cells reinoculated (lower number) while the dashed line shows viable cells at harvest (higher number) and viable cells reinoculated (lower number).

competitors (Meridien Life Science, Cincinnati, OH). Fifty μL of various concentrations (20–2000 ng/mL) of tumor markers were incubated with 50 μL of supernatants of MABs D2 and F11 (diluted to yield 50% of maximum binding) for 2 h at 37°C. The assay procedure followed the direct ELISA method as described for the affinity studies. Standard curves were normalized by expressing experimental absorbance values (B), as $(B/B_0) \times 100$, where B_0 is the absorbance value at zero tumor antigen concentration. The cross-reactivity was evaluated as the percentage of B/B_0 versus concentration of tumor markers.

SDS-PAGE analysis

The protein profile of the supernatants (unpurified MAB) and purified MAB were monitored by SDS-PAGE analysis performed under reducing conditions (5 min of boiling in samples buffer with 2-mercaptoethanol). Five μL of each purified sample D2 and F11 were diluted 1:20 and 1:10, respectively, with deionized water before they were loaded on to a precast gel (4% stacking gel, 12% separating gel) and run with

Broad Range Prestained SDS-PAGE molecular weight standards from Bio-Rad Laboratories as molecular weight markers. Gels were stained with Coomassie G 250 and visualized by Quantity One 1D analysis software (Bio-Rad Laboratories).

Determination of cell-binding ability

The ability of MABs to bind to breast cancer cell lines was tested by ELISA methods. Breast cancer cell lines MCF-7, MDA 231, ZR75-1, and T 47-D and control Chang liver and rat adipose cells (3T3 F442A) were used. Cells were removed from liquid nitrogen storage, thawed, and expanded in culture flasks in RPMI or DMEM supplemented with 10% FBS, 1% antibiotic (streptomycin-penicillin), and 1% Glutamax (Gibco) in a humidified 5% CO_2 , 37°C incubator using standard cell culture procedures. After harvest, 100 μL of cells at a concentration of 2×10^5 cells/mL in the appropriate media were added to each well of sterile 96-well culture plates (Sigma). The cells were grown until confluent. The media were then decanted and cells were fixed in methanol (200 μL) for 10 min at room temperature. The wells were washed with 0.02 M phosphate buffer containing 0.05% Tween-20 and blocked with 100 μL 0.2% BSA in 0.02 M phosphate buffer for 1 h at room temperature. Purified MABs at concentrations that gave readings of approximately 2 $\times$ absorbance value of blanks were chosen as working concentrations. One hundred μL of each appropriately diluted purified antibody solution were added into wells of a 96-well plate in triplicate and incubated for 1 h at 0°C. One hundred μL of 0.02 M phosphate buffer were also added to dedicated wells in triplicate to act as controls and to determine non-specific binding (NSB). Following washing, the MABs that were bound to the cells were quantified using HRP-conjugated anti-IgG and TMB, as described in the ELISA methods.

Results

Immunization

Serum IgG titers increased in all five mice that were immunized indicating a positive response to CA 15-3 challenge

TABLE 1. SUMMARY OF CROSS-REACTIVITY STUDIES BETWEEN MABS F11 AND D2 AGAINST DIFFERENT TUMOR MARKERS

Tumor markers	% Cross reaction	
	F11	D2
CA 125	< 1	< 1
Cyfra	< 1	< 1
PSA	< 1	< 1
Ca 72-4	< 1	< 1
CA 19-9	< 1	< 1
CA 15-3	100	100

Monoclonal antibody at 50% displacement were incubated with antigens as indicated and binding of mAbs were detected with HRP-conjugated goat anti-mouse IgG and detected by ELISA using TMB as substrate. Cross-reactivity with CA 15-3 at 50% displacement was measured as 100%.

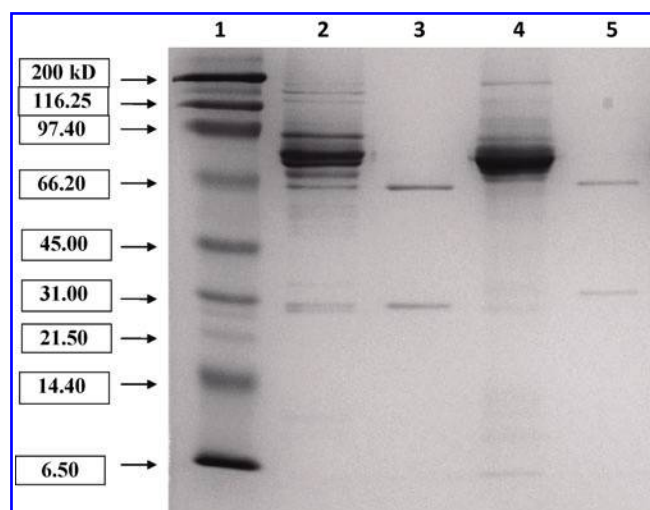


FIG. 4. SDS-PAGE with Coomassie Blue staining of unpurified and purified monoclonal antibodies from D2 and F11. Lane 1, molecular weight standards; lane 2, unpurified MAb D2; lane 3, purified MAb D2; lane 4, unpurified MAb F11; lane 5, purified MAb F11.

(Fig. 1). Serum titers were judged to have achieved high enough levels for splenocyte harvest by 12–16 weeks after initial immunization.

Generation of hybridomas secreting antibodies against CA15-3

From the 5 fusions carried out, growing colonies were found in more than 700 wells when they were screened by indirect ELISA. Only 37 growing colonies showed OD values of higher than 1, indicating a strong probability that they produced MAb that bound to the coated CA15-3 in the wells. After several rounds of recloning eight stable clones were obtained. Two of the stable clones, designated D2 and F11,

were used for initial laboratory-scale production in CELLLine CL350 bioreactors.

Scaling up production in CELLLine CL 350 bioreactors

Both hybridomas D2 and F11 were successfully propagated in CELLLine CL 350 bioreactors. Cells proliferated throughout the interval between harvests as indicated by the accumulation of total cells (Figs. 2 and 3). Viable cells at harvest were about 40–50% of total from the second harvest onwards. The total purified MAb antibodies collected after the 45-day run were 39.25 mg and 18.125 mg from D2 and F11, respectively.

Characterization of MABs

Isotyping of MABs. Both MABs were of IgG1 subtype with light chain belonging to the kappa isotype.

Affinity. The affinity constants (K_a) of D2 and F11 were 2.7×10^{-8} and 1.33×10^{-8} M, respectively.

Specificity. The MABs from D2 and F11 were highly specific for CA15-3, with <1% cross-reactivity with the other tumor markers examined (Table 1).

SDS-PAGE analysis

The purified MABs of D2 and F11 migrated at molecular masses of 55 kDa and 29 kDa, representing the IgG1/k isotype heavy chain and light chain, respectively (Fig. 4).

Reactivity of MABs to cells in vitro

Results show that the OD values in the wells containing breast cancer cell lines, MCF-7, MDA 231, ZR75-1, and T 47-D, were significantly higher in wells where cells had been exposed to the anti-CA15-3 MABs D2 and F11 as opposed to those that were not. The OD values in the latter were considered to be non-specific binding or NSB (Figs. 5, 6). The excess binding over NSB was presumably due to the specific

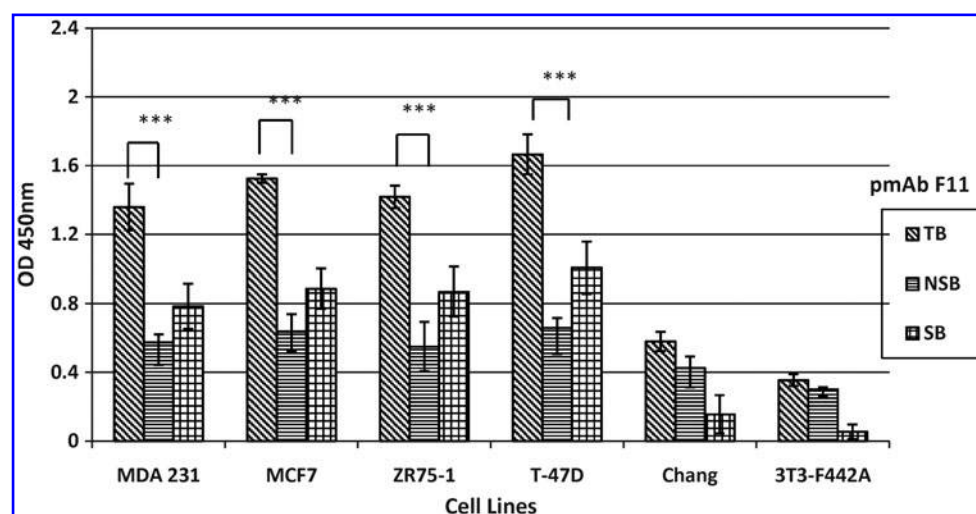


FIG. 5. Binding patterns of purified MAb F11 on breast cancer cell lines (MCF-7, MDA 231, ZR75-1, and T-47D) and control cells (Chang and 3T3-F442A). Reactivity of MAb F11 with these cell lines was determined by indirect ELISA. TB, total binding; NBS, non-specific binding PBS (0.1% BSA); SB, specific binding. The values were represented as mean $\pm$ SD of four replicates. *** p < 0.001 (TB vs. NSB).

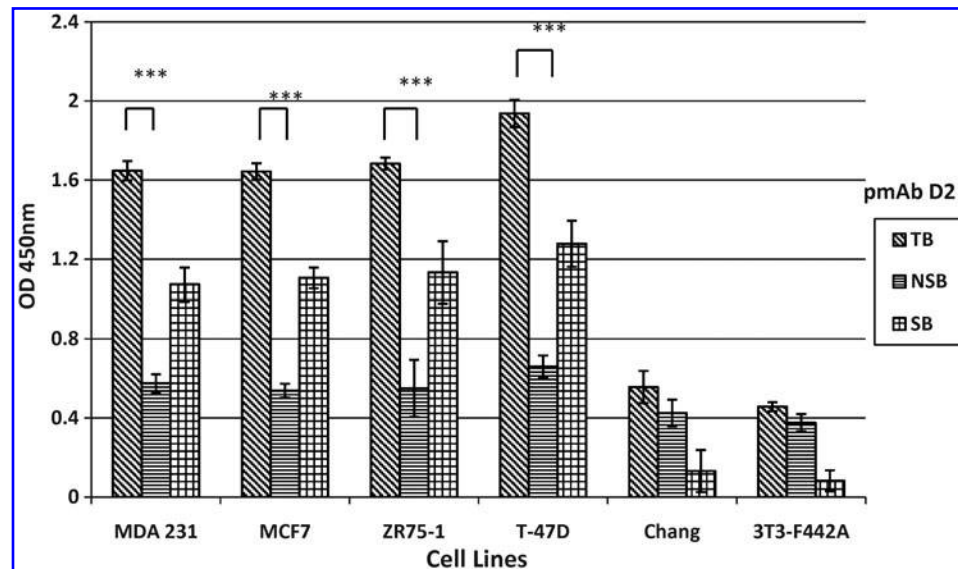


FIG. 6. Binding patterns of purified MAb D2 on breast cancer cell lines (MCF-7, MDA 231, ZR75-1, and T-47D) and control cells (Chang and 3T3-F442A). Reactivity of MAb D2 with these cell lines was determined by indirect ELISA. TB, total binding; NBS, non-specific binding PBS (0.1% BSA); SB, specific binding. The values were represented as mean $\pm$ SD of four replicates. *** $p < 0.001$ (TB vs. NSB).

binding of MAbs to the MUC1 antigens on the breast cancer cell lines. There were lower degrees of total binding and specific binding in control Chang cells and little apparent specific binding in fat cells.

Discussion

MUC1, a large molecular weight glycoprotein, is a member of the mucin family that is normally present on the luminal surface of secretory glands. However in many adenocarcinomas, MUC1 is aberrantly expressed in the hypoglycosylated form⁽¹⁵⁾ and is shed into the circulation in breast cancer patients. The presence of the soluble form of MUC1 (CA15-3, CA27.29 antigen) in serum correlates with the metastasis of mammary carcinoma.^(16,17) Therefore, soluble MUC1 is a well-known marker for diagnosis of breast cancer.

The clinical utility of measurement CA15-3 in monitoring the response to therapy and for early detection of recurrence of metastatic breast disease has been described.^(18,19) Studies have shown that different antibodies used to detect the MUC1 mucin differ in their affinity and epitope specificity for the mucin. Therefore we have undertaken to generate MAbs with high specificity and affinity against CA15-3 with the aim of developing applications for local use in patients and for research purposes.

In this study, we successfully developed eight stable hybridomas expressing MAbs against CA15-3. Only two clones are reported in this study. The clones D2 and F11 are stable, grow in tissue culture flasks through repeated passages, and can be scaled up in CELLline CL 350 bioreactors.

The MAbs D2 and F11 were shown to bind to CA15-3 with high specificity and affinity. It also bound highly to breast cancer cells and less to control Chang cells and fat cells. It is not surprising that there is some binding to Chang cells as MUC1 is expressed in many cells. However, the high degree of binding in the breast cancer cell lines showed that these MAbs can detect overexpression of MUC1 in these cells.

Therefore D2 and F11 MAb can be useful for specific detection of breast cancer. Further research is being conducted to develop applications using these MAbs.

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Author Disclosure Statement

The authors have no financial conflicts to disclose.

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