Antibodies to a Surface Membrane Marker from Human Mammary Carcinoma Cell Line

Mikio Kamiyama, George A. Hashim, Ali Abdelaal, and Lorenzo Araujo

The Jane Forbes Clark Surgical Research Laboratories, Departments of Surgery and Microbiology, St. Luke's-Roosevelt Hospital Center and Columbia University, New York, New York 10025

ABSTRACT

A tumor surface antigen (BTA-BT20-68K) was isolated from a human mammary carcinoma cell line (BT-20). The antigen (Mr, 68,000) induced the formation of high titer antibodies which recognized BTA-BT20-68K as a cell surface marker by the immune adherence hemagglutination test and recognized the soluble antigen by solid phase radioimmunoassay. The antibodies which failed to recognize human β-2-microglobulin, α-fetoprotein, and carcinoembryonic antigen were cytotoxic to the parent BT-20 tumor cells at high serum dilutions. The antibodies recognized a similar tumor surface marker isolated directly from human breast adenocarcinomas, but failed to recognize human lymphocyte antigens isolated from BT-20 cells or bound to human lymphocytes bearing human lymphocyte antigen markers in common with those of BT-20 cells. Added to BT-20 tumor cells in culture and in the absence of complement, antibody-dose-related inhibition of tumor cell growth was documented. In the presence of complement, the antibodies were highly cytotoxic to the parent cells. These results demonstrate the presence of a unique tumor surface marker with chemical and immunological properties in common with that isolated directly from human breast adenocarcinomas.

INTRODUCTION

Tumor-associated antigens are potential cell surface markers for several kinds of malignancies. Difficulties are often encountered by the limited amount of tumor tissue when one tries to isolate, characterize, and document the properties of a particular antigen from a particular tumor type. Studies from our laboratory have focused upon surface antigens from mammary tumors obtained from single patients. Realizing the limitation incurred in doing work with a limited amount of tissue from a single patient (1), and to insure a continuous supply of antigenic material, we turned our attention to tumor cell lines in culture (2). In both of these studies, we noted the presence of a BTA antigen with a molecular weight around 68,000, found in both human mammary carcinoma and in the SW527 mammary tumor cell line. The Mr, 68,000 antigen isolated from human mammary carcinoma could not be distinguished electrophoretically on SDS-PAGE or immunologically using specific antibodies, from the Mr, 68,000 antigen isolated from the SW527 tumor cell line (2). Antibodies prepared against BTA from human tissue recognized BTA isolated from SW527. Conversely, antibodies prepared against BTA from SW527 cells recognized BTA prepared from freshly isolated human mammary carcinoma tissues.

In this report, we extend our studies to yet another human breast tumor cell line, BT-20 [HTB-19; American Type Culture Collection (ATCC), Rockville, MD] and show that M, BTA-BT20-68K present in BT-20 tumor cells grown in culture, has chemical and immunological properties similar to BTA (BTA-Hu-68K) isolated directly from mammary carcinomas obtained from single patient tissue sources. The study further shows that antibodies prepared against purified BTA-BT20-68K are complement fixing, cytoxic to BT-20 tumor cells, and in the absence of complement, inhibit the growth of BT-20 tumor cells in culture.

MATERIALS AND METHODS

Isolation of Tumor-associated Antigens. Breast tumor antigens used in this study were isolated either from the BT-20 tumor cell line maintained in culture in our laboratory or from human tumor tissue following radical mastectomy of invasive ductal carcinoma with a number of positive axillary lymph nodes. The frozen human tumor tissue dispersed by sonication or washed cultured BT-20 cells were extracted with detergent and processed as described earlier (1, 2). Briefly, membrane-bound BTA was solubilized during 15 min of extraction with 0.5% Nonidet P-40 in PBS at room temperature. Repeated extractions even with 1% Nonidet P-40 in PBS did not release any additional antigenic material. The extracts were then filtered on Bio-Gel A-50 columns. The elution pattern showed two well-separated peaks (1, 2). The first peak, Bio-Gel A-50, contained the BTA. This peak was isolated, concentrated by pressure filtration, and lyophilized. The lyophilized material was used for further purification on preparative 7.5% PAG (1–3). Tumor antigens from either of the above-mentioned sources were purified to homogeneity by PAGE according to the procedure described by Davis (3). Briefly, tumor cells or tumor tissue extracts were electrophoresed on PAG without SDS. The desired antigens were located after staining representative gels with Coomassie brilliant blue. Slices of the gels containing the desired bands were homogenized with a Polytron homogenizer (Brinkman Instruments, Westbury, NY) in 0.02 M Tris-HCl, pH 8.6, eluted and concentrated by microconcentrator (Centricon 30; Amicon Corp., Danvers, MA) and lyophilized.

Molecular Weight Determination. Molecular weights of purified antigens were estimated by SDS-PAGE according to the method of Weber and Osborn (4) using a mixture of standard proteins with molecular weights ranging from 14,400 to 94,000 (Pharmacia Fine Chemicals, Inc., Piscataway, NJ).

Preparation of Anti-BTA Antibodies. Antibodies were prepared either in New Zealand white rabbits or in strain 13 guinea pigs.

Antibodies against either of the two BT25 isolated from fresh frozen human breast adenocarcinoma tissue were prepared in rabbits immunized with 200 µg purified antigen emulsified with complete Freund's adjuvant containing 0.5 mg M. butyricum and injected s.c. The immunized rabbits were boosted every week for 4 weeks with 100 µg antigen emulsified with incomplete Freund's adjuvant and injected s.c. on the flank. The immunized rabbits were bled every week.

Antibodies against BTA isolated from cultured BT-20 human tumor cells were similarly prepared in strain 13 guinea pigs. Guinea pigs were immunized with 0.2 ml emulsion containing 100 µg BTA and 0.25 mg M. butyricum in Freund's complete adjuvant. Each guinea pig was boosted with the same emulsion every week for 3 weeks and then terminated. The serum from individual animals was isolated and stored at −80°C in small aliquots.

Antibody Assays. The development of antibodies against BTA was...
cells were counted under the microscope using a hemocytometer. For the measurement of antibody reactivity to crude and purified antigens, the immune serum was first decomplexed at 56°C for 30 min and stored in small volumes at -80°C. Serial dilutions of each serum (25 μl) were made in microplates using veronal buffer containing 1% gelatin at pH 7.5 (GVB). The antigen (4.0 μg protein) was then added to each well and mixed. The plates were incubated for 60 min at 37°C. Guinea pig complement (2.5 μl of 1:80 dilution) was added, mixed on a microplate mixer, and incubated for an additional 40 min at 37°C. At this time, 25 μl of 0.3% dithiothreitol solution in 0.04 M EDTA-GVB buffer was added, mixed, and followed by the addition of 25 μl of human type O erythrocytes (1.2 × 10⁸ cells/ml suspended in 0.04 M EDTA-GVB). The reaction mixture was then centrifuged at 4°C and the supernatant was applied to a Sephadex G10 column. Fractions of 0.5 ml were collected. The radioactivity bound to the protein antigen which eluted at the void volume of the column, was collected and used for further studies.

Antibodies. To this end, the purified antigens (BTA) from either source were labeled with radioiodine (New England Nuclear, Boston, MA). In a glass centrifuge tube, 10 μg of protein antigen were incubated with 50 μl of 0.217 M phosphate buffer, pH 7.2, to which was added 0.5 mg of enzymobead reagent (25 μl) (Bio-Rad Labs, Richmond, CA), 50 μl of Na iodine-125 and 25 μl of 1% glucose, mixed and incubated for 25 min at room temperature. The reaction mixture was then centrifuged at 4°C and the supernatant was applied to a Sephadex G10 column (0.8 x 18 cm) equilibrated and eluted with VBS. Fractions of 0.5 ml were collected. The radioactivity bound to the protein antigen which eluted at the void volume of the column, was collected and used for further studies.

To 100 μl of diluted antiserum were added 125I-labeled BTA (50 μl) and 100 μl VBS containing 0-10 μg unlabeled antigen. The mixture was then incubated at 37°C for 2 h. After the incubation, 25 μl of the incubated mixture was removed and added to 150 μl of goat anti-rabbit or rabbit anti-guinea pig immunoglobulin-coated immunobeads. After incubation with the second antibody for an additional 2 h, the immunobeads were removed by centrifugation at 1000 × g for 5 min, washed three times with VBS and the radioactivity bound to the beads was determined in a Beckman gamma counter.

Complement-mediated Cytotoxicity Test. The cytotoxicity test was carried out according to the procedure originally described by Gorser and O’Gorman (6) and modified by Terasaki and McClelland (7). Suspension of cells, 5 × 10⁶ cells/ml, were prepared together with 5 μl of antibody dilutions in microtiter plates and incubated at room temperature for 60 min. The cells were washed three times with McCoy's medium and resuspended in 10 μl of rabbit complement, diluted 1:5 in McCoy's medium and the incubation continued for an additional 40 min. At this time, the cells were washed three times in McCoy's medium, 10 μl of 0.4% Trypan blue dye was added, and the percentage of viable cells were counted under the microscope using a hemocytometer.

BT-20 Cell Culture. Detailed characteristics of the BT-20 cell lines and culture conditions were described by others (8). For the purpose of this study, cells were properly thawed and washed. They were then placed in Falcon flasks (no. 3032) and cultured in the presence of Eagle's minimum essential medium supplemented with nonessential amino acids (Eagle's BSS) and 5% fetal bovine serum. The culture medium was changed two to three times weekly. When the monolayer of cells covered about 80% of the bottom of the flasks, they were released from the flask with 0.25% trypsin, washed, and harvested. The monolayer of cells which covers 80% of the bottom of the culture flask contained approximately 8.75 × 10⁶ cells by actual cell count in the hemocytometer. The number of viable cells reported in this study are the percentages of the ratio of actual cell count divided by 8.75 × 10⁶ cells. All cultures were carried out in duplicates.

RESULTS

BTA from Human Tumor Tissue and from BT-20 Cultured Cell

Analytical polyacrylamide disc gel electrophoresis of the human breast tumor tissue extracts showed two well-separated protein bands with characteristic slow migration toward the anode. These were designated BTA-Hu-68K and BTA-Hu-43K, respectively. The purified BTA-Hu-68K or the BTA-Hu-43K migrated as a single homogenous band when reelectrophoresed on SDS-PAGE and localized in the region of M, 68,000 and 43,000, respectively (Fig. 1). The SDS-PAGE patterns for extracts from BT-20 breast tumor cells grown in our tissue culture laboratory showed a single protein band in the region of M, 68,000 which was designated BTA-BT20-68K (Fig. 1). The absence from the BT-20 cell extracts of BTA-BT20-43K was clearly noted. Staining of BTA-BT20-68K, BTA-Hu-68K, and BTA-Hu-43K by the method of Zacharius et al. (9) suggested the presence of carbohydrates; however, analysis of purified BTA-BT20-68K by
gas chromatography following hydrolysis in 4 N HCl, failed to reveal the presence of any carbohydrate components.

Recovery of BTA

Approximately 15 mg of pure BTA-BT20-68K, estimated by the Lowry et al. (10) method, were obtained from a batch of 1 x 10^6 cultured BT-20 cells. Smaller amounts of pure antigen were isolated from frozen human breast tumor tissues. Although the tumors obtained from both patients were rather large, about 2 mg BTA-Hu-68K and 1 mg BTA-Hu-43K were isolated from 50-g samples of crude tissues.

Immunological Properties of BTA

Preparation of Anti-BTA Antibodies. Both BTA-Hu-68K and BTA-Hu-43K isolated from either of the two patients were antigenic and induced antibody formation in rabbits. Moderate antibody titers were obtained for each of the two antigens. Using the IAHA test, reciprocal serum titers of 2000 and 1000 were obtained for 250 ng of BTA-Hu-68K and BTA-Hu-43K, respectively. Similarly, anti-BTA-BT20-68K antibodies, prepared in guinea pigs, gave a reciprocal antibody titer of over 4000 for 250 ng of BTA-BT20-68K. Further, decomplemented preimmune sera from either rabbits or guinea pigs did not show any reactivity by the IAHA test even at 1:2 dilutions.

Immunological Reactivity. Immunological reactivity and cross-reactivity of antibodies are shown in Table 1. Using the IAHA test, complete recognition of BTA-BT20-68K and BTA-Hu-68K by their respective antibodies was observed. In contrast, neither of the three antigens nor BTA-Hu-43K isolated from the two patients was recognized by antibodies prepared against carcinoembryonic antigen, α-fp, or B2m.

The lack of reactivity of anti-BTA antibodies with purified HLA is clearly noted (Table 1) and the reactivity of the crude extracts with anti-B2m confirms the presence of HLA as well as B2m in extracts from patient’s tissues and from BT-20 tumor cells. Also, the results presented in Table 1 show the presence of the M, 68,000 and 43,000 antigens in extracts from patient’s tissues, in contrast to only one (M, 68,000) which was isolated from BT-20 cells. Antibodies prepared against BTA-Hu-68K recognized not only the parent human M, 68,000 antigen but also the M, 68,000 antigen prepared from BT-20 cells. Further, anti-BTA-Hu-43K antibodies which recognized the purified M, 43,000 antigen failed to recognize the M, 68,000 antigen from either of the two sources.

Inhibition of 125I-BTA Binding to Antibodies by BTAs from Different Sources. Anti-BTA-BT20-68K and anti-BTA-Hu-68K antibodies recognized both BTA-BT20-68K and BTA-Hu-68K but not BTA-Hu-43K by the solid phase radioimmunoassay (Fig. 2). Similarly, anti-BTA-Hu-43K antibodies which recognized the immunizing antigen failed to react with BTA-Hu-68K or BTA-BT20-68K.

Using rabbit anti-guinea pig or goat anti-rabbit immunoglobulin-coated immunobeads, the percentage of 125I-BTA-BT20-68K bound to anti-BTA-BT20-68K antibodies decreased with increased amounts of unlabeled BTA-BTHu-68K added to the incubation mixture (Fig. 2). Similar results were obtained when unlabeled BTA-BT20-68K was added to anti-BTA-BT20-68K antibodies in the presence of constant amounts of labeled antigen (Fig. 2B). In Fig. 2C, the percentage of binding of 125I-BTA-Hu-68K to respective antibodies was decreased as a function of added unlabeled homologous antigen. The results presented in Fig. 2C further show that the addition of unlabeled BTA-Hu-68K did not alter recognition of BTA-Hu-43K by its antibodies nor did it compete with BTA-Hu-43K for the antibody binding sites. Similar results are presented in Fig. 2D. Antibodies prepared against BTA-Hu-43K recognized the immunizing antigen. The percentage of binding to anti-BTA-Hu-43K decreased as a function of added unlabeled BTA-Hu-43K.

In contrast, the percentage of binding of BTA-Hu-68K to its specific antibodies was unaffected when increasing concentrations of BTA-Hu-43K were added to the reaction mixture.

Cytotoxicity of Anti-BTA-BT20-68K Antibodies. Anti-BTA-BT20-68K antibodies, prepared in guinea pigs, were cytotoxic to viable BT-20 cells. Using the microcytotoxicity assay and in the presence of guinea pig complement (1:125 dilution), BT-20 cells were unable to exclude trypan blue dye during the 30 min of incubation at 37°C (Table 2). In contrast, the viability was unaffected when BT-20 cells were similarly incubated in the presence of decomplemented preimmune guinea pig sera and in the presence or absence of added complement.

The results shown in Table 2 further show that the anti-BTA-BT20-68K antibodies plus complement were not cytotoxic to either normal human lymphocytes or to human myeloma cells (SK007) by the complement-fixing microcytotoxicity assay. Viable human lymphocytes, bearing one or two HLA markers in common with those established in our laboratory for BT-20 cells, were tested against normal preimmune guinea pig serum, in the presence or absence of anti-BTA-BT20-68K antibodies.

### Table 1 Immunological reactivity and cross-reactivity of anti-BTA antibodies by the IAHA test

<table>
<thead>
<tr>
<th>Tumor source</th>
<th>Antigen used</th>
<th>Preimmune sera</th>
<th>BTA-BT20-68K</th>
<th>BTA-Hu-68K</th>
<th>BTA-Hu-43K</th>
<th>CEA*</th>
<th>α-fp</th>
<th>B2m</th>
</tr>
</thead>
<tbody>
<tr>
<td>BT-20</td>
<td>Crude extract</td>
<td>-</td>
<td>1:512</td>
<td>1:256</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>BTA-BT20-68K</td>
<td>-</td>
<td>1:4096</td>
<td>1:2048</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>HLA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1:8</td>
</tr>
<tr>
<td>Patient A</td>
<td>Crude extract</td>
<td>-</td>
<td>1:256</td>
<td>1:256</td>
<td>1:256</td>
<td>-</td>
<td>-</td>
<td>1:2</td>
</tr>
<tr>
<td></td>
<td>BTA-Hu-68K</td>
<td>-</td>
<td>1:2048</td>
<td>1:1024</td>
<td>1:2048</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>BTA-Hu-43K</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1:16</td>
</tr>
<tr>
<td>Patient B</td>
<td>Crude extract</td>
<td>-</td>
<td>1:256</td>
<td>1:256</td>
<td>1:256</td>
<td>1:4</td>
<td>1:2</td>
<td>1:2</td>
</tr>
<tr>
<td></td>
<td>BTA-Hu-68K</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>BTA-Hu-43K</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1:1024</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>HLA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1:8</td>
</tr>
</tbody>
</table>

*CEA, carcinoembryonic antigen.

*+, absence of reaction at 1:2 dilution of serum added to the listed antigens. In addition, immune serum which did not cross-react with other antigens served as second control serum. The amount of antigen or extract used was held constant at 250 ng protein.
with or without added complement. The results presented in Table 2 clearly show that the anti-BTA-BT20-68K antibodies were not cytotoxic to human lymphocytes even if their HLA phenotypes included either HLA-A2 or both HLA-A2 and HLA-A5. Both HLA-A2 and HLA-A5 are common to both the human lymphocytes used and the BT-20 tumor cells. The same results were obtained with SK007 human myeloma cells of unknown HLA phenotype. These cells were equally resistant to the complement-fixing and cytotoxic anti-BTA-BT20-68K antibodies.

Effect of Antisera upon BT-20 Cell Growth in Culture. The results presented in Fig. 3 show growth curves of BT-20 cells in the presence of either preimmune (control) or immunized sera and in the absence of complement.

In control cultures, to which control sera were added (Fig. 3), the number of BT-20 cells increased steadily during the 5 days of culture. At day 5, the monolayer of cells covered 80–90% of the flat bottom of the culture flasks and gave an average cell count of $8.75 \times 10^6$. Although maximum growth occurred on day 7, the choice of 5-day culture provided better cell counts and prevented cell-stacking due to overgrowth. In contrast, the addition at the start of the cultures of immunized sera prevented cell growth and proliferation (Fig. 3A). Immunized serum, diluted 1:1000, permitted growth of BT-20 cells but the number of cells on day 5 was significantly lower (65%) than that of control (Fig. 3A). At 1:500 and 1:250 dilutions, the number of cells was reduced to about 20 and 5%, respectively, and complete inhibition of cell growth was noted with 1:125 dilutions of anti-BTA-BT20-68K antibodies (Fig. 3A).

Effect of Immune Complexes

Neither cell adhesion nor cell proliferation was affected when BT-20 cells were cultured in the presence of immune complexes prepared by incubating anti-BT20-68K antisera with BTA-BT20-68K (Fig. 3B). BT-20 cells, incubated in the presence of immune complexes and under conditions of antigen excess (1:500 plus Ag), adhered to the flasks, divided, and the degree of cell division was similar to that obtained with cells that were incubated in the presence of preimmune serum. Similarly, in cultures which were incubated with immune complexes under conditions of antibody excess, cell adhesion was delayed and the extent of cell expansion was about 20% less than that of

Table 2 Cytotoxicity of anti-BTA-BT20-68K antibody to BT-20 cells, human lymphocytes, and myeloma cells

For controls, wells contained a mixture of cells plus antibodies without guinea pig complement. Additional control wells contained heat-inactivated preimmune serum (normal g. pig serum) in the presence of complement and in the absence of anti-BTA-BT20-68K antibodies.

<table>
<thead>
<tr>
<th>Target cells</th>
<th>HLA-type</th>
<th>Antiserum (1:60)</th>
<th>Complement (1:125)</th>
<th>Percentage of viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>BT20 tumor cells</td>
<td>A2</td>
<td>Normal g. pig serum</td>
<td>Absent</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>A9</td>
<td>Normal g. pig serum</td>
<td>Present</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>B5</td>
<td>Anti-BTA-BT20-68K</td>
<td>Absent</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-BTA-BT20-68K</td>
<td>Present</td>
<td>0</td>
</tr>
<tr>
<td>Normal human lymphocytes, donor no. 1</td>
<td>A2</td>
<td>Normal g. pig serum</td>
<td>Absent</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>A10</td>
<td>Normal g. pig serum</td>
<td>Present</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>B7</td>
<td>Anti-BTA-BT20-68K</td>
<td>Absent</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>B14</td>
<td>Anti-BTA-BT20-68K</td>
<td>Present</td>
<td>100</td>
</tr>
<tr>
<td>Normal human lymphocytes, donor no. 2</td>
<td>A2</td>
<td>Normal g. pig serum</td>
<td>Absent</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>A26</td>
<td>Normal g. pig serum</td>
<td>Present</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>B35</td>
<td>Anti-BTA-BT20-68K</td>
<td>Absent</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>CW3</td>
<td>Anti-BTA-BT20-68K</td>
<td>Present</td>
<td>100</td>
</tr>
<tr>
<td>Normal human lymphocytes, donor no. 3</td>
<td>A2</td>
<td>Normal g. pig serum</td>
<td>Absent</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>A31</td>
<td>Normal g. pig serum</td>
<td>Present</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>B5</td>
<td>Anti-BTA-BT20-68K</td>
<td>Absent</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>B40</td>
<td>Anti-BTA-BT20-68K</td>
<td>Present</td>
<td>100</td>
</tr>
<tr>
<td>Human myeloma cell line, SK007</td>
<td>Unknown</td>
<td>Normal g. pig serum</td>
<td>Absent</td>
<td>100</td>
</tr>
</tbody>
</table>

* HLA phenotype in common with BT-20 tumor cells.
TUMOR SURFACE MARKERS

Preimmune serum

Fig. 3. The growth of BT-20 breast tumor cells in culture is shown in the presence of preimmune or immune sera. In A, the cells were incubated with either preimmune serum or dilutions of anti-BTA-BT20-68K antiserum. In B, the anti-BTA-68K was preincubated either without or with excess antigen before the mixture was added to the cells. In C, the effect of anti-BTA-Hu-43K antiserum upon BT-20 growth in culture is compared to cells grown in the presence of either preimmune serum or anti-BTA-Hu-68K.

controls (Fig. 3B). In contrast, cells treated with antisera dilutions and incubated in the absence of added antigens did not adhere or grow (1:125). Reducing the concentration of antibodies by 4-fold (1:500) permitted some cells to adhere but the extent of cell division was limited to about 20% of controls during the 5-day culture period.

Effect of Antibodies Prepared against BTAs from Human Tumor Tissue

Antibodies against BTA-Hu-43K failed to inhibit either cell adhesion or to prevent cell division when BT-20 cells were cultured in the presence of 1:125 dilution of anti-BTA-Hu-43K antiserum (Fig. 3C). In the presence of the antibodies, BT-20 cells adhered to the flask within 2–3 h after plating. This was followed by cell division and expansion of the area covered by the monolayer of cells. In contrast, anti-BTA-Hu-68K antiserum (1:125 dilutions) inhibited both cell adhesion and expansion in a manner similar to those documented for anti-BTA-BT20-68K antiserum (Fig. 3, A and C).

Effect of Antibodies on the Growth of Adherent Cells

Anti-BTA-BT20-68K antiserum arrested BT-20 cell growth and division when the antiserum (1:125 dilution) was added after the BT20 cells were cultured for 24 h (data not shown). It should be noted that cells adhere to the flask within 2–3 h after plating. In the presence of the antibody, the cells remained adherent and viable as was shown by their ability to exclude trypan blue dye, but failed to continue to divide and the monolayer of cells remained within the arrested boundaries during the 5-day culture period. In this experimental design, removal of excess antibodies by washing (3×) the adherent cells with antibody-free medium, allowed cell growth and division and within 5 days of culture the monolayer of cells covered over 80% of the bottom of the flasks.

DISCUSSION

The study shows the presence of two distinct antigens with molecular weights of 68,000 and 43,000 in human breast cancer tissue. Neither of the two antigens was detected in normal human breast tissue, human red cells, or human lymphocytes; however, large amounts of the M, 68,000 antigen were found in breast tumor cells (BT-20 cell line) grown in culture. The study further shows the surface location of the M, 68,000 antigen and its chemical and immunological properties; the M, 68,000 antigen is a protein and a unique cell surface marker for breast tumor cells. The M, 68,000 and 43,000 antigens from either of the two sources stained red with basic fuchsin on SDS-PAGE. Staining with basic fuchsin was restricted to the M, 68,000 and 43,000 antigens from either of the two sources stained red with basic fuchsin on SDS-PAGE. Staining with basic fuchsin was restricted to the M, 68,000 and 43,000 antigens from either of the two sources stained red with basic fuchsin on SDS-PAGE. Staining with basic fuchsin was restricted to the M, 68,000 and 43,000 antigens from either of the two sources stained red with basic fuchsin on SDS-PAGE. Staining with basic fuchsin was restricted to the M, 68,000 and 43,000 antigens from either of the two sources stained red with basic fuchsin on SDS-PAGE. Staining with basic fuchsin was restricted to the M, 68,000 and 43,000 antigens from either of the two sources stained red with basic fuchsin on SDS-PAGE. Staining with basic fuchsin was restricted to the M, 68,000 and 43,000 antigens from either of the two sources stained red with basic fuchsin on SDS-PAGE. Staining with basic fuchsin was restricted to the M, 68,000 and 43,000 antigens from either of the two sources stained red with basic fuchsin on SDS-PAGE.

Factors responsible for staining with basic fuchsin were not investigated further and neither was a role ascribed to the carbohydrates in any of the documented properties of the M, 68,000 and 43,000 antigens.

Complete recognition by the anti-M, 68,000 antibodies of the M, 68,000 antigen from either of the two sources suggested that the two antigens bear common antigenic properties. The
failure of anti-carcinoembryonic antigen, anti-a-fp, and anti-B2m to recognize either of the two human or the BT-20 antigens suggests that BTA isolated from human tissue material and BTA isolated from the BT-20 tumor cells do not share properties in common with fetal antigens and/or HLA markers. This suggestion gains support from the fact that the BT-20 cell line was originally derived from human mammary ductal-cell carcinoma tissue (8) and that the chemical and immunological properties of the M, 68,000 antigen are not shared with those of antigens described by others. In this regard, the absence of carbohydrate distinguishes the M, 68,000 antigen from the epithelial membrane glycoproteins (11) and from the human erythrocyte glycoprotein A (12). The absence of the M, 68,000 and 43,000 antigens from normal human breast tissues and the failure of the anti-M, 68,000 antibodies to agglutinate human erythrocytes in the presence or absence of complement support the conclusion that the antigens are unique to human breast tumor cells.

Monoclonal antibodies against a variety of animal (13–16) and human (17–20) breast cancer tissues have been prepared in syngeneic and allogeneic hosts. The antigenic material used included whole cells, metastatic cells, and/or respective membrane preparations. Antibodies prepared against these antigens presented a high degree of biological diversity (21), by demonstrating antigenic heterogeneity (21–23) and phenotypic drift in metastatic and cell surface properties (22–24). In view of this, one questions not only the prognostic values of these antibodies and their respective antigens but also their usefulness in immunodiagnosis and immunotherapy. The question of biological diversity and antigenic heterogeneity was not experienced with the M, 68,000 antigen described in this study. The anti-M, 68,000 antibodies, albeit polyclonal, are highly specific for human breast adenocarcinoma cell surface markers which were prepared against a purified antigen. The presence of the M, 68,000 antigen in human breast adenocarcinoma tissue and the documented immunological and molecular properties in common with this antigen from BT-20 cells suggest that the M, 68,000 but not the 43,000 antigen has not been subject to antigenic drifts and lacks apparent antigenic heterogeneity.

The molecular weight of the M, 43,000 antigen was estimated by SDS-PAGE and because of its molecular size, similarities with HLA were suggested; however, electrophoretic mobility and antibody reactivity failed to demonstrate homology or antigenic properties in common with HLA. The absence of B2m and the failure of the M, 43,000 antigen to recognize any of the antibodies used for screening, including the anti-BTA-Hu-68K antisera, support the conclusion that the BTA-Hu-43K is a unique molecule, immunologically distinct from the BTA-Hu-68K and HLA present on human breast tumor tissues. The BTA-Hu-43K was distinctly absent from BT-20 cells. The SDS-PAGE patterns of BT-20 cell extracts failed to demonstrate any traces of BTA-Hu-43K. Further, anti-BTA-Hu-43K antibodies failed to demonstrate its surface presence on BT-20 cells by the complement-mediated red cell agglutination test (results not shown) and/or to prevent cell adhesion and division of BT-20 cells in culture. Also, treatment of BT-20 cells with mercaptoethanol (2) destroyed the ability of BT-20 cells to recognize either the anti-BTA-Hu-68K or the anti-BTA-Hu-43K antibodies, to fix complement, or to agglutinate red cells. Clearly, BTA-Hu-43K is not a fragment of BTA-Hu-68K generated during the isolation procedure, since polyclonal antibodies prepared against the purified M, 43,000 antigen failed to recognize the M, 68,000 antigen. Conversely, antibodies against the M, 68,000 antigen failed to recognize the M, 43,000 antigen. The failure of BT-20 cells to express the M, 43,000 antigen in contrast to its expression in human tumor tissues is not an unusual finding in studies of BT-20 and similar cell lines (25). Using specific monoclonal antibodies, studies have shown that at contact inhibition, MCF-7 cells expressed less antigens on their surfaces than during active proliferation (25). Also, HLA markers which were detected by monoclonal antibodies were no longer detectable after a specific number of passages (26, 27). In the case of BT-20, the B14.2 and B6.2 positive markers were lost after 319 and 320 passages, respectively (25). It should be noted that the BT-20 cells used in our studies were kept below 290 passages.

Considering the reactivity and cross-reactivity between the anti-BTA-BT20-68K and the anti-BTA-Hu-68K and the lack of recognition of the anti-BTA-Hu-43K antibodies by either of the M, 68,000 antigens, several questions were asked concerning the biological properties of the BTA molecules and their respective antibodies. Using the BT-20 cell culture line, the antibodies prepared against either of the M, 68,000 antigens were complement fixing and cytotoxic to BT-20 cells. In contrast, the antibodies, even in the presence of complement, were not cytotoxic to either normal human lymphocytes, to human myeloma cell line (SK007), or to human lymphocytes bearing one or two HLA antigenic markers in common with the BT-20 cells. These results support the conclusion that the complement fixing antibodies are specific for tumor surface markers that exclude the participation of HLA found on the surface of BT-20 cells.

Antibodies prepared against the M, 68,000 antigen from tumor tissue or from the BT-20 cell line were not cytotoxic to BT-20 cells in the absence of complement; however, the addition of anti-BTA-BT20-68K antibodies to BT-20 cells in culture and in the absence of complement prevented cell adhesion and in vitro expansion of these cells. Clearly, at 1:125 and 1:250 serum dilutions the BT-20 cells remained floating, unable to adhere to the bottom of the flask or divide but remained viable during the 5 days of culture. At 1:500 antisera dilution, a small percentage (20%) of cell-expansion was observed and at 1:1000 dilution, cell expansion was about 70% of control cultures.

The ability of the antibodies to prevent cell adhesion and expansion of BT-20 cells in culture was reversed by prior incubation of the antibodies with purified BTA. These results demonstrate the specificity of the antibodies to the target antigen present on the surface of BT-20 cells and that prior treatment of the anti-M, 68,000 antibodies with specific BTA, but not with preimmune serum or anti-BTA-Hu-43K antiserum prevented inhibition of cell adhesion and therefore cell growth; however, the presence of BTA-antibody complexes (or immune complexes) in the incubation medium did not alter the ability of BT-20 cells to adhere, grow, or divide.

Further investigation of the adhesion-growth-inhibition phenomenon revealed that pretreatment of BT-20 cells with anti-BTA-antibodies before culture prevented cell adhesion and therefore cell growth and proliferation; however, cell growth and proliferation were inhibited even after the BT-20 cells were allowed to adhere during a 24-h culture period and, before the antiserum dilutions were added. The degree of inhibition was similar to BT-20 cell pretreated with antibodies prior to cell culture. Under either of these two experimental conditions, the cells remained viable despite treatment with antibodies since washing the 5-day culture with antibody-free medium permitted cell growth and division to proceed. Since cell adhesion is a requirement for BT-20 cells to grow and divide, it is possible
that BT-20 cell adhesion is mediated by the BTA. Should this be the case, the BTAs may serve not only as cell surface markers but as adhesion molecules to mediate metastasis. The fact that the anti-BTA antibodies are complement fixing and cytotoxic to BT-20 cells support the conclusion that BTAs and their respective antibodies may play a role in diagnosis, metastasis, and therapy of breast cancer.

ACKNOWLEDGMENTS

We are grateful to Dr. Yoritaro Inada, Department of Medicine, St. Luke’s-Roosevelt Hospital Center, New York, NY, for the immune adherence hemagglutination test and to Dr. M. Moscarello of the University of Toronto for his analysis of amino and neutral sugars. We thank Nippon Microtec, K.K., EISAI Co., of Tokyo, Japan, for their generous supply of microtiter plates and guinea pig complement, respectively, and to Rosemary B. Edge for typing the manuscript.

REFERENCES


TUMOR SURFACE MARKERS
Antibodies to a Surface Membrane Marker from Human Mammary Carcinoma Cell Line


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/47/9/2433

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.