IMMUNOLOGIC REACTIVITY IN HUMAN BREAST CANCER AGAINST CULTURED HUMAN BREAST TUMOR CELLS

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Sera from women with breast cancer and appropriate controls were tested for immunologic reactivity against a cultured human breast tumor cell line or surface components from the cells. Immunofluorescent tests against living or fixed tumor cells indicated that sera from controls (no breast disease) or from women with breast diseases (fibroadenoma, cystic mastitis) were positive in up to 10% of the cases, while sera from breast cancer patients were positive in 42% of the cases. Hemagglutination tests using erythrocytes tanned with serum-free growth medium from the breast cancer cells showed that sera from controls or from women with breast diseases were positive in up to 8% of the cases, while sera from breast cancer patients were positive in 68% of the cases. The results indicate that the cultured human breast tumor cells contain surface components reactive with serum from breast cancer patients. The cells or surface components from the cells should be valuable in tests monitoring the initiation or metastatic spread of human breast cancer.

INTRODUCTION

Adenocarcinoma of the breast is the most frequent cause of terminal cancer in American women. Due to the complexities of the tumor-host interaction, there is little information about the biochemical and immunologic interactions in breast cancer. Since the survival rate in breast cancer has not significantly improved in the last 20 years, an increased knowledge of the tumor cell and the host reaction to the tumor cell may provide methods for early diagnosis of both primary and metastatic disease. Studies using whole tumor tissue, while providing important information, have been hampered by the limited amount of tissue from a single tumor and by the presence of contaminating, non-tumor tissue. As an alternative source of material for study, investigators have used cultured breast tumor cells obtained from the original tumor tissue or from metastatic foci. Although there are reports of cultured human breast tumor cells, recent cytogenetic studies cast doubt as to the nature and origin of many of the cell lines (1). BOT-2 cells, isolated by Nordquist et al. (2) from an infiltrating ductal cell carcinoma, were reported to be one of the few continuous human breast cell lines (1). We now report the results of our studies on the surface components of BOT-2 cells and the reactions of breast cancer and control patients' sera with BOT-2 cells and their surface components.

MATERIALS AND METHODS

Cell culture. BOT-2 cells (2) were grown on Eagle's minimal essential medium containing 10% heat-inactivated fetal calf serum. Cells were grown at 37 C in plastic flasks and fed twice weekly. To culture BOT-2 cells without serum, light monolayers were fed Eagle's medium with 500 mg % glucose but containing no added macromolecules. The cells were fed twice weekly.

Antigen Extraction. BOT-2 cells, grown with serum, were rinsed with phosphate-buffered saline (0.01*M* Na_2^- or K_2HPO_4, pH 7.2; 0.15*M* NaCl) and scraped from the culture flask. The cells, in 5 m*l* of PBS, were sonicated in a tank sonifier at 4 C for 60 min. The cells were removed by centrifugation and the supernatant fluid was clarified by centrifugation for 60 min at 140,000 *g*. The surface components were concentrated to 5 mg/m*l* by Sephadex G-200 hydration. Proteins were determined by a colorimetric assay (3) using bovine serum albumin as a standard.

Naturally Shed Molecules. After 2 weeks of growth on serum-free medium, the culture fluid from BOT-2 cells was decanted and

centrifuged at low speed to remove cells. The naturally shed molecules in the culture fluid were concentrated by pressure dialysis using a 50,000 MW cutoff membrane (Amicon Corp., Leington, MA.).

Chromatography. BOT-2 cell surface components from cells grown with or without serum were separated by gel filtration chromatography using Sephacryl S-200 in PBS at 4 C. The column was 49 × 2.7 cm. Fractions (3 m*l*) were collected and the absorbancy at 280 *nm* determined. The immunologic reactivity of the pooled fractions against human breast cancer serum or rabbit anti-BOT-2 serum was determined by immuno-precipitation (4).

Antiserum. Rabbit antiserum against living BOT-2 cells was prepared using Freund's complete adjuvant. The gamma G fraction was isolated by DEAE-Sephadex chromatography and absorbed with cross-linked fetal calf serum, human tissue extracts, and packed HeLa cells (5). As shown by immunofluorescence, this antiserum reacted specifically against BOT-2 cells or breast tumor tissue. No reactivity was observed when the antiserum was tested against other human cancer cells or normal breast cells within a breast tissue specimen.

Immunofluorescent Methods. Methods for fixed cell immunofluorescent assays (FCF) and living cell membrane immunofluorescent assays (LCMF) have been described (6). Briefly, prepared cells were incubated with patients serum, rinsed in PBS, and coupled with fluorescein isothiocyanate (FITC)-labelled goat antihuman gamma globulins, washed in PBS, and observed under a fluorescent microscope using a FITC interference filter.

Indirect Hemagglutination: For hemagglutination tests (HA), surface components from BOT-2 cells, grown with or without serum, were coupled to tanned sheep erythrocytes exactly as described (7). Two-fold dilutions of patient's serum were added to an equal volume of the antigen-sensitized erythrocytes and the mixture was incubated for 60 min at 21 C. Hemagglutination was observed by visual inspection. For control experiments, patient's serum was tested against erythrocytes sensitized with fetal calf serum or HeLa cell extracts. The average maximum dilution of reactive patients' sera, after subtracting the control value, was 1: 8.

RESULTS

Preliminary studies using immunofluorescent techniques indicated that BOT-2 cells contained surface molecules which reacted with serum from breast cancer patients (6). To isolate these components, BOT-2 cells were sonicated and the surface components were clarified by centrifugation. After sonication, BOT-2 cells remained viable as shown by quantitative plating experiments. Surface components, after concentration, were chromatographed on Sephacryl S-200. As shown in Figure 1, three peaks of material were found. The first eluted peak was in the column void volume while the second (middle) peak eluted ahead of bovine serum albumin (MW 69,000). The last peak eluted contained molecules in the molecular weight range of 25,000 daltons. Immunoprecipitation studies using rabbit antibody against BOT-2 cells showed that almost all of the reactive material was in the second peak from the Sephacryl S-200 column.

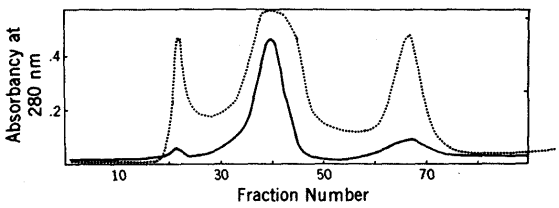

FIGURE 1. Sephacryl S-200 chromatography of surface components from BOT-2 cells grown on serum and growth medium from BOT-2 cells grown without serum. Eight mg of protein extracted from serum-grown cells (----) or 7 mg of protein from serum-free BOT-2 cell growth medium (——) was separated as described in Materials and Methods.

To determine if BOT-2 cells naturally shed surface macromolecules, the cells were cultured in serum-free medium, which was collected and concentrated. Chromatography of this growth medium on Sephacryl S-200 revealed only 1 major peak of material (Figure 1). This peak corresponded exactly to the middle peak of material extracted from sonicated cells grown on serum. Immunoprecipitation studies revealed that this peak of material from cells grown without serum formed a precipitate with rabbit anti-BOT-2 serum. In addition, when serum from breast cancer patients was tested by immunoprecipitation against the chromatographed culture medium from

BOT-2 cells grown with or without serum, only the middle peak reacted.

The immunologic reactivity of sera from breast cancer and control patients against BOT-2 cells or surface components was tested by three methods. As shown in Table 1, sera from women without breast diseases (controls) had low reactivity when tested against BOT-2 cells (FCF, LCMF) or against surface components from BOT-2 cells grown in serum (HA). The reactivity of serum from women with fibroadenoma or cystic mastitis varied considerably depending on the test. However, sera from women with breast cancer were positive in 46-79% of the cases depending on the test.

TABLE 1. *Fluorescent and agglutination tests*[a] *for antibodies*[b] *against BOT-2 cells or surface components from BOT-2 cells grown in serum.*

Diagnosed disease	Number of patients	FCF postive	LCMF positive	HA positive
None (control)	26	4%	4%	8%
Fibroadenoma	32	87%	3%	19%
Cystic mastitis	40	50%	40%	17%
Breast cancer	76	79%	46%	47%

[a]FCF, fixed-cell immunofluorescence; LCMF, living cell membrane immunofluorescence; HA, indirect hemagglutination
[b]Serum from women with diagnosed, untreated breast diseases

Because of the variability in immunologic reactivity of sera from women with fibroadenoma and cystic mastitis, the FCF and LCMF tests were both performed on the same serum samples. As shown in Table 2, the use of both the FCF and LCMF tests greatly reduced the number of positive reactions in fibroadenoma and cystic mastitis to only 4-10% respectively. Controls were positive in 0% of the cases, while sera from women with breast cancer were positive in 42% of the cases.

TABLE 2. *FCF and LCMF tests on the same serum samples.*

Diagnosed disease	Number of patients	FCF and LCMF positive
None (control)	24	0%
Fibroadenoma	27	4%
Cystic mastitis	30	10%
Breast cancer	45	42%

Since chromatography experiments indicated that the growth medium from BOT-2 cells contained mainly one peak, we coupled this material to tanned erythrocytes for the HA test. As shown in Table 3, the naturally shed components from BOT-2 cells grown without serum showed a high degree of specificity for breast cancer patients. Sera from women without breast diseases were positive in 0% of the cases while sera from patients with fibroadenoma and cystic mastitis were positive in 7-8% of the cases, respectively. However, the sera from women with breast cancer were positive in 68% of the cases.

TABLE 3. *HA test for antibodies against serum-free growth medium from BOT-2 cells.*

Diagnosed disease	Number of patients	HA positive
None (control)	10	0%
Fibroadenoma	15	7%
Cystic mastitis	12	8%
Breast cancer	19	68%

DISCUSSION

Because breast cancer is a frequent occurrence in American women, it is important to have sensitive tests indicating the onset of cancer and the metastatic spread of the tumor cells. We and others have shown that the use of cultured human breast tumor cells provides a unique and meaningful way to study the complex events in human breast cancer (8, 9, 10, 11). Our previous studies indicated that about 45% of women with diagnosed breast cancer have antibodies which react with BOT-2 cells (6, 10). Further, chromatography of surface components from BOT-2 cells yielded macromolecules similar to breast tumor antigens isolated from whole tumor tissue (10).

In the present study, our data indicated that the surface components of BOT-2 cells grown in serum contained three peaks of absorbing (280 nm) material. However, only the middle peak reacted with serum from breast cancer patients and with antibody prepared against living BOT-2 cells.

When BOT-2 cells were cultured for several weeks without calf serum, the growth medium contained molecules which reacted with sera from breast cancer patients and rabbit antibody against BOT-2 cells. Chromatography of the BOT-2 cell preparations on Sephacryl S-200 showed that the antibody-reactive molecules were in the broad molecular weight range of 100,000 daltons. This value is similar to that described by others for breast tumor antigens obtained from cancerous human breast tissue (12).

In the present study, our primary interest was to determine the immunologic reactivity of sera from women with breast cancer and appropriate controls against BOT-2 cells or surface components from BOT-2 cells. The data in Table 1 extend our previous data (6, 10) to include 174 patients and controls. The immunologic reactivity of patients without breast diseases was quite low (4-8%). However, the reactivity of sera from women with benign breast tumors (fibroadenoma) was 87% using the FCF test. The reactivity of sera from women with non-malignant breast disease (cystic mastitis) was 50% in the FCF test and 40% in the LCMF test. These high values may be due to common surface components shared by tumor and disease tissue or may be indicative of premalignant cell changes (6, 10, 13, 14). In these tests, the reactivity of sera from breast cancer patients varied from about 45% (LCMF, HA) to 80% (FCF) positive.

In an attempt to reduce the high percentage of positive reactions from women with fibroadenoma or cystic mastitis, we performed both immunofluorescent tests on the same serum samples. The results (Table 2) showed a dramatic reduction in the number of positive reactions. Whereas a single test showed about 50% positive, the use of both tests showed a maximum of 10% positive. These results, in conjunction with results using control sera (0% positive) and breast cancer patient sera (42% positive), indicate that the immunofluorescent tests may be very useful in detecting breast cancer.

The growth medium from BOT-2 cells cultured without calf serum contained only one major band and was not contaminated with normal calf serum macromolecules. When this preparation was used in the HA test, the results (Table 3) were quite impressive. Sera from controls or women with breast disease were positive in only 0-8% of the cases, respectively. In contrast, sera from breast cancer patients were positive in 68% of the cases.

Our data indicate that the surface components from the cultured human ductal carcinoma cells, BOT-2, are quite useful in the immunologic monitoring of breast cancer. Combining the FCF and LCMF tests, or using the HA test (with concentrated serum-free medium), resulted in positive reactions in a high percentage of breast cancer patients and negative reactions in most controls or breast disease patients. BOT-2 cells are a unique line of human breast carcinoma, not only for their immunologic reactivity, but for their basic biochemical and immunologic reactions. We have previously shown that these cells rapidly shed surface components in the presence of cytotoxic human antibody (8). Also, the surface components appear to contain proteins which have both antibody and lectin binding patterns similar to those of minor blood group antigens (9) which have been found by similar methods in the membranes from human breast tumor tissue (15). We are currently investigating the nature of the BOT-2 cell surface components as well as the immunologic reactivity of patients' sera against the components and the intact BOT-2 cells.

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