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A serologic study of cultured breast cancer cell lines: lack of antibody response to tumour specific membrane antigens in patients

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SUMMARY

Humoral antibodies to tumour associated membrane antigens of cultured human breast cancer cell lines were studied using the immune adherence (IA) test. Sera from 353 post-operative breast cancer patients and from twenty-five patients immunized by allogeneic breast cancer cells were tested against the MDA-MB-436 cell line.

Fifty-five (15·6%) sera samples from the non-vaccinated group and 131 (77·3%) of 168 sera samples from the immunotherapy group were IA-positive to this cell line after absorption with bovine erythrocytes to exclude antibody to heterologous membrane antigens (HM Ag). Forty-five of the 55 positive-sera from the non-immunized group and 113 of the 131 positive sera from the immunized group became IA-negative after further absorption with lymphoblastoid cells autologous to MDA-MB-436.

Subsequently, the twenty-eight positive sera remaining were tested for oncofetal antigens (OFA). After absorption with OFA rich tissues (fetal brain and M14 melanoma cells), no reactivity remained in the sera samples.

In order to identify antibodies specific to breast cancer antigens, the 129 sera samples from non-immunized patients were tested against four other breast cancer cell lines; MDA-MB-157, MDA-MB-231, MCF-7 and UCLASO-B1. Four sera which reacted to more than three of the cell lines were identified. The reactivity of three of the four was due to anti-OFA antibody. The last serum sample was reactive to anti-HLA antibodies.

These results indicate that sera of patients with breast cancer contain antibodies to OFA, but do not detect breast histologic type specific antigens as tested by IA using five breast cancer cultured cell lines.

INTRODUCTION

A number of studies have suggested the existence of an immune response to the tumour-associated antigens of breast cancer. Observations of *in vivo* responses include (1) the patients who have a ten to twenty year tumour-free interval after operation and then have a sudden rapid growth of tumour (Alford, 1963); (2) lymphoid infiltration of the primary tumour of sinus histiocytosis of regional lymph nodes that can be correlated to patient survival (Berg, 1971; Black & Speer, 1958; Black & Asire, 1969; Black, Cutler & Barkley, 1972); and (3) patients with breast cancer who respond specifically to the extracts of breast cancer tissues with delayed hyper-sensitivity skin reactions (Roberts & Williams, 1968; Summer, Edwards & Baum, 1972; Hollinshead *et al.*, 1974). *In vitro* tests to measure cell-mediated immune functions

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suggested the existence of host immune defense against breast cancer, which included lymphocyte migration inhibition (Cochran *et al.*, 1974), colony inhibition, cytotoxicity of autologous lymphocytes (Hellström *et al.*, 1971) and blastogenesis of lymphocytes to cancer extracts (Fisher *et al.*, 1969). In general, data reported indicate that breast carcinomas possess tumour-associated common antigens which provoke cellular immune responses.

Serological assays such as complement fixation tests (Taylor & Odili, 1970; Chan *et al.*, 1971), an acetone-fixed immunofluorescence (Priori *et al.*, 1971; Edynak *et al.*, 1972), an indirect immunofluorescence (Richman, 1976) and combined complement fixation and immunodiffusion (Humphrey *et al.*, 1974) have been used to define tumour-related antigens which are associated with humoral immune responses in patients with breast cancer.

The literature contains no mention of antibodies to tumour-associated antigens located on membranes. Three possibilities for this lack of information come to mind: (1) the tumour-associated membrane antigens of breast cancer are not capable of inducing humoral antibodies in patients, or (2) although the patient produces humoral antibodies, the titres are too low to be detected by serological assays used in the previous studies, (3) the serological assays in use cannot define breast cancer specific membrane antigens. We considered the latter two possibilities in our effort to identify serologically tumour-specific membrane antigens on breast carcinoma cells using the patient's serum as the antibody source. In this study, we introduced three major technical improvements over earlier assays: (1) exclusion of non-tumour associated antibodies from the tested patients' sera, (2) a particularly sensitive humoral assay, immune adherence, was used, and (3) newly established human breast cancer cell lines were used.

MATERIAL AND METHODS

Cell lines. Five human breast cancer cell lines were used to detect tumour-associated membrane antigens. Three, MDA-MB-436, MDA-MB-157 (Young *et al.*, 1974) and MDA-MB-231 were derived from pleural effusions of patients with stage III breast carcinoma. These lines were established by Dr Relda Cailleau, MD, Anderson Hospital aud Tumor Institute, University of Texas, Houston, Texas, in January 1976, December 1972, and October 1973, respectively, and were characterized as being of breast cancer origin. MCF-7 (Soule *et al.*, 1973) was established by Dr Mervin Rich at the Michigan Cancer Foundation, Detroit, Michigan, from a pleural effusion of a patient with breast carcinoma. This line retains several characteristics of differentiated mammary epithelial function, including the specific cytoplasmic oestrogen receptor, the capability of forming domes, and produces alphalactoalbumin. Furthermore, the human origin of this line was confirmed from ribosomal RNA molecular weight determinations on cells and human glucose-6-phosphatase isozyme B. UCLASO-B1 was derived from the ascitic fluid of a patient with breast cancer and was established in our laboratory. These cell lines were maintained in L-15 Media (Flow Laboratories, Rockville, Maryland 20852, USA) containing FCS (North American Biological Company, Torrance, California), L-Glutamine (Flow Laboratories, Rockville, Maryland 20852, USA), Insulin (Eli Lilly and Company, Indianapolis, Indiana 46206 USA), Solu-Cortef (The Upjohn Company, Kalamazoo, Michigan 49001, USA), Gentamycin (Schering Corporation, Kenilworth, New Jersey, 07033, USA) and Fungizone (Grand Island Biological Company, Grand Island, New York, USA).

Lymphoblast cell lines. Normal peripheral lymphocytes from the donors of MDA-MB-436 and UCLASO-B1 were transformed by infecting them with Epstein-Barr viruses (EBV) and were established as permanent cell lines. The EBV source was the supernatant of the B-95-8 cell line (EBV-producing marmoset lymphoblastoid cells) (Miller & Lipman, 1973; Miller *et al.*, 1972). These lines were maintained in RPMI 1640 (Flow Laboratories, Rockville, Maryland 20852, USA) containing 20% fetal calf serum and antibiotics (streptomycin, kanamycin, and fungizone).

Patients' sera. Post-operative sera from 353 patients with breast carcinoma were collected. One hundred and sixty-eight serum samples from twenty-two patients immunized with allogeneic breast cancer cell lines were collected from two weeks to thirty months after the immunization. The immunization schedule was as follows: tumour cell vaccine (TCV) containing 10^8 of equal cell numbers of MDA-MB-157, MDA-MB-231 and MCF-7 was started three weeks post-operatively, given every week until week, and 13 then every other week to week 106. For the first four weeks TCV was irradiated prior to the administration. For the following two weeks, 70% of the cells were irradiated, and thereafter whole, living tumour cells were administered.

All sera were heat inactivated at 56°C for thirty min and stored at −70°C until used.

Immune adherence (IA) test. The IA test devised for monolayer cultured cells by Tachibana & Klein (Tachibana & Klein, 1970) was modified slightly for this study. Ten ml of the cell suspension containing $1{\cdot}0 \times 10^6$ cells was seeded into wells of microtest plates (#3040, Falcon Plastics, Oxnard, California). The covered plate was then incubated for 18–24 hr in a humidified 5% CO_2 atmosphere. Each well was washed three times with L-15, using a trumpet-shaped glass capillary tube. The covered plates were incubated at 37°C for 60 min and again washed three times with L-15. Then the indicator system

(25 μl of 1×10^7/ml human O-type erythrocytes and guinea-pig complement diluted 1:30 with RPMI without glutamine (Flow Laboratories, Rockville, Maryland 20852, USA)) was added to each well. The plate was covered and incubated at 37°C for thirty min. After incubation, the plate was inverted for thirty min at 37°C, washed once with L-15 and evaluated under the microscope. The IA patterns were graded from 1 to 4. If heavy rosettes were formed or if rosettes were attached to all target cells, the well was a grade 4; if there was a moderate rosette formation, or if erythrocytes were attached to 75–100% of target cells, it was a grade 3; if a few erythrocytes attached to 50–75% of target cells, the well was a grade 2; and if only a few erythrocytes attached to 25–50% of the target cells, it was a grade 1. More than grade 2 was considered a positive IA response. The IA absorption test was performed as follows: sera were mixed with an equal volume of cell suspension adjusted to an appropriate number, incubated at 37°C for one hr, then at 4°C overnight, and centrifuged at 800 *g* for ten min. Each supernatant fluid was titrated by IA against target breast cancer cells.

RESULTS

Detection of membrane antigens of MDA-MB-436 cell line

The MDA-MB-436 cell line, passages eight to twenty-two, was selected as the target cell because the autochthonous lymphoblastoid cell line was available. Both the tumour cell line and the lymphoblasts were tested for HLA typing. The lymphoblastoid cell line expressed A1, A2, A3 and B7, and the tumour cells expressed A1 and A3. Therefore, the autochthonous lymphoblasts could be used to absorb antibodies to HLA antigens on MDA-MB-436 cells from patients' sera.

Antibodies of non-immunized breast cancer patients

Sera were collected from 353 post-operative breast cancer patients who did not receive immunotherapy. After the sera were absorbed by bovine erythrocytes to remove antibodies to the heterologous membrane antigens (HM Ag) (Irie, Irie & Morton, 1974a, b) the IA reactivity of these sera against MDA-MB-436 cells was tested. The sera were diluted to 1:2 and to 1:20 for the test. Of 353 sera, fifty-five showed positive reactivity. To exclude the HLA reactivity, these sera were retested after further absorption with matched lymphoblasts. The number of the lymphoblastoid cells required to absorb anti-HLA antibodies was determined as follows: a serum with the highest titre to alloantigens of MDA-MB-436 cells (the titre was 1:256) was selected from among the 353 sera. This serum was absorbed by various numbers of lymphoblasts from $2{\cdot}5 \times 10^6$ to 4×10^7 and tested against MDA-MB-436 cells by IA. As shown in Fig. 1, the antibody activity was completely absorbed by 4×10^7 cells per 0·1 ml of serum. Therefore, we used this amount to absorb anti-HLA antibodies from the other sera samples. Of fifty-five sera, only ten continued to show IA reactivity to MDA-MB-436 cells after this absorption. The maximum titre was 1:16. Most of the reactivity was found to be due to antibodies to HLA antigens and/or alloantigens. Subsequently, tumour specificity of these ten sera were examined. Since this breast carcinoma

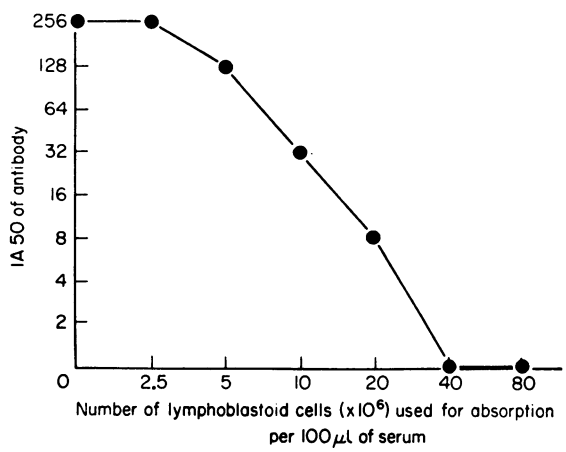

FIG. 1. Quantitative absorption of a serum (# 70) to alloantigens of MDA-MB-436 cells by autologous lymphoblastoid cells. Antibody was tested by the immune adherence (IA).

TABLE 1. IA reactivity of sera from postoperative breast cancer patients to MDA-MB-436 cell line after sequential absorptions by HM Ag, alloantigens and oncofetal antigens

Sera absorbed by (antigen sources)	# Sera positive/ # sera tested	%	Titre (IA50)
Bovine erythrocytes (HM Ag)	55/353	15·6	2–256
Bovine erythrocytes +lymphoblasts (alloantigen)	10/55	18·2	4–16
Bovine erythrocytes +lymphoblasts +fetal brain (OFA)	0/10	0	< 4
Bovine erythrocytes +lymphoblasts +fetal liver (OFA-negative fetal tissue)	10/10	100	4–16

cell line was known to express oncofetal antigens (OFA) (Irie, Irie & Morton, 1976), membrane antigens common to several histologic types of human cancer and fetal brain, we first investigated these sera for anti-OFA antibodies. One hundred microlitres of serum were mixed with 100 μl of packed fetal brain homogenates (second trimester of gestation) to absorb anti-OFA antibodies (Irie *et al.*, 1976). After the absorption, none of the ten sera were reactive by IA against MDA-MB-436 cells, suggesting that the reactivity was OFA associated. There was no reduction of antibody titre observed after absorption with liver tissue from the same fetus (Table 1).

Ab from patients immunized with cultured breast cancer cells

Failing to detect breast-specific tumour antigens with the natural antibodies of patients with breast cancer who had operations only and no immunotherapy we next tested the same cell line using sera from patients immunized with high doses of breast cancer-cell vaccine. The vaccine was composed of three breast cancer cell lines, MDA-MB-157, MDA-MB-231 and MCF-7. A positive result could show that: (1) MDA-MB-436 expressed a tumour-associated antigen that cross reacted with the other breast cancer cells (common antigen), or (2) antigenic stimulation by *in vivo* autochthonous breast cancer cells was too weak to induce natural humoral antibodies. One hundred and sixty-eight serum samples from twenty-two immunized patients were collected from two weeks to thirty months after the onset of immunization. The specificities of these sera were tested against the MDA-MB-436 cell line in the same manner as described above.

The IA reactivity of these sera after sequential absorption by bovine erythrocytes (HM antigens), lymphoblastoid cells (HLA and other alloantigens) and M14 cells (an OFA positive cell line) is shown in Table 2. Of one hundred and sixty-eight sera, 131 were IA positive after HM antigen absorption. Most of the positive sera had an antibody titre greater than 1:64. The one hundred and thirty-one sera were

TABLE 2. IA reactivity of sera from breast cancer patients immunized with breast cancer cells to MDA-MB-436 cell line after sequential absorptions by HM Ag, alloantigens and oncofetal antigen

Sera absorbed by	# Sera positive/ # sera tested	%	Titre (IA50)
Bovine erythrocytes (HM Ag)	131/168	78·0	16–512
Bovine erythrocytes +lymphoblasts (alloantigen)	18/131	13·7	4–48
Bovine erythrocytes +lymphoblasts +M14 (OFA)	0/18	0	< 4

TABLE 3. IA reactivity of 129 sera from breast cancer patients to 5 human breast cancer lines

Sera reacted with	Number of sera
2 lines	10
3 lines	3
4 lines	1
5 lines	0

absorbed by lymphoblastoid cells to remove alloantibodies and only eighteen showed reactivity with titres from 1:4 to 1:48. No additional reduction of the titre could be demonstrated with repeated absorptions with the same cells. Next the eighteen sera were absorbed with M14 cells, and the reactivity was completely abolished, indicating that the reactivity of the eighteen sera was against OFA rather than to tumour-associated antigens on MDA-MB-436.

Search for breast cancer-specific antigens on other breast cancer lines (Table 3)

Because we were unable to detect a breast cancer-specific antigen using the MDA-MB-436 cells as targets, we concluded that either (1) the cell line expressed no breast cancer-specific antigens, or (2) that breast cancer-specific antigens did not induce humoral immune responses in cancer patients. To eliminate possibility # 1, we studied four other breast cancer cell lines, MDA-MB-157, MDA-MB-231, MCF-7 and UCLASO-Bl. Serum samples obtained from 129 patients (non-immunized) were assayed by IA using the four cell lines and MDA-MB-436 as targets. The five cell lines expressed different combinations of HLA-A and −B antigens. Thus, if the reactivity of a serum was against the HLA-antigens on a cell line only, that serum would not react to all of the other targets. However, if a serum reacted with a common breast cancer specific antigen then it should react to more than two of the cell lines. Using this criteria, we found four sera which showed positive reactivity to more than three cell lines. MDA-MB-231 was chosen as the target because it was the most reactive. M14 cells were used to absorb anti-OFA antibodies and the reactivity of three sera was completely abolished. The serum which retained reactivity was the one that had the highest titre of alloantibodies to the MDA-MB-436 cell line. In addition, the HLA-A2 of the MDA-MB-436 cell line overlapped that of the MDA-MB-231 cell line. Thus, it was possible that the reactivity was anti-HLA. The serum was mixed with the lymphoblasts of MDA-MB-436 cell line to absorb anti-HLA antibodies and the reactivity disappeared.

DISCUSSION

In the present study, sera from breast cancer patients were used to identify tumour associated membrane antigens on human breast cancer cells. Using immune adherence techniques, 353 sera from 353 post-operative patients and 168 sera from twenty-two patients immunized with cultured breast cancer cells were tested. After sequential absorption of non-tumour associated antibodies, the patients' sera identified oncofetal antigens on five tissue culture cell lines, MDA-MB-436, MDA-MB-231, MCF-7, MDA-MB-157 and UCLASO-Bl. No histological type-specific tumour antigens were detected on these lines.

The heterologous membrane antigens (HM Ag), a bovine serum component that is incorporated from medium into human cells during tissue culture, was excluded by absorbing the patients' sera with bovine erythrocytes (HM Ag positive cells). The reaction between alloantigens of the target breast cancer cells and patients' sera was avoided by absorbing the sera with lymphoblastoid cells derived from the donor of target cells. The lymphoblastoid cell line was established by infecting peripheral blood lymphocytes with Epstein-Barr viruses. Since HLA antigens on the lymphoblastoid cells were identical to those on the target tumour cells, the EBV-transformed lymphoblastoid cells were useful for eliminating antibody to the alloantigens of target cells. Of fifty-five sera positive to the MDA-MB-436 cell line, forty-five (81·8%) were, in fact, negated by the lymphoblastoid cells.

Similarly, reactivity of 113/131 immunized sera (86·3%) was completely absorbed by the lymphoblastoid cells. A high percentage of the alloantigen–antibody reactivity may have been due to the patients' history of pregnancies and /or blood transfusions at the time of mastectomy. All reactivity remaining after exclusion of HM Ag and HLA-antigens was due to OFA-anti-OFA reactivities.

In this study, we confirmed that breast cancer cell lines possessed OFA. Instead of sera from melanoma patients, breast cancer patients' sera were used as the antibody source. OFA has been defined as a membrane antigen common to several histologic types of human cancer including melanoma, sarcoma and breast carcinomas. Cross-reactive antigen is found in fetal brain tissues. Although we did not verify the existence of tumour-specific antigens on human breast cancer cells, we did detect OFA.

There are several possible reasons why this experimental system did not demonstrate tumour-specific antigens of human breast cancer cells: (1) of five cell lines studied, four were derived from pleural effusions. It is possible that these metastatic cancer cells are more undifferentiated and, as such, lose the breast tissue characteristics which primary tumour cells might possess, and instead acquire fetal substances. (2) It has been well documented that the cancer bearing host is immunologically suppressed in both animal studies and in humans. One might expect that few humoral antibodies would be produced against weakly antigenic autochthonous tumours. Since in our studies we used patients' sera as the antibody source, we may have overlooked the existence of tumour-specific antigens on the cell lines. (3) The possibility of individually specific-tumour antigens, i.e. tumour-specific antigens unique to individual cancer tissues, cannot be neglected. This type of antigen has been identified on chemically-induced animal tumours, and has also been detected on human cancer tissues, such as melanomas and sarcomas. In the human systems, the antigen has been detected by autochthonous sera. Only one of the breast cancer cell lines, MDA-MB-436, could be tested with an autochthonous sample. No reactivity was observed. Sera samples from patients from whom the other four lines were derived were not available. (4) The immune adherence assay is one of the most sensitive serological techniques for detecting tumour-associated membrane antigens. However, this assay requires complement.. Therefore non-complement fixing antibodies, such as IgA and IgG_4 are not detectable by this assay. IA is most sensitive for IgM antibody detection, but it is possible that breast tumour-specific antigens induce antibodies that cannot be assayed by IA.

However, our preliminary results using indirect membrane immunofluorescence assays for the detection of IgM and IgG antibodies indicated that these breast cancer patients' sera detected only OFA on the MDA-MB-436 cell line (unpublished data).

Further studies, in which other cultured cell lines, autochthonous sera and more sensitive serological assays are employed, will be required to confirm whether or not membrane antigens on breast cancer cells induce humoral immune responses in patients.

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