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Surface differentiation antigens of human mammary epithelial cells carried on the human milk fat globule

(cell-type-specific antigens/immunofluorescence/affinity chromatography)

ROBERTO L. CERIANI, KAREN THOMPSON, JERRY A. PETERSON, AND S. ABRAHAM

Bruce Lyon Memorial Research Laboratory, Children's Hospital Medical Center, Fifty-First and Grove Streets, Oakland, California 94609

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ABSTRACT **Rabbit antibodies against components of the human milk fat globule bind specifically to normal human breast epithelial cells and cell lines derived from breast carcinomas, as well as to the outer surface of the human milk fat globule. Variation in indirect immunofluorescence staining in both intensity per cell and percentage of cells stained is observed for the different breast cell lines. Cells derived from other epithelial and other ectodermal tissues, fetal fibroblasts, cells of the blood buffy coat, and even fibroblasts of the breast itself do not bind the antibodies. This suggests that these antibodies are detecting cell-type-specific antigens. These normal breast epithelial cell antigens are on the cell surface and their expression is stable in long-term cultured cell lines, even after much chromosomal variation in a given line. By affinity chromatography, three distinct antigenic components can be isolated from the milk fat globule, one of which contains carbohydrate. These differentiation antigens of the human breast epithelial cell are not only useful as specific cell-type markers, but also can provide a tool to study the role of the cell surface in normal and neoplastic mammary development.**

Mammary epithelial cells have unique differentiated characteristics. They secrete specific proteins (casein, α-lactalbumin), synthesize lactose, and are stimulated by a set of specific hormones (for review, ref. 1). Other highly differentiated tissues like brain (2) or lymphoid tissue (3) have, together with other specific metabolic characteristics, tissue-specific surface antigens. Recently, we have shown that normal mouse mammary epithelial cells have antigens that distinguish them from other cell types (4). These antigens are also expressed in spontaneous and transplantable mouse mammary tumors (unpublished results). However, since the antiserum to mouse mammary epithelial cells was induced in rabbits, by injection of intact cells (4), antibodies could have formed against both intracellular and cell-surface components. There is a special interest in narrowing the specificity of these antibodies to only surface components because they will then provide a tool to study the outermost components of the breast cell that could correspond to surface macromolecules involved in cell recognition, communication, and sorting out.

The production of antibodies directed solely against outer cell surface components is hindered by the difficulties inherent in purifying such cell membranes. Plasma membrane constituents amount to about 2–3% of the total cell protein (5); thus, separation from other cellular components present in much greater amounts is difficult. Moreover, when a source of normal human tissue, other than blood, is needed for purification of normal cell surface antigens, one is faced with an unresolved problem. Because the milk fat globules are secreted into milk by the breast epithelial cell—by envelopment of fat droplets by the plasma membrane (6)—they represent an accessible source of enriched plasma membrane. Additional information that enzyme markers of the plasma membrane are found in the milk fat globule membrane (7) and the fact that churned milk fat globules appear as an almost pure membrane fraction when examined by electron microscopy (8) make this material the immunogen of choice.

Here we describe the production of an anti-human mammary epithelial cell antiserum (anti-HMEC) raised against the defatted human milk fat globule (HMFG), which specifically identifies mammary epithelial cells from both the normal breast and from human breast carcinoma cell lines. Also, we describe characteristics of the antigenic components present on the HMFG, and procedures for their separation.

MATERIALS AND METHODS

For the preparation of defatted HMFG, the washed cream fraction of human milk (8) was extracted twice with two volumes of chloroform and twice with 1 volume of ether, and then lyophilized.

Electrophoresis was performed in 5% polyacrylamide gels (12 cm long and 0.6 cm in diameter) in the presence of 0.1% sodium dodecyl sulfate, 7 M urea, and 0.1 M sodium phosphate buffer, pH 7.2, with samples dissolved in 1% sodium dodecyl sulfate. Gels, run with 100 and 500 μg of protein, respectively, were stained with Coomassie blue and periodic acid–Schiff (9). Protein was determined by the method of Lowry *et al.* (10).

Anti-HMEC was prepared from rabbits immunized by repeated injections of 5 mg of protein of defatted HMFG emulsified with Freund's complete adjuvant. Gamma globulin fractions (4) of anti-HMEC and nonimmunized rabbit sera were absorbed with ½ volume of washed, sedimented human blood cells to remove species-specific antibodies.

Affinity chromatography was performed with anti-HMEC conjugated by the cyanogen bromide method (11) to Sepharose 4B (2–3 mg of protein per ml). Because the defatted HMFG was difficult to dissolve, affinity chromatography was performed with HMFG material, hereafter referred to as S-HMFG, prepared by the sodium dodecyl sulfate/mercaptoethanol method (12). (The HMFG used to prepare S-HMFG was obtained from different donors than those used to isolate defatted HMFG used as immunogen.) Five milligrams of protein of the S-HMFG, dissolved in phosphate-buffered saline and 1% (vol/vol) Triton X-100, were applied to a 10 ml anti-HMEC-Sepharose-4B column that was equilibrated with 1% Triton X-100 in phosphate-buffered saline, and allowed to bind for 45 min at room temperature. Unbound S-HMFG was rinsed from the column and collected. The column was then washed with three volumes of 1% Triton X-100. The fraction of S-HMFG that bound was eluted with 1 M acetic acid in 1% Triton X-100, dialyzed against distilled water, lyophilized, and finally analyzed by polyacrylamide gel electrophoresis as given above.

Abbreviations: HMFG, human milk fat globule; S-HMFG, human milk fat globule material prepared by the sodium dodecyl sulfate/mercaptoethanol method (12); HMEC, human mammary epithelial cell.

Affinity chromatography of ^{125}I-labeled S-HMFG, prepared by the chloramine-T method (13), was performed as above except that 0.1% bovine serum albumin was added to all solutions. The specifically bound material was eluted and run in polyacrylamide gel electrophoresis. Gels were sectioned and radioactivity distribution was determined (14).

Human cell cultures were grown, unless otherwise indicated, in plastic culture dishes under standard culture conditions in Waymouth's medium plus 10% fetal calf serum with 100 units of penicillin and 100 μg of streptomycin per ml. Human cell lines 734B (15), BT-20 (16), Hs578T, A704 (17), A498 (17), A427 (17), HT-29 (18), SH-4 (19), Hs906TCE, HT1417*, and Hs578Bst were kindly provided by A. Hackett, Naval Biomedical Research Laboratory, Oakland, Calif. Cell line MCF-7 (15) and normal human mammary cells from breast fluid, obtained from a normal woman 1 year after weaning and cultured for 3 weeks (20), were donated by G. Buehring, School of Public Health, University of California, Berkeley, Calif. Cell lines WI-38 (21) and MDA-MB-157 (22) were provided by E. M. Jensen, Mason Research Institute, Rockville, Md. Normal human mammary fibroblasts were obtained from normal breast tissue removed during elective cosmetic surgery.

In preparation for immunofluorescence staining, the cells in monolayer cultures were dispersed in 0.05% trypsin/0.02% EDTA and then suspended in growth medium, layered on top of a 0.5% agar substratum, also made up in growth medium, and incubated for 48 hr. The cells did not attach to the agar substratum but remained in suspension individually or in small clumps.

Indirect immunofluorescence staining was carried out as described previously (4) using an amplifying layer of fluorescein-conjugated goat antiserum to rabbit gamma globulin (Antibodies, Inc., Davis, Calif.). A gamma globulin preparation from a nonimmunized rabbit served as a control for each assay (see Fig. 2I). All staining reactions were carried out on cells in suspension except for cells from human breast fluid that were stained in culture dishes *in situ* and then gently removed with a rubber policeman for observation.

RESULTS

Polyacrylamide gel electrophoresis revealed that the defatted HMFG is composed of four main proteinaceous components (Fig. 1B). Other minor components were also seen in individual gels; however, their presence was not detected routinely. Since some membrane components do not stain well with Coomassie blue (12), other components may not have been detected. Two of the four Coomassie-blue-positive components are positive with periodic acid–Schiff stain, suggesting the presence of glycoproteins (Fig. 1A).

The antigenicity of the HMFG was tested by affinity chromatography. S-HMFG that had an electrophoretic profile similar to that of the defatted HMFG was applied to an anti-HMEC-conjugated Sepharose 4B column. Approximately 10% of the S-HMFG material added bound to the column and was eluted with 1 M acetic acid. This eluate contained three of the four major components found in HMFG (Fig. 1C). In order to verify that the acid-eluted material was indeed HMFG material and not immunoglobulins or fragments thereof that disassociated from the column matrix, a similar experiment was done using ^{125}I-labeled S-HMFG. All four major components of the HMFG were labeled with ^{125}I, and the same three components as with the unlabeled S-HMFG were found to bind. In this experiment approximately 5% of the added ^{125}I-S-HMFG bound, whereas in a control experiment with goat anti-rabbit gamma globulin conjugated to Sepharose, only 0.1% of the added ^{125}I-S-HMFG bound.

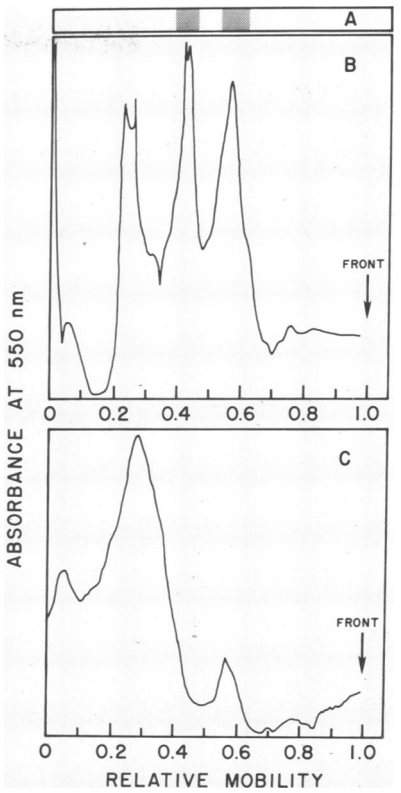

FIG. 1. Polyacrylamide gel electrophoresis of human milk fat globule (HMFG) material and products of its fractionation by affinity chromatography with Sepharose-4B-anti-HMEC. (A) Sketch of gel containing defatted HMFG stained for carbohydrate with periodic acid–Schiff reagent (origin at left). (B) Densitometry tracing of gel containing defatted HMFG stained for protein with Coomassie blue. (C) Densitometry tracing of gel stained with Coomassie blue containing products of S-HMFG (see *Materials and Methods*) that bound specifically to a Sepharose-4B-anti-HMEC column and eluted with 1 M acetic acid.

The anti-HMEC raised against HMFG stained by indirect immunofluorescence only cells of mammary epithelial origin (Table 1). Cells collected from normal human breast fluid (23) stained specifically on their surface (Fig. 2C). Our observation that only 50% of these cells appear to be epithelial (Table 1) is consistent with reports of others (23) that other cell types are present in breast fluid, e.g., foam cells, histiocytes, lymphocytes, and neutrophils. The surface of the stained normal cells was covered with blebs as seen in phase contrast (Fig. 2D), and it was some of these blebs that stained intensely (Fig. 2C). In contrast, a more regular perimeter membrane staining was observed on most cells derived from mammary carcinomas (Fig. 2G). On some cells in the same preparation staining covered the entire surface, and on others, it appeared in patches. This perimeter pattern of membrane staining was observed with all five breast carcinoma cell lines assayed (Table 1). Electron microscopy of four of these cell lines—namely 734B, MCF-7, BT-20, and MDA-MB-157—reveals the presence of desmosomes, duct-like vacuoles, and tonofibrils, characteristic of epithelial cells (24).

The percentages and intensities of fluorescence staining varied with the cell lines tested (Table 1). Nevertheless, apparent similarities of staining for line 734B and MCF-7 can be

* Isolated by S. Rasheed, University of Southern California, Los Angeles, Calif.

Table 1. Indirect immunofluorescent staining of human cells with anti-HMEC

Cells	Passage level	Tissue of origin	Specific fluorescent intensity*	Percentage of cells stained
Normal breast	0	Breast fluid	+4	50
734B	12	Breast carcinoma	+4	70
MDA-MB-157	96	Breast carcinoma	+3	60
BT-20	40	Breast carcinoma	+2	70
	247	Breast carcinoma	+2	70
MCF-7	160	Breast carcinoma	+4	70
Hs578T	9	Breast carcinosarcoma	+2	40
A498	22	Kidney carcinoma	0	0
A704	19	Kidney carcinoma	0	0
A427	34	Lung carcinoma	0	0
HT-29	134	Colon carcinoma	0	0
Hs578Bst†	8	Breast ‡	0	0
WI-38†	15	Normal embryonic lung	0	0
HMF†	2	Normal breast	0	0
HT1417	24	Lymphoma	0	0
SH-4	105	Melanoma	0	0
Hs906TCE	4	Melanoma	0	0
Leukocytes	0	Blood, buffy coat	0	0

* Intensity was judged on scale of 0 to +4 and specificity was evaluated by comparing anti-HMEC to normal rabbit gamma globulin.
† Typical fibroblastic morphology; HMF is normal human mammary fibroblasts.
‡ Hs578Bst was derived from connective tissue adjoining the breast carcinosarcoma from which cell line Hs578T originated.

correlated with the fact that they originated from the same pleural effusion. However, these two cell lines had distinctly different chromosome numbers (15) and different passage levels *in vitro* (Table 1). In addition, BT-20 cells stain with the same percentage and with equal intensity at two different passage levels (Table 1).

The HMFG was stained in a perimeter-type fashion (Fig. 2A). The free fat droplets that appear as birefringent spheres in phase contrast microscopy (Fig. 2B) do not fluoresce, indicating that the anti-HMEC does not bind to HMFG fat or substances dispersed in it.

These normal cell surface antigens present on the HMFG, on normal mammary epithelial cells, and on cell lines derived from mammary carcinomas, are not detectable on fibroblasts derived from normal breast tissue (Fig. 2E), from breast tissue adjoining a carcinosarcoma (Hs578Bst), or from embryonic lung (WI-38); all of them have characteristic fibroblastic morphology. Also, these HMFG membrane antigens are not expressed on human white blood cells. Furthermore, they are not found on cells derived from two kidney carcinomas (A498 and A407), a lung carcinoma (A427), a colon carcinoma (HT-29) (Fig. 2K), two melanomas (SH-4 and Hs906TCE), or a lymphoma (HT1417) (Table 1). Absorption of anti-HMEC with 2 volumes of packed colon carcinoma cells (HT-29) did not diminish binding to MCF-7 cells.

In order to test whether or not the antigens recognized by anti-HMEC are located on the cell surface, mammary carcinoma cells of cell line 734B were treated with 0.25% trypsin for 30 min at 37° before staining. The antigenic components were completely removed by this treatment, while the cells remained intact. Moreover, pre-absorption of anti-HMEC with 734B cells or the HMFG prevented the subsequent staining of 734B cells.

DISCUSSION

The unique characteristics of the HMFG make it the best choice for the procurement of human mammary cell surface antigens. The present communication demonstrates that antibodies raised against defatted HMFG stain by immunofluorescence cells derived from breast epithelium, and do not stain cells originating from other tissues. Thus, this antibody preparation appears to be organ-specific. The specific antigens binding anti-HMEC are not associated with epithelial-like morphology, because they do not bind to epithelial-like cells derived from kidney, lung, and colon. Also, these antigens are not shared with other cells of the ectodermal layer such as those of melanomas. In addition, the antibodies may well be cell-type-specific, because they do not stain breast fibroblasts.

The antigens detected by anti-HMEC are located on the breast epithelial cell surface as demonstrated by: (*a*) the perimeter-type of immunofluorescent staining; (*b*) their removal by trypsin treatment; and (*c*) the binding of anti-HMEC to the membrane of the milk fat globule and not to its contents (Fig. 2A and B). Moreover, the antigens detected on the breast cell surface were shown to be the same as those located on the HMFG membranes, since antisera absorbed with these membranes no longer stained breast cell lines.

Because viable, intact cells were used in the present experiments rather than fixed cells, the possibility that the surface antigens detected by anti-HMEC are also present in intracellular membranes cannot be ruled out. Penetration of the anti-HMEC into intact cells cannot be ensured. For this same reason, if small quantities of intracellular material were present in the defatted HMFG (25, 26) that was used as a source of immunogen and they gave rise to subpopulations of antibodies in anti-HMEC against intracellular antigens, the latter antigens would not be detected.

These normal breast differentiation antigens are stable characteristics, because they continue to be expressed in breast carcinoma cell lines even after many passages *in vitro* and much variation in chromosome number. The first point is demonstrated by the similar staining of BT-20 cells at 40 and 247 passages, and of 734B and MCF-7 cells, which arose from the same pleural effusion (15) but have 12 and 160 passage levels, respectively. Also, these antigenic characteristics continue to be expressed in metastases of breast carcinomas as exemplified

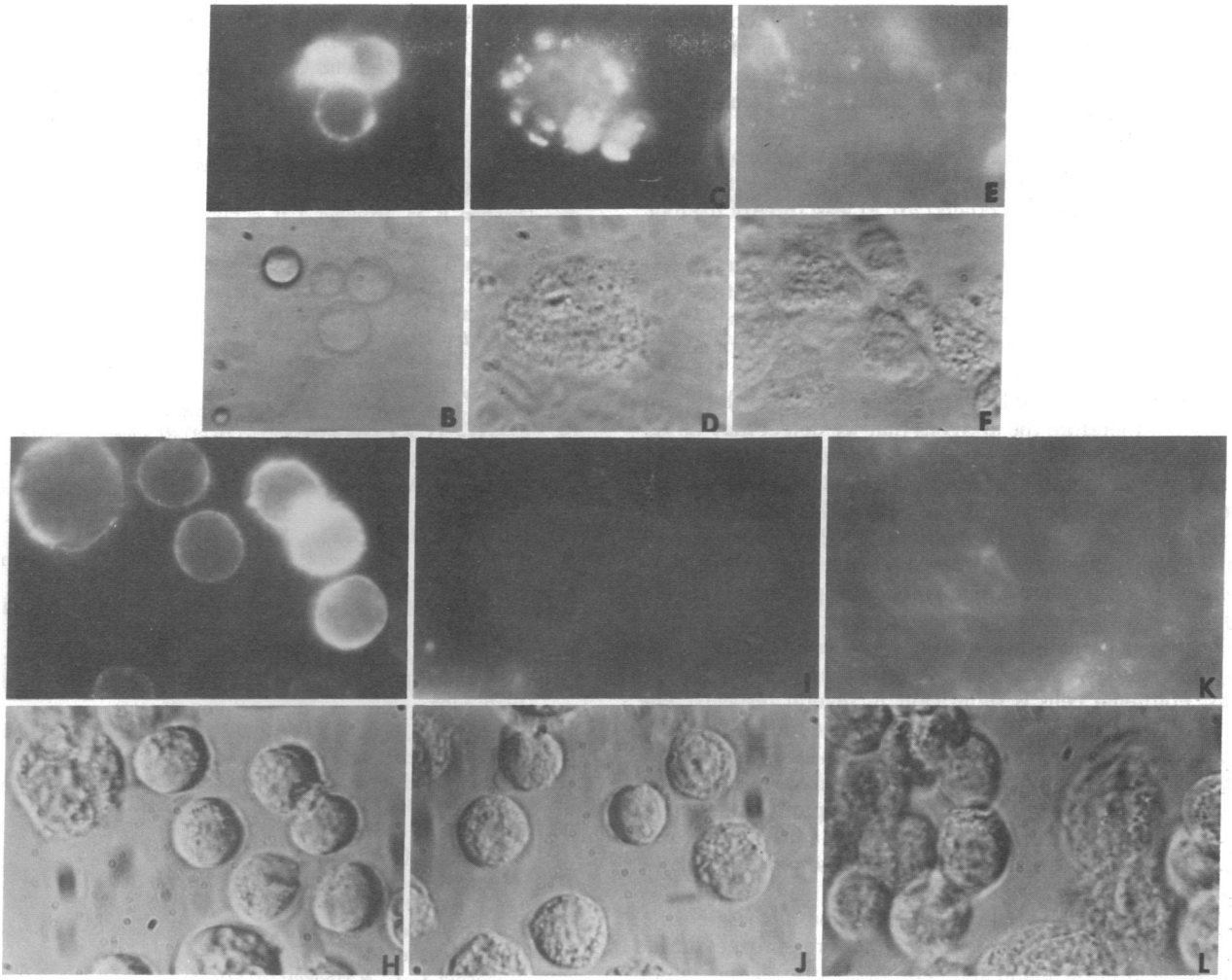

FIG. 2. Indirect immunofluorescence staining with anti-HMEC; (A and B) human milk fat globule; (C and D) a normal human epithelial cell from breast fluid; (E and F) normal human breast fibroblasts; (G and H) breast carcinoma cell line (MDA-MB-157) with (I and J) normal rabbit gamma globulin control; (K and L) colon carcinoma cell line (HT-29). (A, C, E, G, I, and K) Fluorescence microscopy. (B, D, F, H, J, and L) Phase contrast microscopy. ×400

by the staining of cell lines 734B, MCF-7, and MDA-MB-157, as well as by being present on cell lines (BT-20 and Hs578T) from primary solid breast tumors. It would appear that chromosomal variations do not affect antigenic specificity because all of the breast carcinoma cell lines that stained with anti-HMEC are heteroploid (27).

In spite of this antigenic persistence after neoplastic transformation in the breast cell lines, the antigenic expression appears different for each breast tumor cell line (Table 1). These differences could be due either to loss of one or more components, or to quantitative changes. Phenotypic variation of differentiated characteristics is commonly observed in neoplastic tissue (28). Also in other systems, tumor cell membranes have been found to continue to carry differentiation antigens, such as TL antigens (alloantigens characteristic of mouse thymus cells) found in murine leukemias (29) and NS-1 (nervous system antigen-1) detected on mouse glioma cells (2).

The HMFG provides an excellent source of normal breast epithelial cell antigens that can be used as a normal reference in monitoring variation in antigenic expression after neoplastic transformation. In contrast, antibodies produced against tumor cells that react with normal antigens, as in the case of the brain-specific antigen NS-1 (2), could correspond to only part of the spectrum of antigens carried by the normal cell.

Histocompatibility-type antigens are probably not the major antigenic components detected here, because: (*a*) breast carcinoma cells originated from different donors and anti-HMEC recognizes all of them (albeit at different intensities); (*b*) anti-HMEC failed to stain breast fibroblasts (Hs578Bst) derived from the same individual as the breast carcinosarcoma cells (Hs578T) that it did stain [fibroblasts express the same HL-A antigen profile as ectodermal cell lines (melanomas) (30)], and (*c*) anti-HMEC absorbed with HT-29 colon carcinoma cells bound as well to MCF-7 breast carcinoma cells as did the unabsorbed antiserum. Further, the HMFG material used for immunization was obtained from a pool of milk donors and the anti-HMEC was absorbed with human blood cells to remove species-specific reactivity.

Mucoprotein(s) obtained from the bovine milk fat globule are antigenic (31); however, they differ from the HMFG antigens in that antibodies against the former do not bind to normal human breast but do bind to different human neoplastic and fetal tissues (31). Others who have immunized with the bovine milk fat globule generated antibodies that hemolyzed homologous red blood cells (7). In our studies, populations of antibodies in the anti-HMEC that reacted with erythrocytes were removed by absorption. Electrophoretic patterns of glycoproteins and proteins of human erythrocyte ghosts were

different from defatted HMFG patterns in terms of the number of bands and relative mobilities (unpublished results), suggesting that different components are present. This supports the idea that anti-HMEC recognizes differentiation antigens of the breast epithelial cell.

The presence of multiple proteinaceous components on the surface of the human breast epithelial cell is evident from the present results, and those of others (25). At least three of the four major components of the HMFG are antigenic (Fig. 1) and it is conceivable that more refined techniques could separate each into several distinct antigens. With several specific antigens on the surface of the normal breast epithelial cell, it is possible that, in different breast tumors and cell lines, significant and detectable variation in their expression can occur.

The HMFG is derived from the apical surface of the breast cell (6) and therefore the antigens that we detect may be restricted to this specialized surface. Enzymes that are markers for cell plasma membranes are found in high concentrations in milk fat globule preparations (7, 26) and in mammary cell fractions enriched for plasma membranes (32). A stronger proof than these enzyme studies that the outer envelope of the HMFG is derived from the plasma membrane is the presence of similar antigens on both of them, as shown here.

The present demonstration that antibodies can be made that recognize cell-type-specific antigens on the surface of human breast epithelial cells is significant in several respects. The specificity of anti-HMEC will allow for the identification of normal and neoplastic breast epithelial cells in mixed primary cultures and in isolated metastases with unknown carcinoma of origin (33), and provide a means for the purification of this cell type from mixed cell suspensions. Also, anti-HMEC could be used as a vector to carry cytotoxic substances to neoplastic breast tissue (33).

The study of the variations in expression of these normal differentiation antigens (at least three) of breast tissues during development of the gland and in preneoplastic and neoplastic lesions could provide an insight into their role in regulation of growth, morphogenetic arrangement, and possible metastatic ability of breast cells. Further, this approach could permit the establishment of precise breast epithelial cell antigenic patterns for high-risk populations in terms of mammary carcinogenesis.

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