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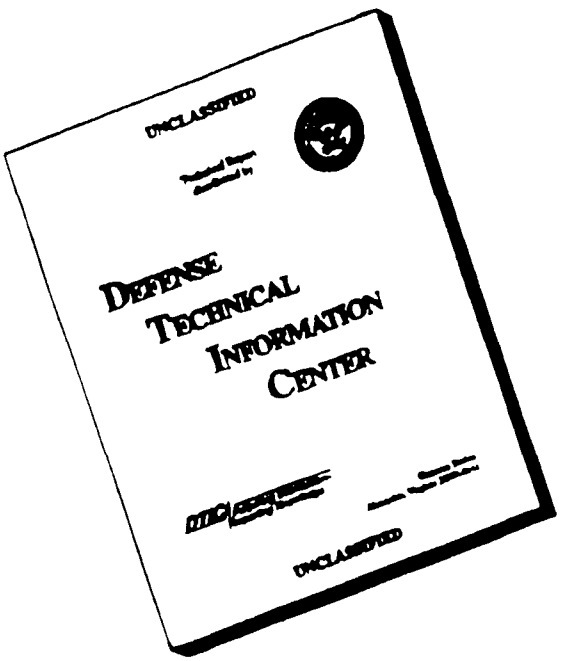

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We are attempting to identify integrin-specific signalling events that may contribute to malignant transformation in mammary epithelium. We are focusing on the extracellular matrix protein laminin-5r, which is located in mammary epithelial basement membrane. We hypothesize that binding of integrins to laminin-5r elicits downstream signalling events that may help define the normal phenotype of mammary epithelial cells; disruption of these signals may contribute to the development of malignancy. To date, we have established that normal and malignant cell lines plated on laminin-5r differ in at least three ways: normal and malignant cells use different integrin receptors to bind laminin-5, malignant cells migrate while normal cells do not, and normal and malignant cells exhibit different patterns of tyrosine phosphate-containing proteins following binding to laminin-5. The integrins that mediate these responses have been identified in two cell types, and we hypothesize that in these two cells, the conversion from normal to malignant phenotype involves the activation of the $\alpha 3\beta 1$ integrin. Our goals for the upcoming year are to further elucidate the signalling pathways associated with the $\alpha 3\beta 1$ integrin following binding of laminin-5r, and to identify functionally active regions of laminin-5r responsible for eliciting these effects.

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Introduction

Extracellular matrix (ECM) proteins play a critical role in regulating cell growth, migration, and differentiation. During malignant transformation, cells deregulate their adhesion to ECM molecules and become highly migratory. Cells bind ECM molecules through specific receptors, a majority of which belong to the integrin family of cell surface adhesion molecules. Integrins are heterodimers, consisting of one α and one β subunit; at present, at least 14α and 8β integrin subunits have been identified, which organize into at least 20 different receptors that bind a wide variety of ECM and cell surface molecules (1).

How specific ECM molecules and their integrin receptors contribute to malignant transformation in vivo remains largely unknown. Previous studies have suggested that changes in expression of ECM molecules in situ may contribute to malignany in breast, although the functional significance of these changes is not yet known [e.g., (2-9)]. Likewise, α2, α3, and α6 integrins may play a role during mammary tumorigenesis, but the correlation between expression of these integrins and tumor progression is not well established [e.g., (10-19)].

In this study we examined the adhesion and migration of normal (HUMEC) and malignant (MCF-7, MDA-MB-231, MDA-MB-435) mammary epithelial cells on one member of the laminin family of ECM proteins, rat laminin-5 (LM-5r) . We found that while both HUMEC and MCF-7 cells preferentially adhered to LM-5r through the α3β1 integrin receptor in rapid adhesion assays, only MCF-7 cells migrated on LM-5r in Transwell and colloidal gold displacement assays; MDA-MB-231 and MDA-MB-435 cells bound LM-5r through a β1 integrin receptor that is resistant to antibodies that block the function of α1, α2, α3, α4, and α6 integrin subunits. Both HUMECs and MCF-7 cells exhibit equal strength of adhesion to LM-5r in centrifugal detachment assays. Stimulation of HUMECs with either serum factors or the β1 integrin activating antibody TS2/16 increased haptotactic migration 2-3 fold, and this migration was inhibited by anti-α3 integrin antibodies. Together these findings suggest that α3β1-mediated adhesion to LM-5r may play a role in maintenance of the normal phenotype as well as malignant progression in breast, and that activation of the α3β1 integrin or expression of novel integrins may contribute to development of the the malignant phenotype in mammary epithelium.

Body:

Materials and Methods

Cells. HUMECs (ninth passage) were purchased from Clonetics (San Diego, CA), maintained in Mammary Epithelial Growth Medium (Clonetics) a serum-free medium, and used by passage 12. Cells were passaged using the Clonetics Reagent Pack as indicated by the manufacturer. MCF-7, MDA-MB-231, and MDA-MB-435 cells were maintained in RPMI supplemented with 10% fetal calf serum (Gemini, Irvine CA) and 2 mM glutamine/penicillin G (100 units/ml)/Streptomycin sulfate (100 μg/ml) (BioWhittaker, Walkersville MD), and routinely passaged using trypsin/EDTA (Biowhittaker). MCF10A cells were maintained and passaged in DFCI medium according to Band and Sager (20). Rat 804G cells were passaged and maintained under conditions identical to those for MCF-7 cells except that DMEM was substituted for RPMI medium. All cells were maintained at 37°C in a humidified incubator containing 10% CO_2. 804 cell conditioned medium was collected after 3 days of culturing and was clarified by centrifugation at 1500 x g.

Antibodies. Rat monoclonal antibody GoH3 against the α6 integrin was purchased from Immunotech (Westbrook ME). Mouse monoclonal antibodies against α2 (clone P1E6), α3 (clone P1B5), and β1 (Clone P4C10) were purchased from Gibco (Gaithersburg, MD). Mouse antibody MOPC 31c as well as goat anti-mouse and anti-rat secondary antibodies coupled to fluorescein isothiocyanate were purchased from Sigma (St. Louis, MO). Mouse monoclonal antibodies TR1, FM3, and CM6 against LM-5r were isolated and purified from ascites fluid as described elsewhere (21). Mouse monoclonal antibody PY20 against phosphotyrosine was purchased from Transduction Laboratories (Lexington KY). Rat monoclonal antibody 9EG7 was purchased from Pharmingen (San Diego CA). Mouse monoclonal antibodies against α1 and α4 integrins were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Mouse monoclonal antibody TS2/16 was kindly provided by Martin Hemler (Dana Farber Cancer Institute, Boston MA).

Adhesion assays. Untreated 96 cell plates (Sarstedt, Newton, NC) were coated for at least two hours at room temperature with rat or mouse laminin-1 (Gibco), human laminin-2 (placental laminin, Gibco), anti-LM-5r monoclonal antibodies (all at 20 μg/ml) or with human fibronectin or human vitronectin (both at 40 μg/ml, Gibco). All proteins were diluted in 100 mM carbonate buffer, pH 9.3. Plates were then washed twice with phosphate buffered saline (PBS) containing 0.2% Tween 20 (PBST) and blocked overnight at 4°C with blotto (5% nonfat dried milk in PBST). Following two washes with PBST, wells containing anti-LM-5r antibodies were incubated for 1 hour at room temperature with 804G cell conditioned medium, thereby allowing for "capture" of soluble laminin-5r, then washed twice with PBST. For CM6 antibody blocking experiments,

wells were incubated with indicated concentrations of blocking antibodies diluted in blotto. As controls, wells were blocked with blotto alone, irrelevant antibody MOPC 31c, or anti-laminin-5r monoclonal antibody FM3. Wells were washed twice with PBST.

In some experiments, cells were allowed to attach to 804 cell matrix or wells coated with 804G cell conditioned medium. To prepare 804G cell matrix, cells were grown to confluency in Sarstedt 96 well plates, the culture medium was removed and the cells were washed in sterile PBS. The cells were removed according to the method of Gospodarowicz (22) by incubating them 2 x 5 minutes in 20 mM sterile NH_4OH. The wells were extensively washed with PBS and distilled water and blocked with blotto as described above. Additional wells were coated for 2 hours with 804G cell conditioned medium, then washed and blocked as described above.

Cells were collected by brief trypsinization, blocked with either serum-containing medium or Tryspsin Inhibitor solution (Clonetics), washed twice with DMEM/1% bovine serum albumin, then plated (1.2×10^5/ well) in DMEM/1% bovine serum albumin/25 mM HEPES, pH 7.2. For anti-integrin antibody blocking experiments, cells were incubated at room temperature with blocking antibodies for 30 minutes prior to addition to plates; blocking antibodies were present during plating. Plates were kept at 37°C in a humidified incubator containing 10% CO_2 for 30 minutes. To remove unbound cells, wells were then filled with PBS and the plates were inverted in a tank of PBS and allowed to gently shake for 15 minutes. Excess PBS was absorbed from the wells by inverting plates on paper towels. Bound cells were fixed in 3% paraformaldehyde/PBS, then stained with 0.5% crystal violet in 20% methanol/80% H_2O. Wells were washed with water to remove excess dye, then cells were solubilized in 1% SDS and the amount of dye was quantitated using a Molecular Devices plate reader set to absorb at 595 nm.

Centrifugal detachment assays. The detachment assay which measures strength of adhesion is an adaptation of the assays previously described (23,24). Briefly, rectangles of polystyrene were cut to fit a 96-hole silicon gasket (BioRad, Hercules, CA). The wells of the assay plate were affinity coated with laminin-5r using 10 µg/ml TR1 antibody as described above, then washed and blocked with blotto at 4°C overnight. HUMEC and MCF-7 cells labeled overnight with 10 µCi ^{35}S-methionine and cysteine (Translabel; ICN, Costa Mesa, CA) in 90% methionine-free RPMI medium (ICN) were collected as described above, plated (10,000 cells/well) in DMEM/1% BSA, and allowed to bind for 25 minutes at 37°C. Plates were flooded with warm PBS, sealed, inverted and centrifuged for 15 minutes at 80, 1200, 0r 1450 x g. As a control, one set of plates was inverted in PBS for 15 minutes, representing a 1 x g "spin." The entire plate, still inverted, was submerged in cold PBS and then in fixative (3.7% formaldehyde/5% sucrose/0.1% Triton X 100/PBS). After air-drying, the bound radioactivity, representing cell adhesion, was quantified on a

Molecular Dynamics phosphorimager.

Transwell haptotactic migration assays. Transwell filters (8.0 μm pore size, Costar, Cambridge MA) were coated for 4 hours with identical concentrations of extracellular matrix proteins used in adhesion assays, diluted in Hanks Balanced Saline Solution (Biowhittaker). Separate filters were coated for 1 hour with mouse monoclonal antibody TR1, diluted in 100 mM carbonate buffer. Antibody-coated filters were blocked for 2 hours with blotto, then incubated for 1 hour with 804G cell conditioned medium, thereby allowing for "capture" of LM-5r on the filter. For CM6 antibody blocking experiments, filters were incubated with indicated concentrations of blocking antibodies diluted in blotto. As a control, filters were blocked with blotto alone. Following two washes with PBST, filters were inverted and cells (6×10^4 cells/filter) were plated on the uncoated side in migration medium (DMEM/2 mM glutamine/1 mM sodium pyruvate). For anti-integrin antibody blocking experiments, cells were incubated with blocking antibodies in migration medium for 30 minutes prior to plating on filters. Antibodies were also present in migration medium throughout the migration assay. Cells were maintained at 37°C in a humidified incubator containing 10% CO_2 for 18 hours, then filters were fixed and cells stained using the Diff-Quik stain kit (Baxter, McGaw Park, IL). The uncoated side of each filter was wiped with a cotton-tipped applicator to remove cells that had not migrated through the filter. Filters were then cut from their supports, mounted on slides and viewed under bright field optics. To quantitate migration, stained cells were counted in four fields (under 30x magnification) from each of two filters for each condition. Results were expressed as the mean number of cells counted in each field ± the standard deviation.

Colloidal gold uptake motility assays. Colloidal gold motility assays were performed exactly as described by Albrecht-Buhler (25). Colloidal gold particles coated on glass coverslips were coated with LM-5r affinity captured by TR1 as described for adhesion assays. As controls, coverslips coated with gold particles were blocked with blotto and incubated with 804G cell conditioned medium. Cells were collected as for cell adhesion assays and plated (5,000/well) in 6 well plates containing coated coverslips. After 18 hours, cells were fixed in 10% formalin/PBS, viewed under dark field microscopy and photographed using Kodak Gold 200 color print film. Migration was quantitated by digitally scanning prints of photographic images (Scanjet IIcx; Hewlett Packard, Palo Alto CA, USA), and computing the black area (displaced gold) in scanned images using Adobe Photoshop 3.0 (Adobe Systems, Inc., Mountain View, CA, USA) running on a Quadra 950 computer (Apple Computer, Inc., Cupertino, CA, USA.). The results were expressed as the ratio of displaced gold area to the total area of each image ± standard deviation (n=3).

FACS analysis. Cells were typsinized, blocked, and washed as for adhesion assays, then washed twice with ice cold FACS buffer (Hanks

Buffered Saline Solution containing 5% fetal calf serum and 0.02% NaN_3), with each wash followed by gentle centrifugation at 4°C (500x g). All subsequent steps were performed at 4°C. Cells were then incubated for 1 hour with anti-integrin antibodies diluted at the same concentrations used for adhesion and migration assays in FACS buffer, then washed twice with secondary antibody buffer (Hanks Buffered Saline Solution containing 5% goat serum and 0.02% NaN_3). Cells were incubated for one hour with goat anti-mouse or goat anti-rat secondary antibodies coupled to fluorescein isothiocyanate (diluted 1:128 or 1:200, respectively, in secondary antibody buffer), washed twice with FACS buffer, then analyzed on a Beckton-Dickinson FACScan flow cytometer. As a control, cells were incubated with anti-mouse or anti-rat secondary antibodies only.

Polymerase chain reaction. Reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out as described previously (26). Briefly, poly(A)+ RNA from HUMEC and MCF-7 cells was isolated using the Micro-FastTrack mRNA isolation kit (Invitrogen, San Diego CA). First-strand cDNA was prepared by using oligo(dT) primers and the RT-PCR kit (Stratagene, San Diego). Oligonucleotides were synthesized with a Cyclone Plus DNA synthesizer (Millipore, Bedford MA). Primers for α3 were: 3484, 5'-AAGCCAAGTCTGAGACTGTG-3'; and 3485, 5'-GTAGTATTGGTCCCGAGTCT-3', corresponding to nucleotides 2757-2776 and 3393-3413, respectively, of the human α3 sequence (27).

Western blots. Extracellular matrix from HUMEC and MCF-7 cells was isolated as described previously (21) and probed for the presence of laminin-5 by western blot using the polyclonal antibody 0668B as described previously (21).

Immunoprecipitation. HUMEC and MCF-7 cells were surface labeled with biotin using Sulfo-NHS-LC-Biotin (Pierce, Rockford IL) as directed by the manufacturer. Surface labeled cells were solubilized on ice in PBS/2% Renex30 (Accurate Chemicals, Westbury NY)/1 mM PMSF/1 μg/ml aprotinin/1 μM leupeptin/1 μM pepstatin. Lysates were precleared by adding sepharose beads coupled to goat anti-mouse IgG antibodies (Sigma) for 30 minutes at 4°C, and clarified by centrifugation at 4°C. α3 integrin subunits were immunoprecipitated from lysates by adding 10 μl cell lysate, 5 μl P1B5 antibodies and 10 μl antibody-coupled agarose per sample to 200 ml immunoprecipitation buffer (50 mM Tris-HCl/0.5 M NaCl/1 mM $CaCl_2$/ 1 mM $MgCl_2$/0.1% Tween 20) and rocking overnight at 4°C. Agaorose beads were pelleted by centrifugation and washed 5 times with immunoprecipitation buffer, then boiled in reducing sample buffer. The supernatants were separated by SDS-PAGE, transferred to PVDF membranes, blocked overnight with 0.1% Tween 20/0.2% gelatin/PBS at 4°C, then incubated with streptavidin conjugated to horseradish peroxidase (Boehringer Mannheim, Indianapolis, IN) diluted 1:4000 in blocking buffer for 1 hour. Membranes were washed 3x with

PBS/0.2% Tween 20 and developed using the ECL chemiluminescence kit according to the manufacturer (Amersham, Arlington Heights, IL).

Results.

As outlined in the original grant proposal, the specific aims of this study are:

1) To isolate cell surface complexes associated with laminin-binding integrins ($\alpha 2\beta 1$, $\alpha 6\beta 1$) from normal human mammary epithelial cells and from three human mammary cell lines that display varying degrees of metastatic capability.
2) To directly examine tyrosine kinase activity associated with these isolated complexes.
3) To identify tyrosine kinases and/or kinase substrates in isolated integrin complexes whose activity and/or phosphorylation state correlates with the degree of malignancy exhibited in these cells.
4) To identify novel integrin-associated proteins whose presence in these integrin-associated complexes correlates with the degree of malignancy exhibited in these cells; these may serve as therapeutic targets for future drug design.

Work during the first year of this grant focused on characterizing a relatively new laminin isoform, laminin-5r, which was substituted for laminin-1 in this project because it is a more physiologically relevant ligand for mammary epithelial cells. The data obtained during the first year of this grant allowed us to formulate three hypotheses regarding the contribution of laminin-5r and its receptors to development of the malignant phenotype in mammary epithelium. The results obtained for each hypothesis, and the corresponding contribution to the original specific aims, are described below.

Hypothesis 1. Changes in adhesion and spreading on laminin-5r in malignant cells is due to modified expression of integrin receptors for laminin-5r (Specific Aim 1). This hypothesis predicts that a) normal and malignant cells may use different integrin receptors to bind laminin-5r, or b) that normal and malignant cells use the same integrin receptor but that the molecular complexes assembled by this receptor differ significantly.

These predictions were tested using normal human mammary epithelial cells (HUMECs) and three malignant cell lines (MCF-7, MDA-MB-231, and MDA-MB-435) in rapid (30 minute) adhesion assays. HUMECs adhered efficiently to wells coated with LM-5r captured with a specific monoclonal antibody TR1 (Fig. 1), 804G cell extracellular matrix, or 804G cell conditioned medium (not shown) (see Materials and Methods). Rapid adhesion of HUMECs to all other ECM molecules tested was relatively poor (Fig. 1). The most active of these was laminin-2, which supported approximately 20% as much adhesion as LM-5r (Fig. 1). As controls, plates coated with antibody, the blocking agent "blotto", or DMEM/10% FCS did not promote adhesion. All three malignant cell lines adhered best to LM-5r, but, in contrast to HUMECs, they also adhered well to all other ECM molecules tested (Fig. 1).

To investigate the contribution of specific integrins to adhesion of normal and malignant cells to LM-5r, we used blocking monoclonal antibodies that recognize integrin chains reported to be involved in binding to laminins: P1E6 against α2 (28), P1B5 against α3 (28), GoH3 against α6 (29), and P4C10 against β1 integrins (30). In rapid adhesion assays, antibodies against α3 and β1 integrin subunits reduced adhesion of HUMECs and MCF-7 cells by greater than 90% (Fig. 2). In contrast, antibodies against α2 and α6 had no inhibitory effect on the adhesion of either cell type to LM-5r. We conclude that both HUMEC and MCF-7 cells utilize α3β1 integrin receptors to bind LM-5r.

Both MDA-MB-231 and MDA-MB-435 cell lines were inhibited from binding LM-5r by antibodies against the β1 integrin subunit, but antibodies against the α2, α3, and α6 integrin subunits failed to significantly interfere with adhesion in these cell types (Fig. 2). Additional experiments using higher concentrations of anti-α subunit inhibitory antibodies (up to 50 μg/ml) and inclusion of antibodies that block the function of α1 and α4 integrins failed to inhibit adhesion of either cell type (not shown). FACS analysis with these antibodies revealed that each antibody recognized an epitope on the surface of these cells, demonstrating that these integrins are expressed but are not used in adhesion to LM-5r (Fig. 3). In control experiments the same concentrations of GoH3 antibody was able to block MDA-MB-231 and MDA-MB-435 cell adhesion to laminin-1, demonstrating that the α6β1 integrin is functionally expressed on the surface of these cells and that this function is inhibitable by GoH3 (Fig. 4). We conclude that the MDA-MB-231 and MDA-MB-435 cell lines differ from HUMECs and MCF-7 cells in that they do not use the α3β1 integrin to bind LM-5r; rather, they use a β1 integrin that is resistant to α1, α2, α3, α4 and α6 function blocking antibodies.

Because we identified the integrin receptor for LM-5r in HUMEC and MCF-7 cells, we choose to focus our studies on these two cell types in hopes of identifying integrin-specific differences in the behavior of these two cells.

<u>MCF-7 cells use α3β1 integrin to migrate on LM-5r</u>.

To measure haptotactic migration, we plated either HUMECs or MCF-7 cells on the upperside of Transwell filters (8 μm pore diameter) coated on the underside with LM-5r. MCF-7 cells migrated very efficiently through LM-5r coated filters (Fig. 5). In contrast, HUMECs did not: they were as migratory on LM-5r coated filters as on control (BSA) coated filters (Fig. 5). Consistent with our cell adhesion data, migration of MCF-7 cells was

inhibited by the anti-LM-5r antibody CM6 and antibodies directed against α3 and β1 integrins, while antibodies directed against α2 or α6 integrins had no effect (Fig. 5).

To measure random cell motility, cells were plated on colloidal gold particles coated with antibody-captured LM-5r. As cells randomly migrated they displaced the gold particles leaving behind a dark trail easily viewed under dark field optics (25). Consistent with our Transwell assays, MCF-7 cells were actively motile on LM-5r coated gold particles. They formed rounded, multicellular aggregates, appeared to collect LM-5r-coated gold particles on their surfaces, and were loosely adherent to the glass. On control, BSA coated particles, they instead became spread, migrated poorly, and did not aggregate (Fig. 6). In contrast, HUMECs migrated on LM-5r at background levels, approximately equal to that on control, BSA coated gold particles (Fig. 6).

In summary, both HUMECs and MCF-7 cells adhere efficiently to LM-5r through the α3β1 integrin receptor. However, HUMECs are statically adherent, whereas MCF-7 actively migrate on LM-5r. Apparently, the same receptor is responsible for these distinct behaviors.

It is possible that this differential behavior on LM-5r may occur because these cell lines express different structural isoforms of the α3β1 integrin receptor, or different levels of the same isoform. The α3 integrin subunit exists in two variants, A and B, that contain different cytoplasmic domains (26). By RT-PCR analysis we detected a 570 bp band corresponding to the α3A variant in both cell types (Fig. 7a). We detected no 426 bp band corresponding to the α3B variant in either cell type. Immunoprecipitation of cell surface, biotin-labeled α3 integrin with the P1B5 antibody revealed that both cell types expressed α3 subunits of virtually identical size (Fig. 7b). FACS analyses (Fig. 3) revealed that both cell types expressed nearly identical amounts of α3 integrin subunits on their surface. We therefore conclude that both cell types express nearly identical amounts of the same structural variant of the α3β1 integrin receptor.

Western blot analysis with the polyclonal antibody 0668B, which cross-reacts with both human and rat laminin-5, showed that the extracellular matrix of both cell types contained laminin-5 (Fig. 8), in similar concentration and overall band composition. However, it is possible that these cells exhibit differential migration because they bind different portions of the molecule. We recently found that the anti-LM-5r monoclonal antibody CM6 disrupts cell adhesion and hemidesmosome formation in keratinocyte cell lines (31). When tested in rapid adhesion assays, LM-5r captured by CM6 antibody did not support adhesion of either cell type (Fig. 9). Identical results were obtained when CM6 was

added to wells containing LM-5r affinity captured by the antibody TR1 (not shown). CM6 is a slightly more efficient capturing agent than is TR1 (M. Fitchmun and E. Marshall, personal communication), suggesting that adhesion wells coated with CM6 contained at least as much laminin-5r as wells coated with TR1; yet, these wells supported no cell adhesion whatsoever (Fig. 9). Based on these results, we conclude that differential migration is not due to usage of distinct cell attachment sites on LM-5r.

Recent models suggest that cell/substratum adhesive strength acts in concert with cell motile force to regulate cell migration (32,33). Because HUMECs and MCF-7 cells use the same integrin receptor to bind LM-5r, it is possible that their differential behavior following attachment to LM-5r is due to assembly of distinct adhesion complexes; these complexes may exhibit different ability to adhere to LM-5r. To measure the relative strength of adhesion of HUMECs and MCF-7 cells to LM-5r, we used a centrifugal cell detachment assay. Cells were allowed to adhere to LM-5r, and were subsequently exposed to increasing g forces by centrifugation (23). We found that exposure to 1200 x g detached approximately 30% of both adherent HUMECs and MCF-7 cells (Fig. 10). Exposure to 1450 x g removed slightly more of both cell types. We therefore conclude that HUMECs and MCF-7 exhibit nearly equal strength of adhesion to LM-5r.

Like many integrin receptors, the $\alpha 3\beta 1$ integrin may exist in different activation states (34,35). Integrin receptor function may change, depending upon its activation state (36,37). To test the possibility that the behavior of HUMECs and MCF-7 cells following adhesion to LM-5r differs due to alterations in the activation state of the $\alpha 3\beta 1$ integrin, we performed haptotactic migration experiments with HUMECs stimulated either with the $\beta 1$ integrin activating antibody TS2/16 (Fig. 11A) (38) or with complete growth medium containing soluble growth factors (Fig. 11B). In both cases, HUMECs migrated 2-3 fold more than unstimulated cells which were kept in normal migration medium lacking growth factors (Fig. 11). Migration induced by either stimulatory TS2/16 antibody or growth factors was completely inhibited by antibodies against the $\alpha 3$ integrin and by CM6 (Fig. 12), suggesting that both treatments stimulated migration via the $\alpha 3\beta 1$ integrin. In support of this possibility, FACS analysis revealed that HUMECs stimulated by soluble factors exhibit increased reactivity with the monoclonal antibody 9EG7 (Fig. 12), which recognizes an epitope unveiled on the $\beta 1$ chain of activated integrin receptors (35).

Hypothesis 2. Laminin-5r contains at least one adhesion site used by mammary epithelial cells; both normal and transformed cells use the same adhesion site(s) (Specific Aim1). Because the adhesion sites for the $\alpha 1\beta 1$, $\alpha 3\beta 1$, $\alpha 6\beta 1$, and $\alpha 6\beta 4$ integrin receptors have been mapped on

laminin-1, the original proposal did not address the goal of mapping functional sites on laminin. However, with the decision to introduce laminin-5r into the project and the subsequent isolation of a function blocking monoclonal antibody that recognizes laminin-5r (CM6), the opportunity has arisen to map and define a novel functional site on a laminin isoform.

Efforts to map such a site require the successful cloning and expression of the constituent chains of laminin-5r. This has been the goal of a separate project underway in this laboratory. As outlined in the first progress report, our plan was to probe recombinant fragments of LM-5r chains with our function blocking antibody CM6 and to test positive fragments for adhesion- and migration-promoting activity in functional assays. To date, significant portions of the three constituent chains of LM-5r have been cloned in the laboratory. Based on western blotting data (21) and electron microscopic examination of rotary shadowed complexes CM6/LM-5r (31), we believe that the epitope for the CM6 antibody lies on the $\alpha 3$ chain of LM-5r. Three overlapping clones containing the entire open reading frame of the $\alpha 3$ cDNA have been isolated. However, the expression of these clones as a glutathione-S transferase-containing fusion protein (using the pGEX vector) has proven technically troublesome: to date, only one portion, which spans amino acids 291-1064 and contains the epitope recognized by the monoclonal antibody 5C5, has been successfully expressed in sufficient quantities to allow for screening by western blot. While this fragment of the $\alpha 3$ chain reacts with the 5C5 antibody as expected (Fig. 13B), it fails to react with CM6 (Fig. 13A), suggesting that the CM6 epitope may lie in one of the other two cloned fragments of the $\alpha 3$ cDNA. Efforts to increase expression efficiency of the other two cloned portions of the $\alpha 3$ chain, including a switch from JM109 bacterial cells to TOPP2 bacterial cells, are currently under way. Preliminary experiments probing with antibodies against the glutathione-S transferase portion of the fusion protein have suggested that this switch in bacterial strains may prove effective in increasing expression of the fusion proteins (not shown).

3. Adhesion to laminin-5r via specific integrin receptors activates biochemical signalling pathways that are responsible for maintaining the normal phenotype; malignant cells fail to activate these pathways (Specific Aim 2). One of the primary goals of this project is to identify biochemical signalling pathways that help define normal and malignant phenotypes in mammary epithelium. With the introduction of laminin-5 into the project, we also have the opportunity to explore the biochemical signalling responses following adhesion to LM-5r.

The delineation of integrin-associated biochemical signalling pathways using methods described in the original proposal requires extraordinarily large numbers of cells (i.e., in excess of 10^8 cells per experiment). We

therefore feel it is necessary to use established cell lines rather than primary cultures of both normal and malignant cells. To this end, we have devoted significant efforts towards finding a suitable cell line to represent the non-malignant, HUMEC phenotype. Our hope is to increase production of both malignant and "normal" cell lines to maintain regular production of approximately 10^9 cells per week.

Our best candidate cell line so far is MCF-10A, which was derived from a benign, fibrocystic tumor. Like HUMECs, MCF-10A cells adhere well to LM-5r via the $\alpha 3\beta 1$ integrin as determined by antibody blocking in rapid adhesion assays (Fig. 14) but migrate within background values on LM-5r in haptotactic assays and random motility assays (not shown). Like HUMECs, MCF-10A cells can also be stimulated to migrate on LM-5r by soluble growth factors (not shown). For these reasons, we have chosen to focus on MCF-10A and MCF-7 as cell lines representative of the normal and malignant phenotype on LM-5r, respectively.

Preliminary experiments comparing MCF-10A and MCF-7 cells following adhesion to LM-5r have focused on the distribution of tyrosine phosphate in these cells. Distribution of tyrosine phosphate-containing proteins is a generalized reflection of the protein tyrosine kinase activity in these cells, and numerous studies have linked integrin signalling with tyrosine kinase activity (reviewed in original proposal).

Our initial studies reveal distinct differences in the amounts and distribution of phosphotyrosine-containing proteins in MCF-10A and MCF-7 cells (Fig. 15). In particular, the vast majority of tyrosine phosphate-containing proteins in MCF-10A cells are redistributed from a detergent-soluble fraction (Fig. 15, lane 4) to a detergent-insoluble fraction (Fig 15, lane 7) following adhesion to LM-5r. This effect is not seen in MCF-7 cells, which have a majority of tyrosine phosphate-containing proteins in the detergent insoluble fraction regardless of whether cells are plated on LM-5r or a control substrate, poly-L-lysine. This suggests the possibility that cytoskeletal rearrangements following adhesion to LM-5r differ in these two cell types. Also, we have observed that a subset of proteins in MCF-10A cells becomes enriched in tyrosine phosphate following adhesion to LM-5r (Fig. 15, arrows); these same bands contain high amounts of phosphotyrosine in MCF-7 cells as well (Fig. 15, lanes 1 and 5).

Discussion

We examined the contribution of laminin-5 to adhesion and migration of normal and malignant mammary epithelial cells in in vitro assays using integrin-specific inhibitory antibodies and purified LM-5r under defined, serum-free conditions. This approach provided an advantage over previous in vivo metastasis studies and in vitro studies using "reconstituted basement membrane" (e.g., Matrigel) (39-42) in that it allowed us to examine the interactions between a single ECM protein

and its integrin receptors.

One striking finding was that LM-5r was more adhesive for mammary epithelial cells than all other ECM molecules tested. This observation, combined with our localization of LM-5r to the basement membrane of normal rat mammary gland, and identification of laminin-5 in the basement membrane of cultured mammary cells, argues strongly that LM-5r plays a critical role in maintenance of normal mammary epithelium.

Interestingly, neither HUMECs nor the malignant cell lines cells attached to LM-5r when plated in the presence of the anti-LM-5r monoclonal antibody CM6. This was true whether CM6 was used as a blocking agent on affinity captured LM-5r or as a capturing agent itself. CM6 recognizes an epitope on the $\alpha 3$ chain of LM-5r (21). The results obtained from our adhesion assays suggest that the $\alpha 3\beta 1$ integrin receptor may be used to adhere to LM-5r, via an epitope on the $\alpha 3$ chain of LM-5r defined by the monoclonal antibody CM6. This is consistent with the observation that a monoclonal antibody, BM165, directed against the $\alpha 3$ chain of the human isoform of laminin-5, also blocks cell adhesion (43). Interestingly, CM6 does not cross react with the human form of laminin-5 (21), suggesting these cell adhesion sites may be distinct.

Previous studies have attempted to correlate the presence or absence of specific extracellular matrix molecules with breast tumor progression, but these studies often reach conflicting conclusions. Laminin-5 has been localized to invading cancer cells in breast and other organs (44), and malignant MCF-7 cells actively secrete laminin-5 and incorporate it into their basement membrane (Fig. 8). Our observation that LM-5r stimulates both random motility and haptotactic migration through the $\alpha 3\beta 1$ integrin in MCF-7, but not HUMEC cells suggests that LM-5r may also play a role in invasion and metastasis.

How can such divergent cellular phenotypes result from binding LM-5r through the same receptor, the integrin $\alpha 3\beta 1$? Although previous studies have identified changes in expression of integrin subunits in malignant tumors, no conclusive marker for malignant transformation in breast has been identified. Inherent in many of these studies is the assumption that these integrins are fully and/or equally functional when expressed on the cell surface. Recent work from this laboratory and others has demonstrated that cell surface integrin receptors, including $\alpha 3\beta 1$ (34,35) may exist in different activation states, and that integrin receptor function may change, depending upon its activation state (36,37).

It is therefore possible that the $\alpha 3\beta 1$ integrin exists in different activation states in HUMEC and MCF-7 cells, and that this differential activation is

functionally expressed as increased strength of adhesion to LM-5r and a subsequent increase in migration in MCF-7 cells. Cell substratum adhesiveness plays a critical role in modulating cell migration (45), and can vary according to the activation state of integrin receptors. It is also possible that the strength of adhesion of the $\alpha 3\beta 1$ receptor to LM-5r in MCF-7 cells increases when these cells are subjected to detachment by increasing g forces. This occurs, for example, in the $\alpha 4$ integrin-mediated rolling and tethering behavior of lymphocytes, which become more adhesive in the presence of higher tensile shears (46).

Our evidence suggests that normal cells express the $\alpha 3\beta 1$ integrin in a relatively low activation state that allows attachment to, but not migration on LM-5r. Activation of this receptor, either directly via a monoclonal antibody or indirectly through growth factors, stimulates migration of these cells. That CM6 blocks this increased migration suggests that this stimulation affects a LM-5r mediated response rather than stimulating migration through a different mechanism.

Recent studies addressing functional differences between normal and malignant mammary epithelial cells suggest that $\beta 1$-containing integrins mediate adhesion to ECM molecules in both normal and malignant cells [e.g., (10,47,48)]. These studies seemingly aimed at identifying a specific integrin, whose presence would distinguish between normal and malignant cells. Our results, instead, suggest that the functional state, rather or in addition to, expression of a particular integrin, needs to be taken into account. Thus, it is not the level of expression of $\alpha 3\beta 1$ that distinguishes the normal and malignant cell types that we studied. Rather, $\alpha 3\beta 1$ integrin-mediated, strong adhesion to LM-5r, with resulting low-resistance to high g force detachment, no haptotactic migration, and no random motility, may be defining characteristics of normal mammary epithelium.

Conclusions

One of the principle goals of this second year of this project was to test the three hypotheses advanced at the end of the first year of work. The work described in this report demonstrates that progress has been made on all three hypotheses, so that new, more specific hypotheses can be established to guide the work performed in the third year of this project. The results obtained in the second year of this project, their impact on the original hypotheses advanced, and the resulting changes in each hypothesis, are summarized as follows:

The work performed in year two of this project supports two models of integrin expression in normal and malignant cells. First, the identification of the $\alpha 3\beta 1$ integrin as the receptor for LM-5r in both HUMEC and MCF-7 cells suggests that same integrin can function differently in normal and transformed cells. These cells therefore can be used to test the following model: activation of the $\alpha 3\beta 1$ integrin in mammary epithelial cells stimulates biochemical signalling pathways responsible for changes cytoskeletal rearrangements and cell shape that give rise to a migratory phenotype; the a3b1 integrin in non-migratory HUMECs is in a low activation state. By using conditions that we know can or cannot activate $\alpha 3\beta 1$ integrins and support migration, we should be able to establish a direct link between the migratory and non-migratory phenotype and specific integrin-associated biochemical signalling events in a single cell type, the HUMEC. Therefore, our original Hypothesis #1 can be modified as follows: Changes in adhesion and spreading on laminin-5r in malignant cells is due to **modified activation of the** $\alpha 3\beta 1$ **integrin receptor**. This in turn allows for modification of the third hypothesis, by introduction of a specific receptor: Adhesion to laminin-5r **via the** $\alpha 3\beta 1$ **integrin receptor** activates biochemical signalling pathways that are responsible for maintaining the normal phenotype; malignant cells fail to activate these pathways.

The second model of integrin expression resulting from the work performed in the second year is that normal and malignant cells express different receptors for laminin-5. This completely contradicts the first model proposed, yet our observation that high amounts of blocking antibodies directed against a variety of integrin α chains fails to inhibit adhesion of MDA-MB-231 and MDA-MB-435 cells to LM-5r suggests that these cells utilize either a modified form of one or more of these integrins or that they use a different, $\beta 1$-containing integrin to bind LM-5r. In either case, the observation clearly suggests that different integrins may support different phenotypic responses following adhesion to LM-5r. As in the first model, binding to LM-5r via $\alpha 3\beta 1$ may activate a non-migratory biochemical pathway, while binding to LM-5r via another, as-yet-unidentified integrin may stimulate migration through a separate pathway. This model also modifies the third hypothesis advanced in the

first progress report as follows: Adhesion to laminin-5r **via the** $\alpha 3\beta 1$ **integrin receptor** activates biochemical signalling pathways that are responsible for maintaining the normal phenotype. **Malignant cells bind laminin-5r via a different receptor, and each receptor activates its own set of distinct biochemical signalling pathways. Activation of the biochemical signalling pathways associated with adhesion to laminin-5r in the malignant phenotype promotes migration.**

Likewise, the discovery that CM6 blocks adhesion of both cells using $\alpha 3\beta 1$ integrin receptors and cells using a different, unidentified $\beta 1$ integrin receptor strongly supports our second hypothesis: Laminin-5r contains at least one adhesion site used by mammary epithelial cells; both normal and transformed cells use the same adhesion site(s). This hypothesis is equally applicable to both models: HUMECs and MCF-7 cells use the same receptor to bind LM-5r, while HUMEC/MCF-7 and MDA-MB-435/231 cells likely use different receptors, yet all four cell types bind a common region on LM-5r.

The preliminary work on phosphotyrosine distribution following adhesion to LM-5r was performed to test the first of the two models, and clearly demonstrates the applicability of the MCF-10A cell line as a tool for further biochemical analysis of the signalling pathways in normal cells. These results are highly encouraging and should allow us to examine specific signalling activities in normal and malignant cells under clearly defined conditions.

The advantage of both models is that they allow for specific predictions. Model one will allow us to use a single cell type to draw correlations between migration and integrin-associated signalling: Integrin complexes isolated from MCF-10A cells that bind LM-5r in the presence or absence of activating integrin antibodies or soluble growth factors should differ significantly, either in the constituent proteins of these complexes or in the biochemical signalling activity these complexes possess. In addition to providing clues about the differences between the migratory and non-migratory phenotype in a single cell type, this analysis will provide important data on the basic properties of signal transduction following adhesion to LM-5r. Based on our preliminary data, we predict that cells stimulated with either growth factors or $\beta 1$ integrin activating antibodies will exhibit increased tyrosine phosphorylation of cytoskeletal proteins, possibly including integrins. We also expect to find an increase in tyrosine kinase activity and/or a decrease in tyrosine phosphatase activity in these activated cells.

Model one will also allow us to compare the differences between two cell types that utilize the same receptor for LM-5r. Model one predicts that MCF-7 represents a cell that expresses a constituitively activated $\alpha 3\beta 1$ integrin; the integrin-associated signalling in these cells should likewise

be constituitively activated. When compared to non activated HUMECs or MCF-10A cells, we should find supporting evidence for the data we obtain on activated and non-activated MCF-10A cells. Our predictions for this scenario are similar to that for the one cell type system: MCF-7 cells should have increased tyrosine phosphorylation of cytoskeletal proteins, increased tyrosine kinase activity, and/or decreased tyrosine phosphatase activity relative to unstimulated MCF-10A cells.

Model two will allow us to test the hypothesis that distinct receptors activate different signalling pathways that are responsible for static adhesion and migration in normal and malignant cells, respectively. We therefore predict that the signalling pathways activated in MDA-MB-231 and MDA-MB-435 cells following adhesion to LM-5r will be different from those activated in HUMECs, MCF-10A or MCF-7 cells. This difference may be expressed as differences in the types of protein tyrosine kinases activated in each cell type, the types of substrates phosphorylated by these kinases, or the type of adhesion complex assembled by these cells. Methods for distinguishing between these possibilities are outlined in the original proposal.

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Appendix

Figure Legends

Figure 1. Normal and malignant mammary epithelial cells preferentially bind laminin-5r. Normal (HUMEC), MCF-7, MDA-MB-231, and MDA-MB-435 cells were plated on the indicated extracellular matrix molecules for 30 minutes, gently washed to remove unattached cells, then fixed, stained and quantitated as described in Materials and Methods. LM-5r= laminin-5r; LM-2 = human laminin-2; hFn = human fibronectin; rLM-1 = rat laminin-1; hVn = human vitronectin. Data are presented as the statistical mean +/- standard deviation (n=8).

Figure 2. Inhibition of mammary cell adhesion to laminin-5r by anti-integrin antibodies. Normal (HUMEC), MCF-7, MDA-MB-231, and MDA-MB-435 cells were plated on laminin-5r for 30 minutes in the presence of antibodies directed against the following integrin subunits: P1E6 = $\alpha 2$, P1B5 = $\alpha 3$, GoH3 = $\alpha 6$, P4C10 = $\beta 1$. Cells were then gently washed, fixed, stained, and quantitated as for Fig. 1. As a control, cells were plated on laminin-2. Note that adhesion of all cell lines was completely blocked by antibodies against $\beta 1$. LM-5r = laminin-5r.

Figure 3. Mammary epithelial cells express $\alpha 2$, $\alpha 3$, $\alpha 6$, and $\beta 1$ integrins. Normal cells (HUMEC), MCF-7 cells, MDA-MB-231 cells, and MDA-MB-435 cells were stained with the monoclonal antibodies directed against the following integrin subunits: P1E6 = $\alpha 2$, P1B5 = $\alpha 3$, GoH3 = $\alpha 6$, P4C10 = $\beta 1$, followed by secondary antibodies conjugated to fluorescein, and analyzed by flow cytometry. As controls, cells were also stained with secondary antibodies alone. Note that all cell types expressed all four integrins tested. Results are expressed as mean fluorescence intensity (arbitrary units) of 10,000 sorted cells.

Figure 4. The monoclonal antibody GoH3 blocks adhesion of mammary epithelial cells to laminin-1. MDA-MB-231 and MDA-MB-435 cells were plated on laminin-1 in the presence of the indicated antibodies and processed for rapid adhesion activity as described in Fig.1.

Figure 5. Inhibition of mammary cell migration on laminin-5r by inhibitory antibodies. HUMEC and MCF-7 cells were plated on Transwell filters whose reverse sides were coated with bovine serum albumin (BSA) or laminin-5r, in the presence of either CM6 or antibodies directed against the indicated integrin subunits: P1E6 = $\alpha 2$, P1B5 = $\alpha 3$, GoH3 = $\alpha 6$, P4C10 = $\beta 1$. No inhibitory antibodies were included in control wells. After 18 hours, cells were fixed and stained, and migration quantitated as described in Materials and Methods. Results are expressed as statistical mean of number of cells counted per microscopic field (30x magnification) $\pm$ standard deviation (n=8).

Fig. 6. MCF-7 cells migrate approximately 10-fold more than HUMECs on LM-5r coated colloidal gold. **A.** Dark field images of indicated cells plated on colloidal gold particles coated with laminin-5r (LM-5r) or blotto (-CTL). Bar = 40 μm. **B.** Quantitation of cell migration. Photographs of dark field microscopic fields as shown in panel A were digitized and scanned for black areas where cells had migrated. Values are expressed as the mean percentage of black area per field ± standard deviation (n=3).

Figure 7. HUMEC and MCF-7 cells express identical isoforms of the α3 integrin. (A). Expression of the α3A isoform was detected by RT-PCR of mRNA isolated from HUMEC and MCF-7 cells. Primers flanking the sequences encoding the alternatively spliced cytoplasmic domains amplified a 570 bp band corresponding to α3A, but no 426 bp band corresponding to α3B. (B). Immunoprecipitation of α3 subunits from cell surface, biotin-labeled HUMEC and MCF-7 cells. α3 subunits from both cell types migrate at approximately 130 kDa. Migration of molecular weight standards is indicated at left.

Figure 8. HUMECs and MCF-7 cells secrete laminin-5. Extracellular matrix of both cell types was isolated and probed for the presence of laminin-5 by western blot using the polyclonal antibody 0668B. This antibody recognized bands of 150, 145, and 135 kDa in both preparations, corresponding to the α3, γ2, and β3 chains of laminin-5, respectively. As controls, extracellular matrix of rat 804G cells and human SCC25 cells, known sources of rat and human laminin-5, respectively, were probed for comparison. Migration of molecular weight standards is shown at left.

Figure 9. The monoclonal antibody CM6 blocks adhesion of mammary epithelial cells to laminin-5r. HUMEC and MCF-7 cells were plated for 30 minutes on plates coated with laminin-5r affinity captured from 804G cell conditioned medium by the indicated antibodies. Note that no cells adhered to wells coated with CM6. As controls, wells were coated with the anti-laminin-5r antibody TR1, irrelevant mouse IgG_1 (MOPC), or no antibody. Results are expressed as in Fig. 2.

Figure 10. HUMECs and MCF-7 cells exhibit equal strength of adhesion to LM-5r. Metabolically labeled cells were plated on antibody-captured LM-5r for 25 minutes then plates were exposed to the indicated g force by centrifugation for 15 minutes. Cells remaining attached to plates were detected by phosphorimager analysis. Values are expressed as average percentage of maximum cell binding ± standard deviation (n=3).

Figure 11. Activating antibodies and complete growth medium stimulates migration of HUMECs on LM-5r. HUMECs were plated on Transwell

filters as described in Fig. 5, in the indicated media and in the presence of the indicated antibodies. Cells were fixed, stained, and counted as described in Fig. 5.

Figure 12. Activation of HUMECs with complete growth medium stimulates appearance of the 9EG7 antibody epitope. HUMECs were incubated in migration medium (MIG) or complete growth medium (MEGM) for 18 hr, then collected and analysed for appearance of the 9EG7 epitope by FACS analysis. Reactivity with secondary antibody alone (GAR-FITC) is shown as a control.

Figure 13. The CM6 epitope is not contained in the middle fragment of the α3 chain of LM-5r. A cloned fragment of the α3 chain of LM-5r was expressed as a glutathione-S-transferase fusion protein and bacterial lysates were probed by western blot with CM6 antibody (panel A) or the 5C5 antibody (panel B). For each panel, lane 1=lysate of transfected bacteria stimulated with IPTG; lane 2=lysate of untransfected bacteria stimulated with IPTG; lane 3=purified LM-5r. Migration of molecular weight standards is indicated at left.

Figure 14. MCF-10A cells utilize the α3β1 integrin to bind laminin-5r. MCF10-A cells were incubated in the presence of indictated antibodies and evaluated for adhesion to LM-5r in rapid adhesion assays as described in Figure 2. Note that α3 and β1 antibodies blocked adhesion completely. P1E6 = α2, P1B5 = α3, GoH3 = α6, P4C10 = β1.

Figure 15. Differential tyrosine phosphorylation in HUMECs and MCF-7 cells following adhesion to LM-5r. MCF-7 (lanes 1, 2, 5, 6) or MCF-10A (lanes 3, 4, 7, 8) cells were plated on poly-L-lysine (lanes 1-4) or on LM-5r (lanes 5-8) for 30 minutes, collected and extracted with CSK buffer containing 0.5% Triton-X 100. Equal amounts of protein from the detergent-soluble (even numbered lanes) and -insoluble (odd numbered lanes) fractions were separated by polyacrylamide gel electrophoresis and probed for phosphotyrosine-containing proteins by western blot. Migration of molecular weight standards indicated at left.

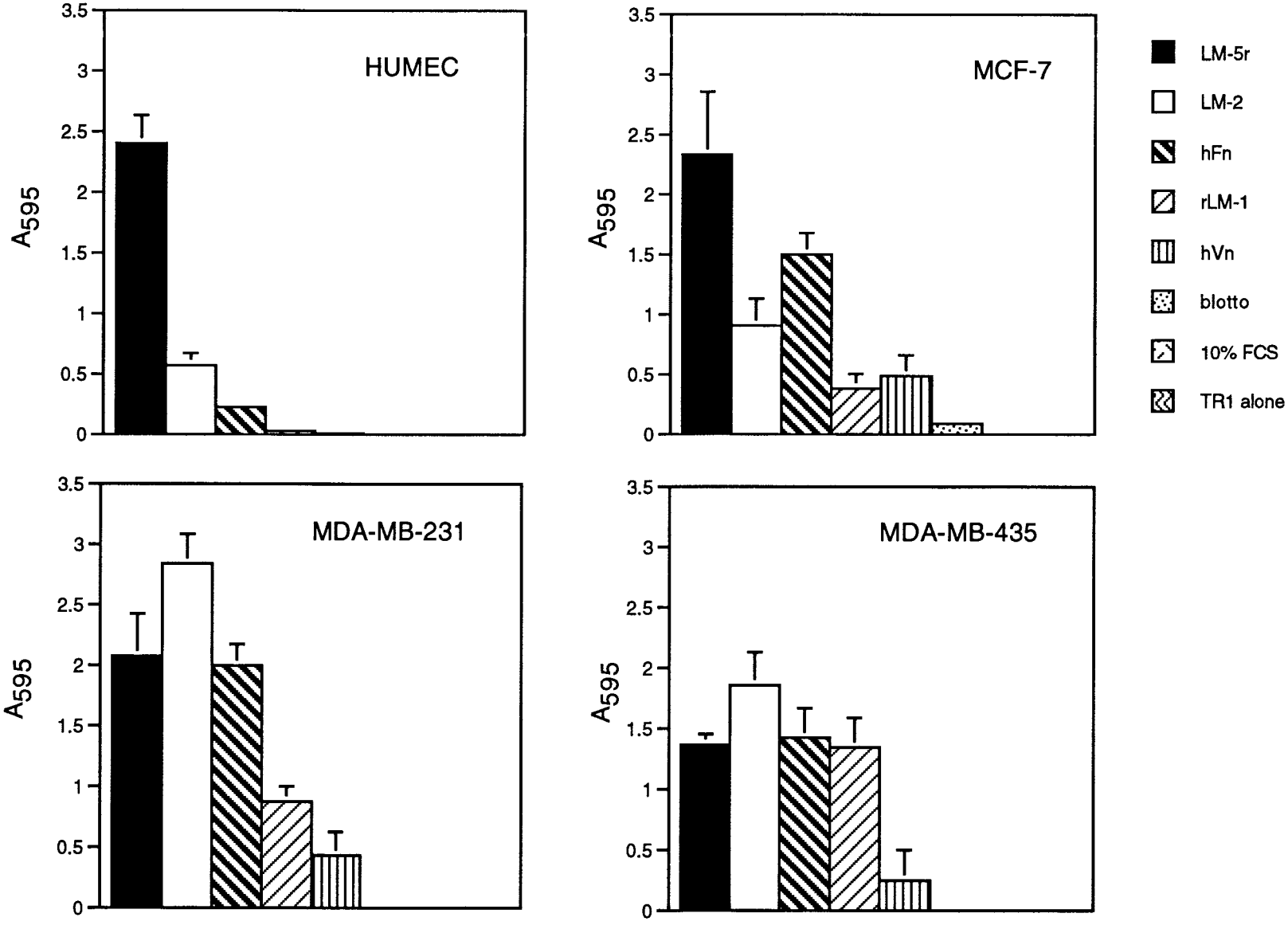

HUMEC
MCF-7
MDA-MB-231
MDA-MB-435
A595
3.5
3
2.5
2
1.5
1
0.5
0
LM-5r
LM-2
hFn
rLM-1
hVn
blotto
10% FCS
TR1 alone

FIGURE 1

FIGURE 2

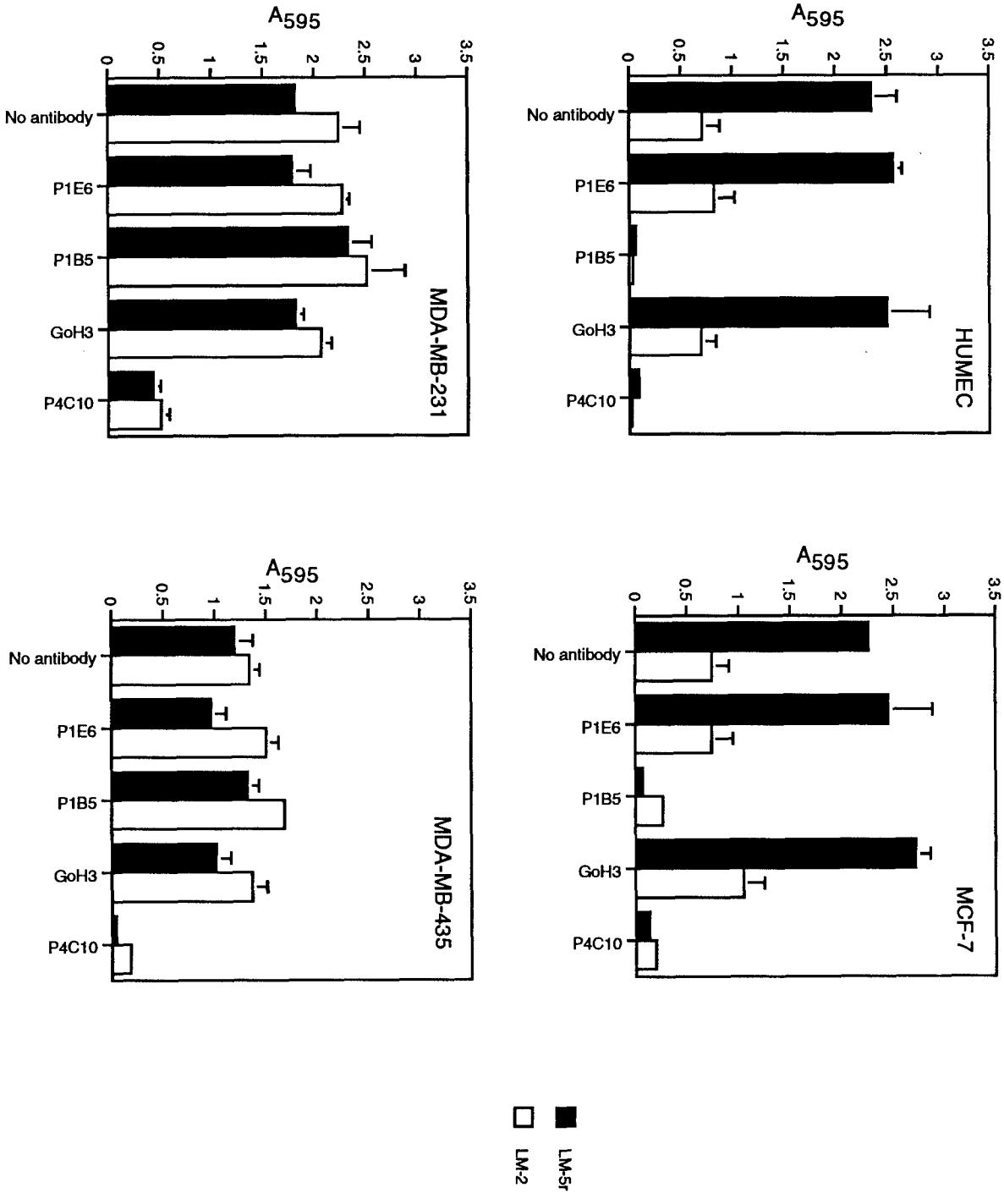

A595
0
0.5
1
1.5
2
2.5
3
3.5
No antibody
P1E6
P1B5
GoH3
P4C10
MDA-MB-231
HUMEC
MDA-MB-435
MCF-7
LM-2
LM-5r

FIGURE 3

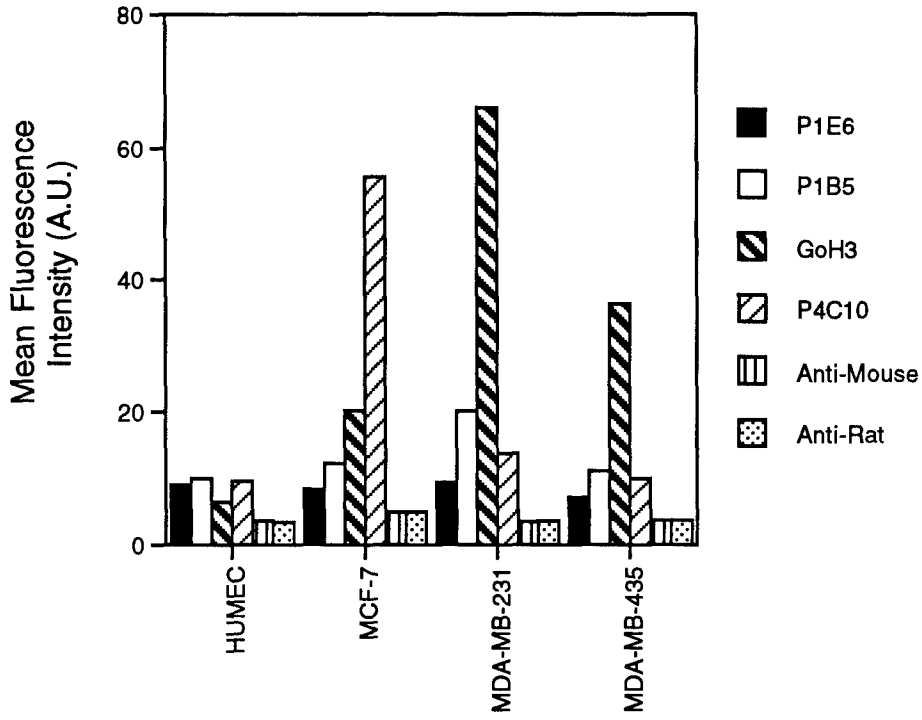

Mean Fluorescence Intensity (A.U.)
80
60
40
20
0
HUMEC
MCF-7
MDA-MB-231
MDA-MB-435
P1E6
P1B5
GoH3
P4C10
Anti-Mouse
Anti-Rat

FIGURE 4

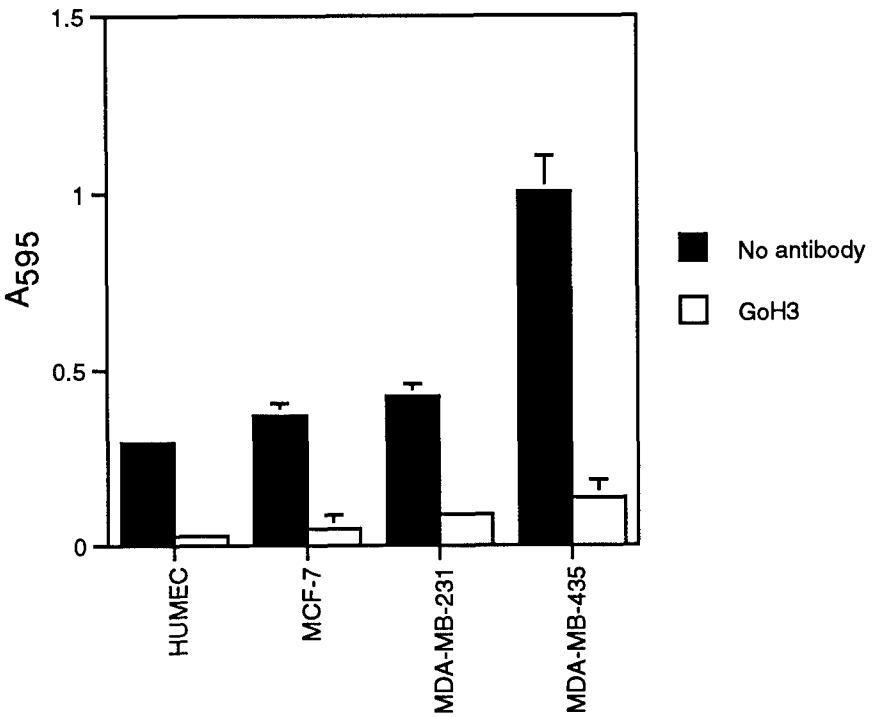


FIGURE 5

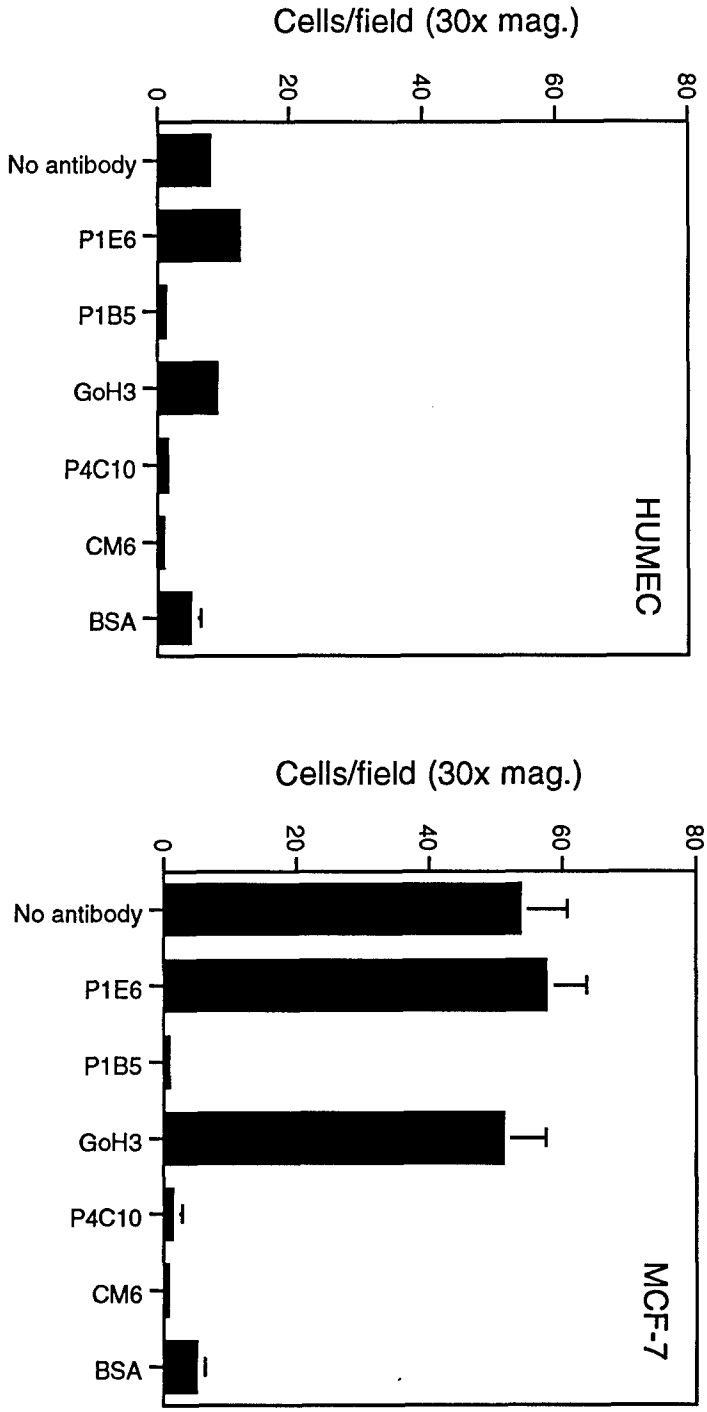
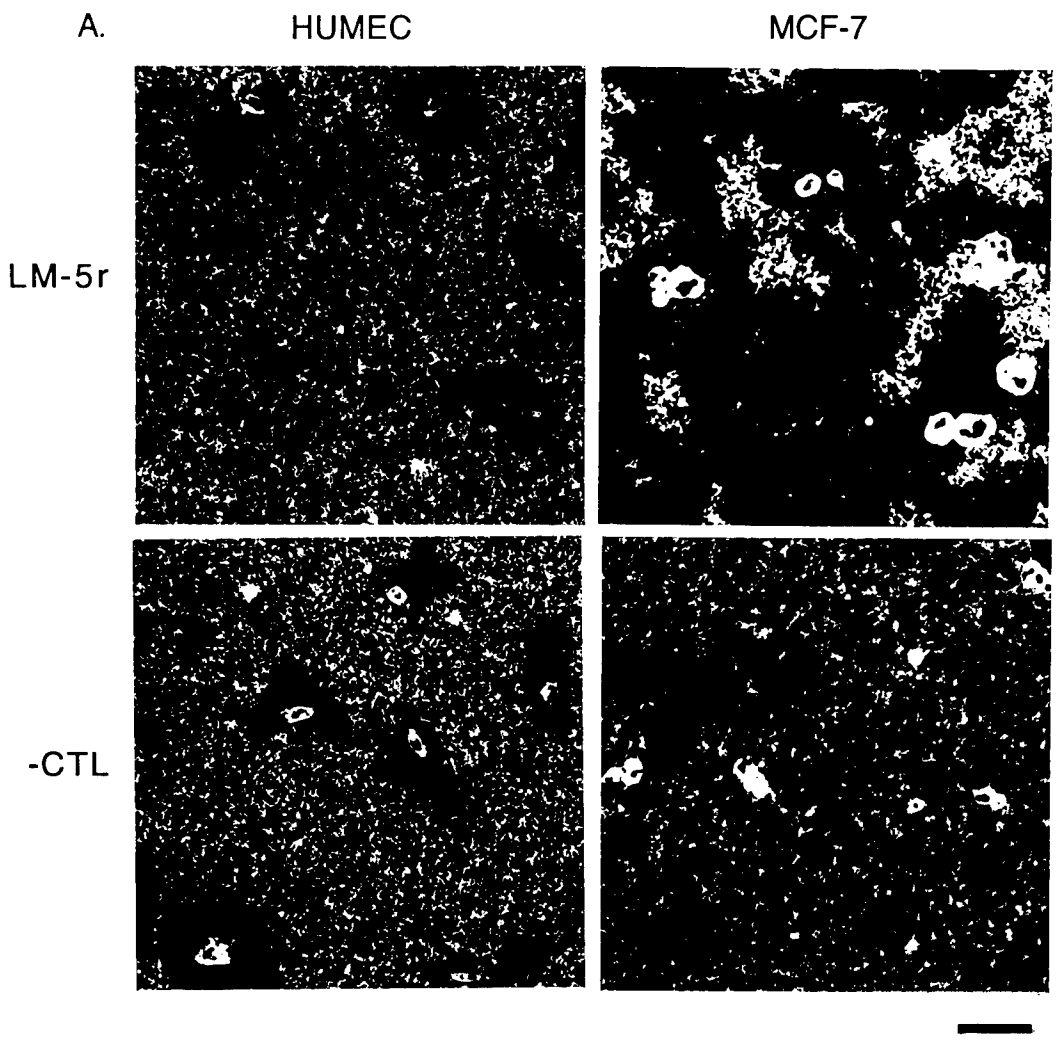

Cells/field (30x mag.)
0
20
40
60
80
No antibody
P1E6
P1B5
GoH3
P4C10
CM6
BSA
HUMEC
Cells/field (30x mag.)
0
20
40
60
80
No antibody
P1E6
P1B5
GoH3
P4C10
CM6
BSA
MCF-7

FIGURE 6


A.
HUMEC
MCF-7
LM-5r
-CTL

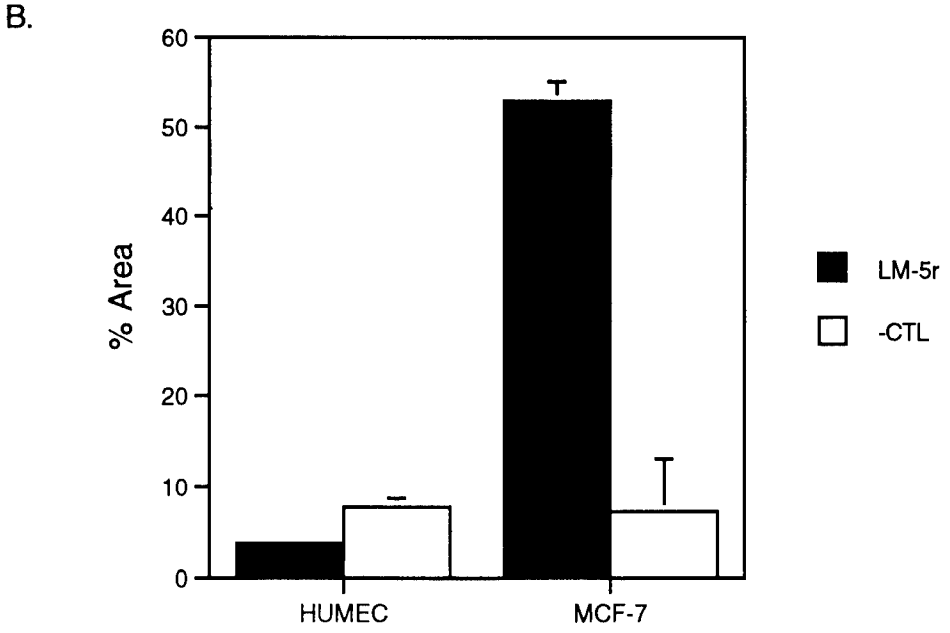

B.
60
50
40
30
20
10
0
% Area
HUMEC
MCF-7
LM-5r
-CTL

FIGURE 7a

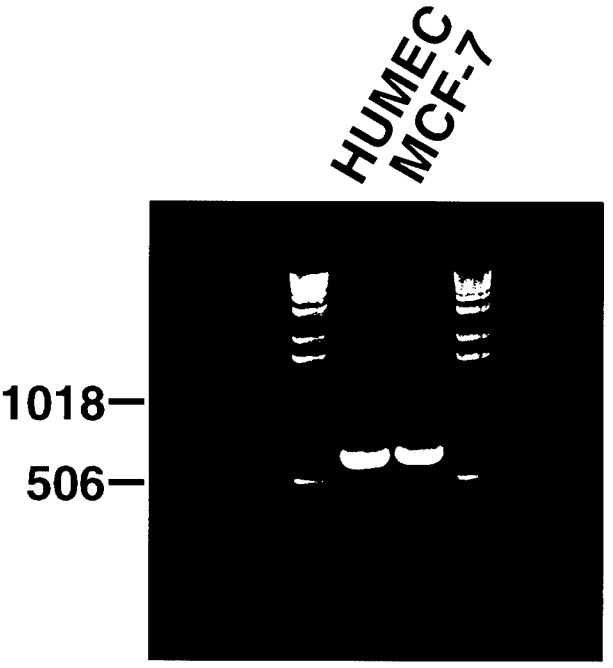


Figure 7b

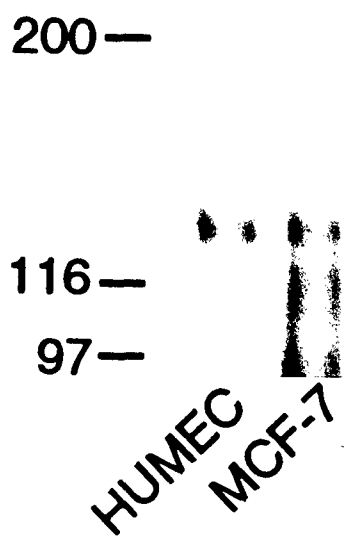


Figure 8

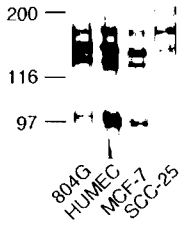


Figure 9

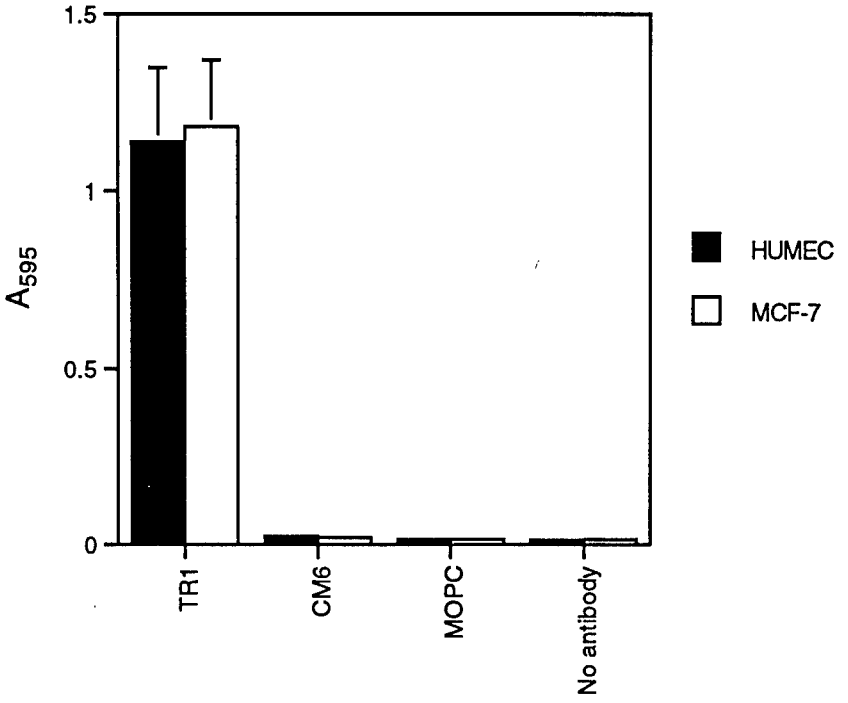


Figure 10

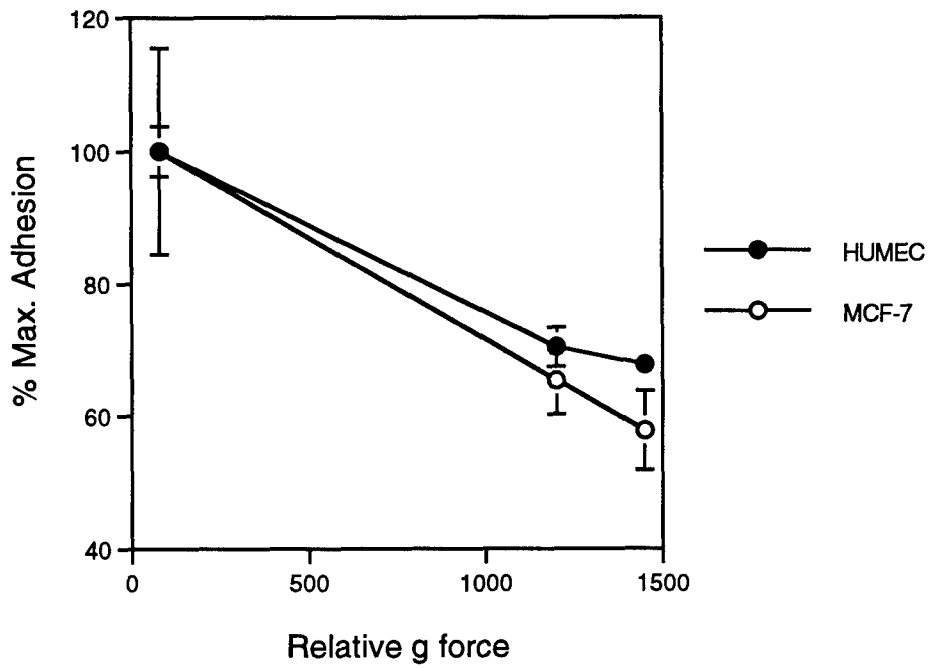

120
100
80
60
40
% Max. Adhesion
0
500
1000
1500
Relative g force
HUMEC
MCF-7

Figure 11a

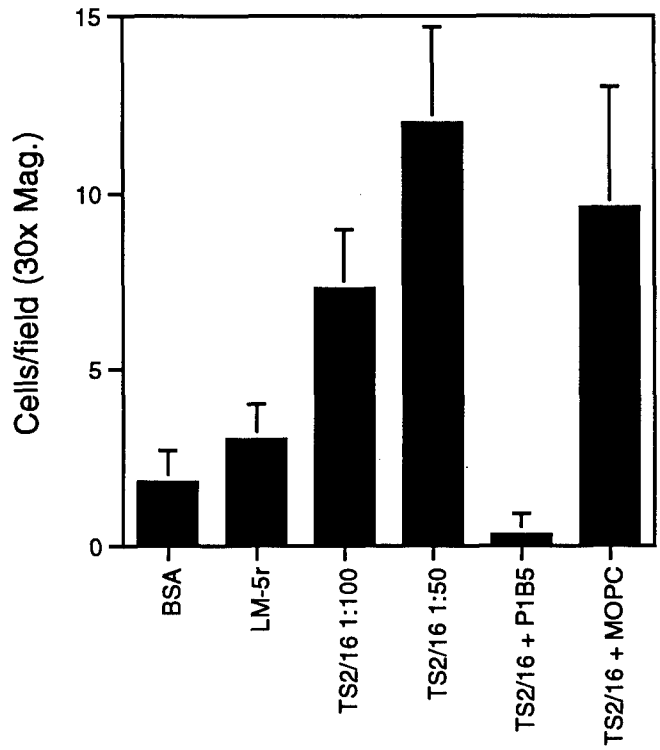

Cells/field (30x Mag.)
15
10
5
0
BSA
LM-5r
TS2/16 1:100
TS2/16 1:50
TS2/16 + P1B5
TS2/16 + MOPC

Figure 11b

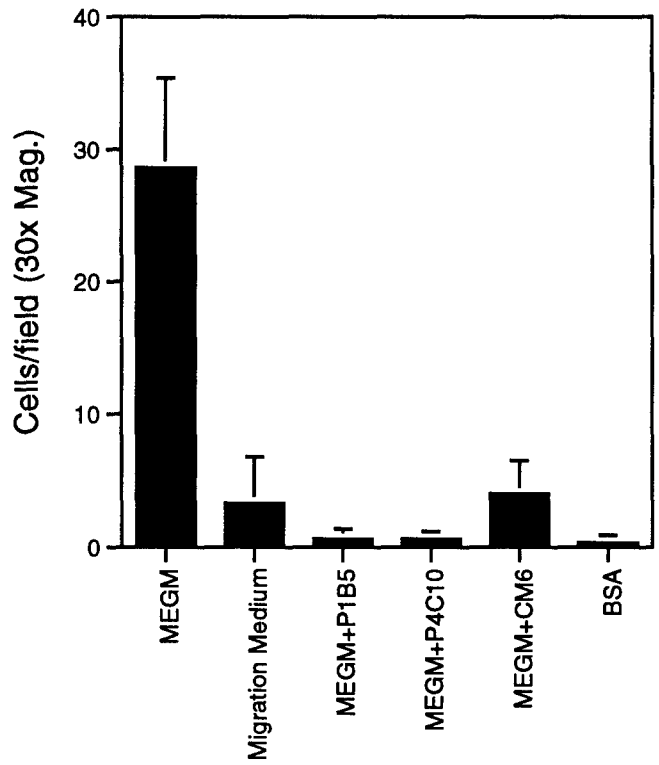

Cells/field (30x Mag.)
40
30
20
10
0
MEGM
Migration Medium
MEGM+P1B5
MEGM+P4C10
MEGM+CM6
BSA

Figure 12

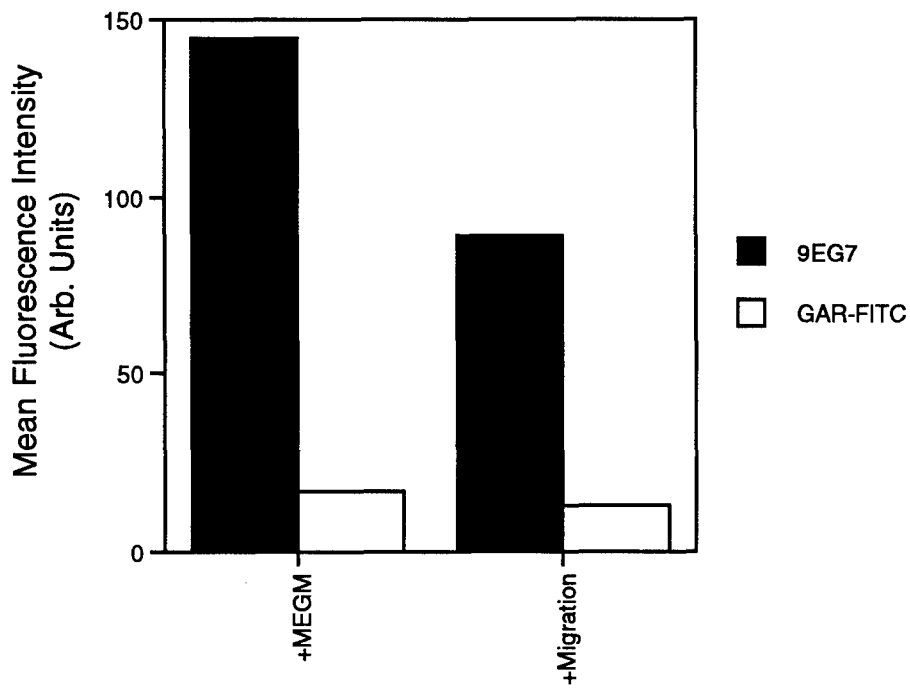

Mean Fluorescence Intensity
(Arb. Units)
150
100
50
0
+MEGM
+Migration
9EG7
GAR-FITC

Figure 13

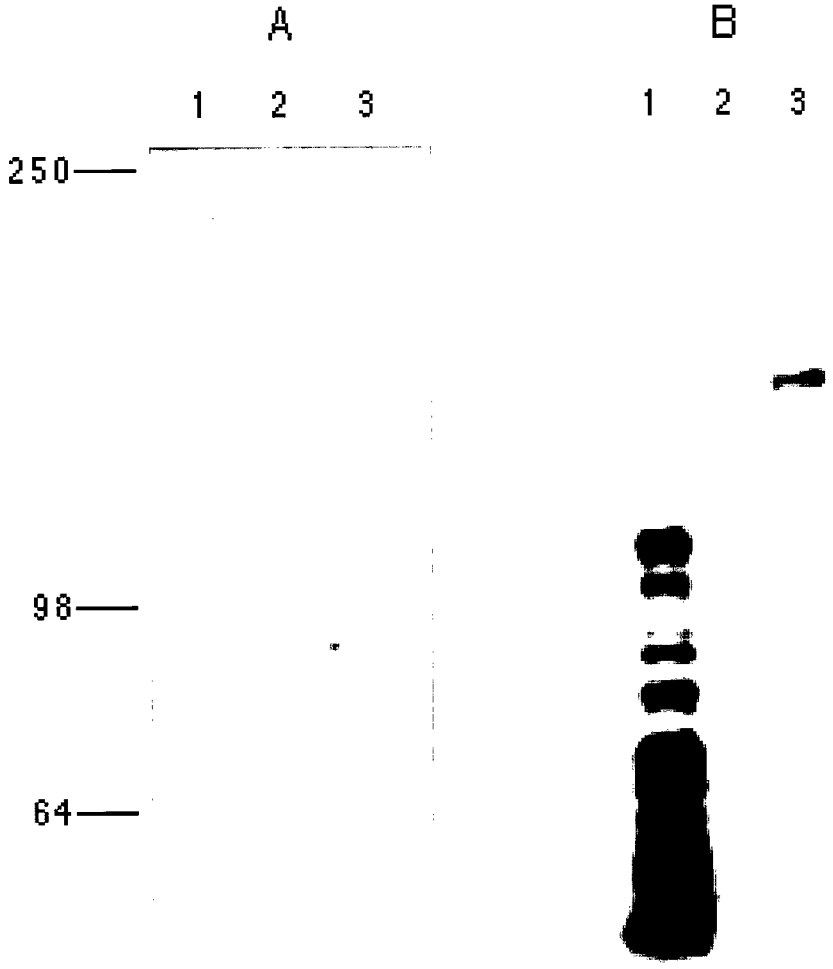
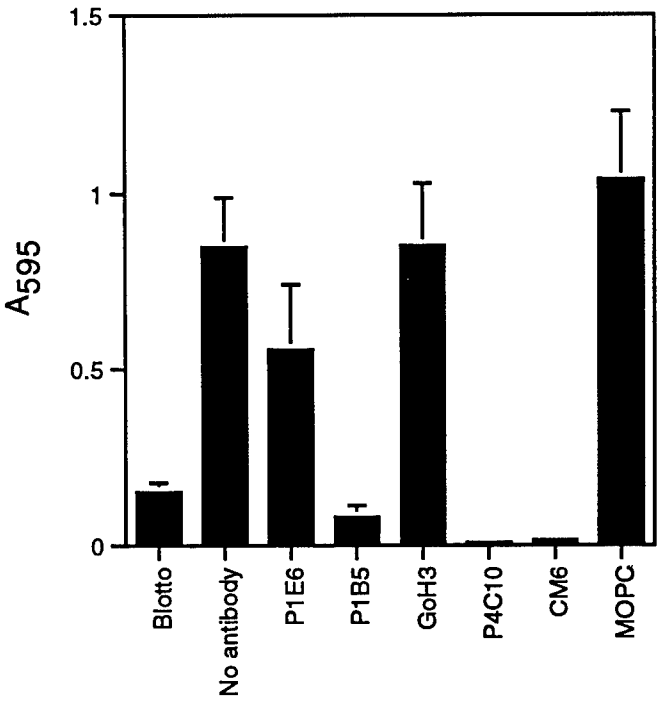


Figure 14


1.5
1
0.5
0
A_{595}
Blotto
No antibody
P1E6
P1B5
GoH3
P4C10
CM6
MOPC

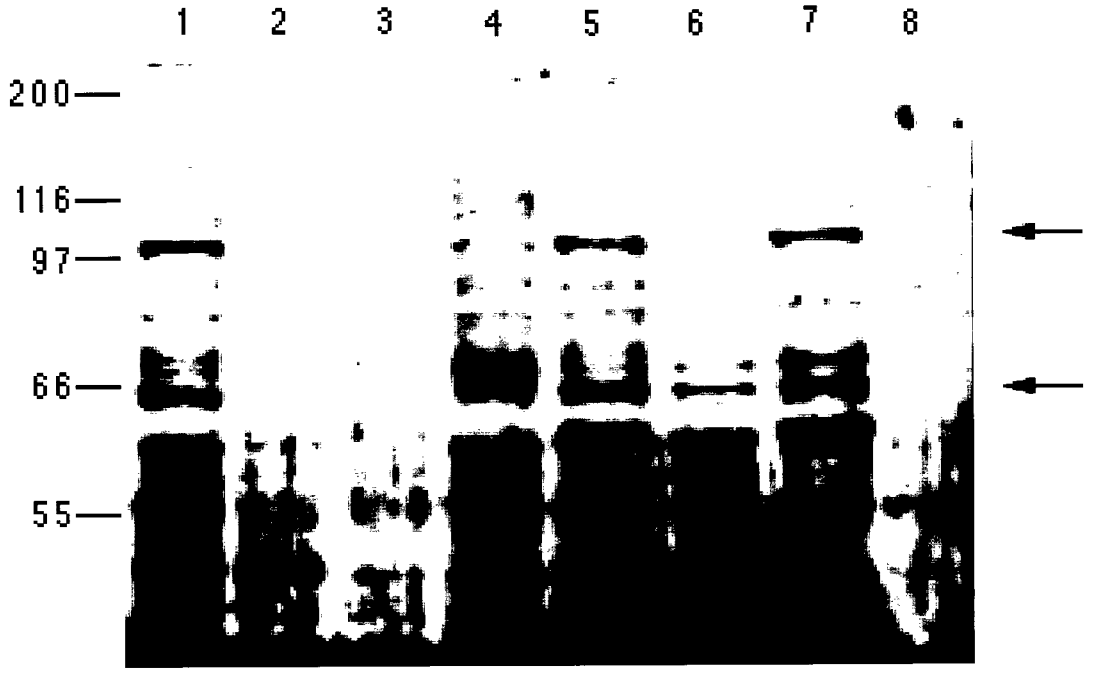


Figure 15

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 6 May 98

MEMORANDUM FOR Administrator, Defense Technical Information Center, ATTN: DTIC-OCP, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for the following contracts. Request the limited distribution statement for these contracts be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

Contract Number	Accession Document Number
DAMD17-94-J-4030	ADB215484
DAMD17-94-J-4138	ADB215863
DAMD17-94-J-4158	ADB215553
DAMD17-94-J-4278	ADB215864
DAMD17-94-J-4267	ADB216187
DAMD17-94-J-4200	ADB216054
DAMD17-94-J-4185	ADB219284
DAMD17-94-J-4172	ADB224562
DAMD17-94-J-4156	ADB216186
DAMD17-94-J-4082	ADB215979
DAMD17-94-J-4053	ADB216052
DAMD17-94-J-4028	ADB218953

2. Point of contact for this request is Ms. Betty Nelson at DSN 343-7328 or email: betty_nelson@ftdetrck-ccmail.army.mil.

FOR THE COMMANDER:

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management