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Single-Chain FV Molecules Capable of Inhibiting the Growth of
Breast Cancer

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13. ABSTRACT (Maximum 200 Words) Antibodies that perturb signal transduction of cancer cells have demonstrated significant utility in the treatment of breast cancer and lymphoma. As signal transduction in the Epidermal Growth Factor Receptor (EGFR) family (EGFR, HER2, HER3, HER4) involves ligand binding and subsequent heterodimerization of two members, the most potent monoclonal antibody (MAb)-based agent would likely be one that also mediates a similar crosslinking event. The fundamental hypothesis underlying this Concept Award Project was that signal transduction through components of the EGFR family could be manipulated through the construction of novel bispecific antibodies that engage multiple epitopes of this family. The goals of this proposal were to develop a novel, rapid methodology to create bispecific single-chain Fv (bs-scFv) molecules using molecular shuffling of two large groups of				
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Introduction

Antibodies that perturb signal transduction of cancer cells have demonstrated significant utility in the treatment of breast cancer and lymphoma. As signal transduction in the Epidermal Growth Factor Receptor (EGFR) family (EGFR, HER2, HER3, HER4) involves ligand binding and subsequent heterodimerization of two members, the most potent monoclonal antibody (MAb)-based agent would likely be one that also mediates a similar crosslinking event. *The fundamental hypothesis underlying this Concept Award Project was that signal transduction through components of the EGFR family could be manipulated through the construction of novel bispecific antibodies that engage multiple epitopes of this family.* The goals of this proposal were to develop a novel, rapid methodology to create bispecific single-chain Fv (bs-scFv) molecules using molecular shuffling of two large groups of scFv (libraries) specific for HER2 and HER3 and to perform preliminary evaluations of the in vitro specificity and anti-tumor effects against cells that over express both target antigens.

Body

Research Accomplishments:

Task 1. Isolate scFv clones from a large human scFv library that are reactive with HER3. We used human HER3 extracellular domain (ECD) as a target to pan a phage display library. A number of scFv were isolated that bind to HER3 (Table 1). PCR fingerprinting revealed that 6 contained unique sequences. Note: while it was not directly identified in the scope of the proposal, we also isolated scFv molecules that bound to HER4 ECD (10 unique clones) and EGFR ECD (33 unique clones).

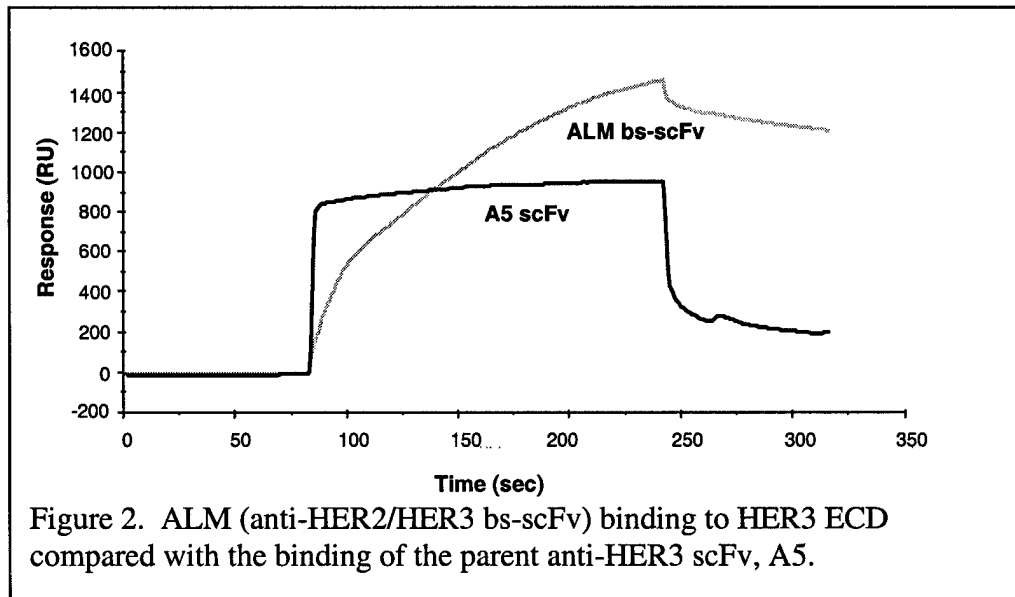
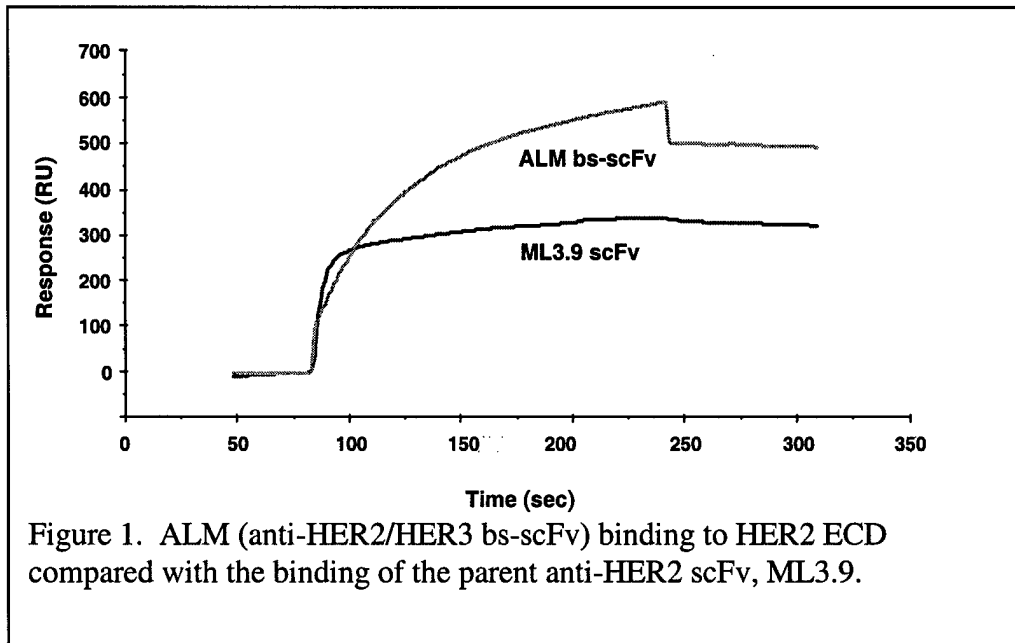
Table 1. Properties of scFv sublibraries.

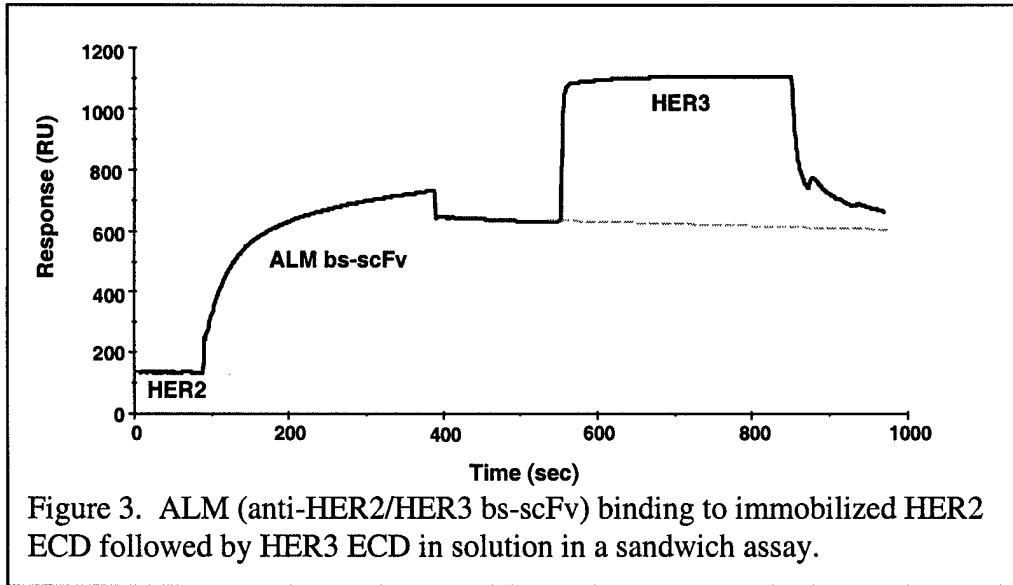
Antigen	# of scFv libraries screened	# Unique scFv clones in sublibrary
HER2	2	2*
HER3	1	6
HER4	1	10
EGFR	3	33

*Note, 75 affinity mutants ranging in affinity from the mM to pM level have been created from one of these clones

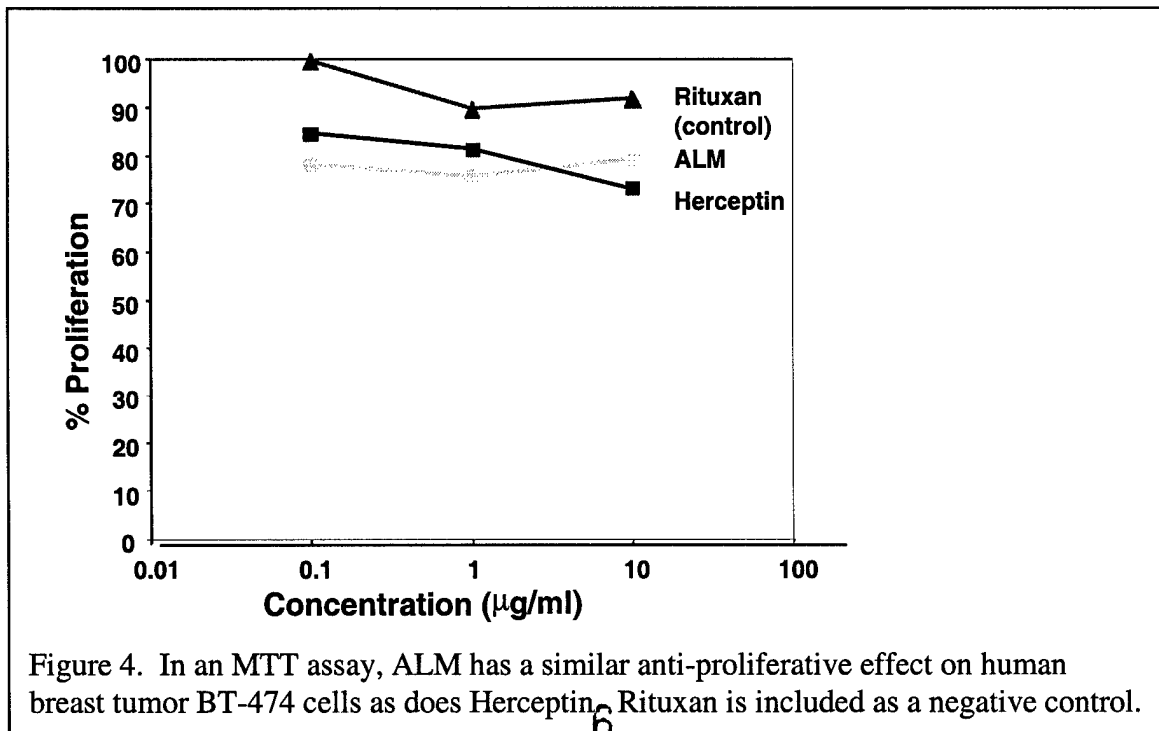
Task 2. Create bs-scFv molecules using scFv shuffling. A unique peptide spacer with the amino acid sequence "NSGAGTSGSGASGEGSGSKL" was designed that would impart flexibility and resistance to serum proteases. Based upon incompatible restriction sites between the scFv clones, scFv shuffling techniques will require significant sight-directed mutagenesis to alter restriction sites. We have begun this process. In the meantime, we opted to select a few anti-HER3 scFv molecules and use these together with the peptide spacer (shown above) and our best anti-HER2 scFv (ML3.9) to create a few bs-scFv for the initial screening process. As a result of this effort, we have now created two bs-scFv that are specific for HER2 and HER3 (ALM and FLM), two homodimeric scFv that are specific for HER3 (ALA and FLF), one homodimeric scFv that is specific for HER2 (MLM) and one heterodimeric scFv that is specific for two epitopes on HER3 (FLM).

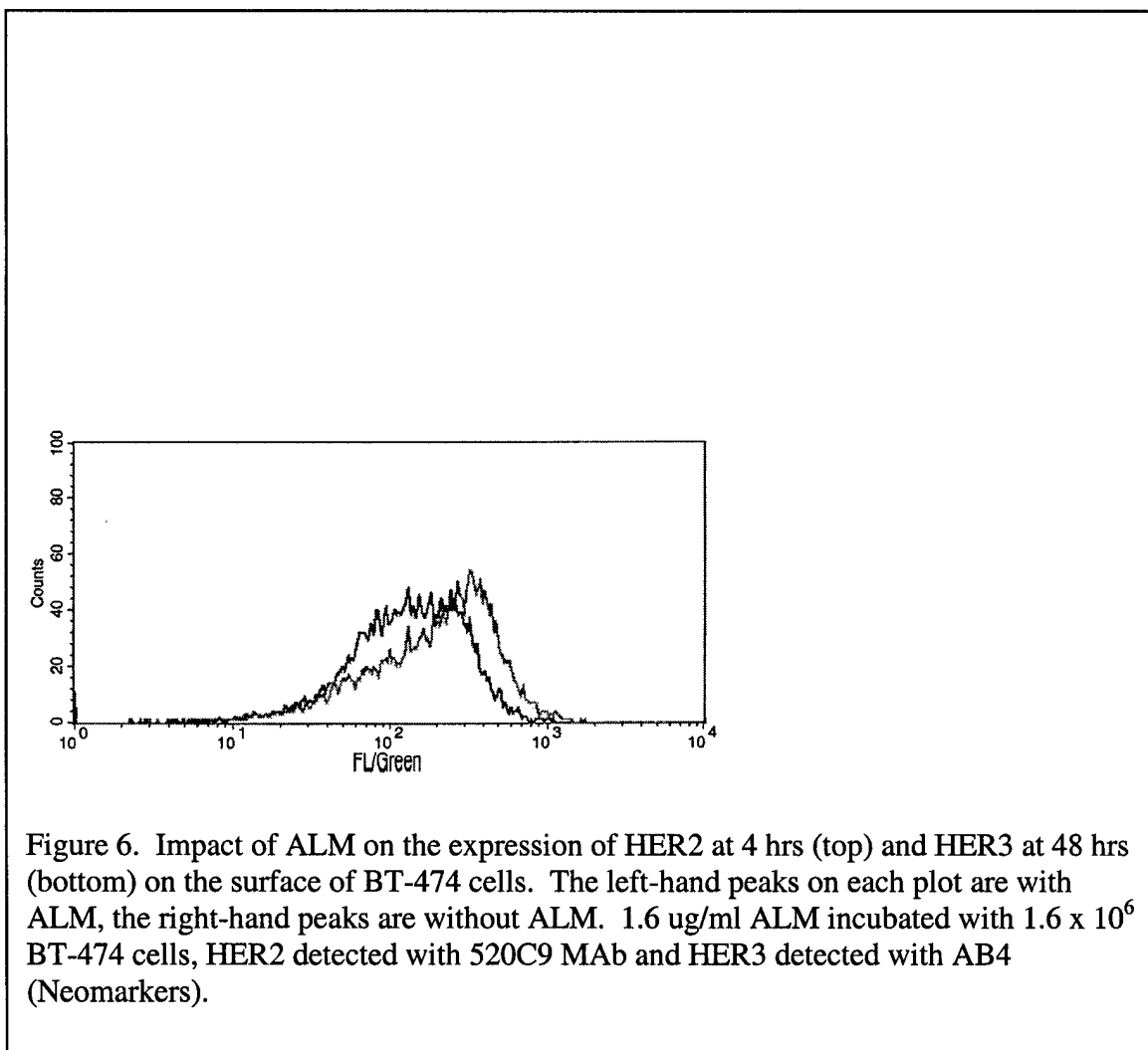
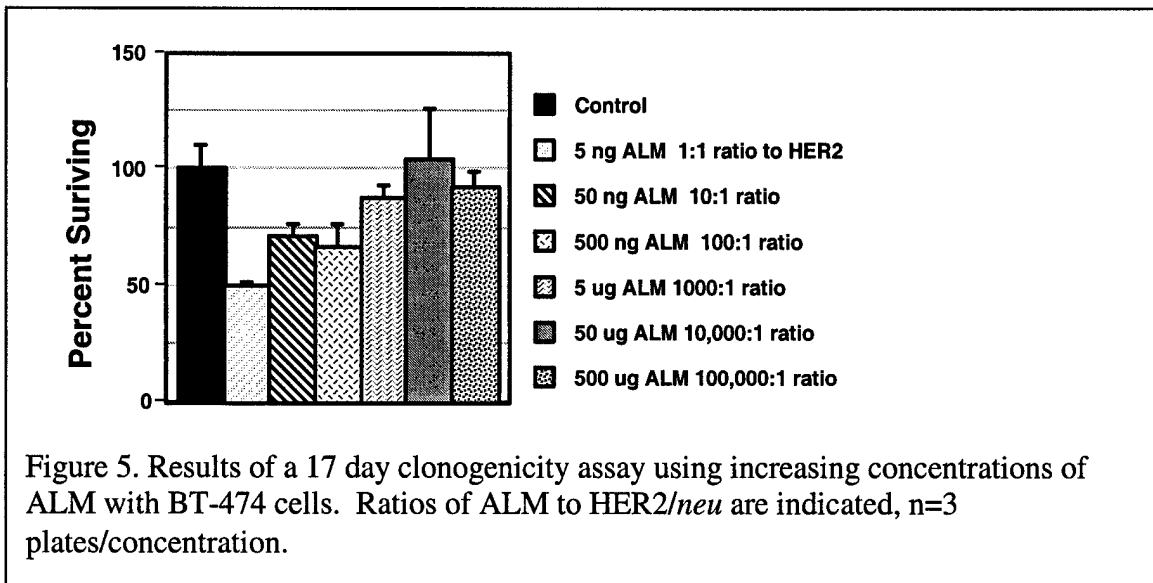
Task 3. Evaluate the specificity of the bs-scFv molecules for both target antigens. The specificity of the first bs-scFv, ALM, for HER2 and HER3 ECDs was evaluated by surface plasmon resonance on the BIAcore instrument. It was found to be capable of binding to HER2 ECD, HER3 ECD and of forming a complex between HER2 and HER3 ECDs in a sandwich assay (Figures 1-3).





Task 4. Evaluate the function of the bs-scFvs. The functional consequences of the binding of first bs-scFv, ALM, to cells that overexpress HER2 and HER3 was evaluated in MTT assays, a clonogenicity assay and by flow cytometry. In a five day MTT assay, which is a measure of impact on cellular proliferation, ALM mediated a similar degree of growth inhibition of human breast tumor BT-474 cells as did Herceptin – a clinically approved MAb that targets HER2 (Figure 4). In a 17 day clonogenicity assay, ALM incubated with BT-474 breast cancer cells at a roughly equimolar concentration to the amount of cell-surface HER2 lead to approximately a 50% reduction in tumor cell survival (Figure 5). Increasing the quantity lead to a decrease in efficacy. Finally, the impact of ALM on HER2 and HER3 expression on BT-474 cells was determined by flow cytometry. It was found that 1.6 $\mu\text{g/ml}$ ALM incubated with 1.6×10^6 BT-474 cells resulted in a reduction of cell-surface HER2 after 4 hr @ 37°C and a reduction in cell surface HER3 after 48 hr @ 37°C (Figure 6).





Key Research Accomplishments

- Isolated scFv from a human phage display library that are specific for HER3, HER4 and EGFR. 6 unique clones that bind to HER3, 10 unique clones that bind to HER4 and 33 unique clones that bind to EGFR.
- Developed a novel peptide spacer that is expected to be protease resistant for use in linking the scFv into a bispecific format (Spacer sequence = NSGAGTSGS GASGEGSGSKL)
- Created the following scFv-based molecules; two bs-scFv that are specific for HER2 and HER3 (ALM and FLM), two homodimeric scFv that are specific for HER3 (ALA and FLF), one homodimeric scFv that is specific for HER2 (MLM) and one heterodimeric scFv that is specific for two epitopes on HER3 (FLM).
- Evaluated the impact of the lead bs-scFv molecule, ALM, on the in vitro growth properties of the human breast tumor cell line, BT-474, that overexpresses HER2 and HER3.
 - In an MTT assay evaluating the impact on cell proliferation, a five day incubation with ALM, Herceptin (positive control) or Rituxan (negative control) lead to comparable inhibition of proliferation with ALM and Herceptin (Figure 4).
 - In a clonogenicity assay, a 17 day incubation with 5 ng to 500 ug concentrations of ALM (ALM to cell surface HER2 ratios of 1:1 to 100,000:1) lead to approximately 50% reduction in cell viability in the group treated with the lowest concentration of ALM (Figure 5). Interestingly, as the concentration was increased, the impact on survival decreased.
 - Have determined by flow cytometry that incubation of BT-474 cells with ALM leads to decreases in the cell surface expression of HER2 and HER3 (Figure 6).

Reportable Outcomes

- American Association for Cancer Research abstract. #4810. Production and Evaluation of Bispecific single-chain Fv molecules that target HER2/neu and HER3. E. Horak, L. Shahied, C. Shaller, A. Tesfaye, H. Simmons, R. Alpaugh, N. Greer, T. Heitner, J. Garrison, J. Marks, L. Weiner and G. Adams. Proc. AACR. 43:971, 2002.
- Presentation at "AntibOZ", An International Forum: Predicting the Next Wave of Protein-Based Therapies and Immunodiagnostics. Heron Island, Queensland Australia. April 8-12th, 2002.
- Provisional Patent filed April 12, 2002.

Conclusions

The generation of a scFv shuffling system for creating multiple bs-scFv partners was more difficult than expected. While progress has been made in this area, we have proceeded to make a two of bs-scFv molecules that target HER2 and HER3 and homodimeric controls that target HER2 or HER3. The lead molecule, ALM, has been evaluated for specificity and efficacy in vitro. We have found that it binds specifically to both targets and mediates a decrease in cell surface expression of HER2 and HER3 and leads to a reduction in cell survival and proliferation. We plan to evaluate the in vivo efficacy of ALM in immunodeficient mice bearing s.c. human breast cancer tumors. If

ALM shows a similar effect in vivo, we will plan on initiating clinical trials as soon as possible.

References

N/A

Appendices

American Association for Cancer Research abstract. #4810. Production and Evaluation of Bispecific single-chain Fv molecules that target HER2/neu and HER3. E. Horak, L. Shahied, C. Shaller, A. Tesfaye, H. Simmons, R. Alpaugh, N. Greer, T. Heitner, J. Garrison, J. Marks, L. Weiner and G. Adams. Proc. AACR. 43:971, 2002.

#4808 Glycopeptide-Related Surface Antigen (GRSA) appears to be a marker of breast ductal carcinoma in situ. William G. North, Brendan P. Keegan, Vincent A. Memoli, and Wendy A. Wells. *Dartmouth Medical School, Lebanon, NH.*

We previously have shown that expression of the vasopressin gene by breast cancer gives rise, not only to biologically active peptide also, to protein products at the cell surface referred to by us as glycopeptide-related surface antigen (GRSA). This name was coined because the antigen is related to glycopeptides raised against the C-terminal half (18 AA) of the vasopressin moiety of vasopressin. Rabbit polyclonal antibodies were previously employed to show by immunohistochemistry (and by Western blotting) that GRSA was present in all of 23 breast cancers examined, but was absent in 21 cases of breast fibrocystic disease (including atypical ductal hyperplasia) and from normal breast tissue. We now report that GRSA was present in almost all (>90%) of 38 cases of cytologically identified ductal carcinoma in situ (DCIS) examined using both our protein A-purified polyclonal antibodies and a new monoclonal antibody (MAG 1). MAG 1 is a mouse IgG1 antibody generated by us using hybridoma technology and purified through affinity chromatography from ascites fluid. Immunohistochemistry was performed on 3-5 mm sections of AMEX-fixed or formaldehyde-fixed tissue sections for the presence of DCIS. Human anterior hypophyseal tissue was employed as a positive control and antibody blocked with peptide as a negative control. Antigen-retrieval was performed on formaldehyde-fixed tissues by treating sections with trypsin for 10 min at ambient temperature. Over 90% DCIS examined were positive for GRSA and this staining was determined to be specific since it was entirely blocked in the presence of peptide antigen. Our data indicate GRSA is a marker of early oncogenic transformations in breast tissues and that MAG 1 can be used as an effective marker of oncogenic transformation in the breast. Identification of GRSA may be useful in discriminating DCIS from ADH, and identifying DCIS cytologically.

#4809 Detection of HPV-infected cells utilizing an on-chip hydrodynamic focusing system. Lauren S. Gollahon, Zhiqiang Du, Nikita Patel, Marc Pimsleur, Diana Contreras, and Darryl Bornhop. *Texas Tech University, Lubbock, TX, and Texas Tech University - Health Sciences Center, Lubbock, TX.*

Cervical cancer is the most common malignant reproductive tract cancer in women from 15 to 35 years of age. It is the third cause of death from cancer in women behind lung and breast cancer. It is estimated that over 1 million cases of invasive cervical cancer cases diagnosed in the U.S. for 2002. Over 1 million women will eventually die of the condition. Annually, 35,000 women are diagnosed with early stage cervical cancer. The progression of disease is directly related to infection by certain strains of human papillomavirus (HPV). Early detection and treatment are critical for the successful therapy of cervical cancer, therefore it is important to identify abnormal cells as quickly and accurately as possible. The most important step in accurate identification of early stage cervical intraepithelial neoplasia (CIN) from collected cells. Cervical samples are routinely collected by Pap smear and microscopically examined for pathologic features. To date, there is no accurate or convenient method of screening for HPV infected cells. The most accurate method is staining the cell preparation. Identification of HPV infection is performed through ancillary techniques that rely primarily on immunofluorescence and screening for cytoplasmic or nuclear proteins or through DNA amplification. Therefore, there is a need for novel methods of cell isolation, particularly those that are sensitive to the capture of abnormal cells. We have applied an antibody-based hydrodynamic focusing microflow system for the capture and enrichment of HPV-infected human cervical epithelial cells. This new antibody-based capture and enrichment system allows sensitive and accurate detection of the presence of HPV in targeted cells without secondary manipulations to the samples. Results show human cervical epithelial (HCE) cells containing HPV were captured from mixed populations of infected and uninfected HCE cells and human cervical stromal (HCS) cells. We are currently modifying this technique for applications to other target specific exfoliated cells from large volumes and/or bodily fluid flushes, e.g. pancreatic brushes, colonic effluents and ductal lavage.

#4810 Production and evaluation of bispecific single-chain Fv molecules that target HER2/neu and HER3. Eva M. Horak, Lillian S. Shahied, Calvin C. Shaller, Abohawariat Tesfaye, Heidi H. Simmons, R. Katherine Alpaugh, Nathaniel B. Greer, Tara Heitner, Jennifer L. Garrison, James D. Marks, Louis M. Weiner, and Gregory P. Adams. *Fox Chase Cancer Center, Philadelphia, PA, and University of California, San Francisco, San Francisco, CA.*

Signal transduction through members of the EGFR Family (EGFR, HER2/neu, HER3 and HER4) is dependent upon the formation of homodimers or heterodimers triggered by the binding of ligand. Overexpression of the members of this receptor family has been correlated with a poor prognosis in a number of types of cancer. Antibodies, such as Herceptin (anti-HER2), C225 and ABX-EGF (anti-EGFR) or small molecules like IRESSA, that perturb signaling through these receptors, have been associated with significant clinical responses. We hypothesize that bispecific single-chain Fv (bs-scFv) that bind to selected pairs of these receptors could prevent ligand induced signaling and trigger cytostatic or cytotoxic effects. We have previously described the production of the extracellular domains of EGF, HER2/neu, HER3 and HER4 receptors and their use as targets for selection of specific binders from a naive human scFv phage display library. We are now producing single gene bs-scFv molecules from these four sublibraries with the goal of functionally selecting bispecific scFv molecules that

target epitope pairs and mediate anti-tumor effects. Our initial focus has been on HER2/neu and HER3. The first bs-scFv we have produced, A5-ML3.9, is capable of binding to both the HER3 and HER2/neu receptors. Its *in vitro* binding kinetics, cell surface retention, *in vivo* tumor-targeting properties and ability to impact the growth of tumor cells expressing both HER2/neu and HER3 receptors will be presented. This research was supported by Concept Award # DAMD17-01-1-0520 from the Department of Defense Breast Cancer Research Program.

#4811 Synthetic immunostimulatory oligonucleotides show anti-tumor activity and increase therapeutic effectiveness of monoclonal antibody and chemotherapeutic agents. Hui Wang, Jie Hang, Mao Li, Zhenqi Shi, Lin Lin, Ekambar R. Kandimalla, Dong Yu, Qiuyan Zhao, Sudhir Agrawal, and Ruiwen Zhang. *Department of Pharmacology and Toxicology, University of Alabama at Birmingham, Birmingham, AL, and Hybridon, Inc., Cambridge, MA.*

It has been suggested that the observed antitumor activities of antisense oligonucleotides that contain CpG dinucleotides could be due to a combination of antisense and immune-mediated effects. Oligonucleotides containing CpG dinucleotides in certain sequence contexts can induce a number of cytokines, including IL-12, IL-6, IFN- γ , and TNF- α . The presence of CpG dinucleotides in antisense oligonucleotides could contribute to the antitumor activity of antisense agents independent of direct inhibition of targeted gene product. In the present study, we used several oligonucleotides containing natural and synthetic immunostimulatory motifs to evaluate the antitumor activity in human cancer xenograft models. The simultaneous administration of these synthetic CpG oligos at various doses significantly inhibited tumor growth in mice bearing xenografts of human cancers of colon (HCT-116 and DLD-1) and prostate (DU145) and glioblastoma (U87MG). The synthetic CpG oligos also increased the therapeutic effects of monoclonal antibody (anti-HER2/neu) and chemotherapeutic agent, paclitaxel in all three models tested (HCT-116, DLD-1 and DU145). These data demonstrate that the oligonucleotides that contain CpG dinucleotides exhibit anticancer activity mainly by immune stimulation. In addition, we demonstrated that synthetic motifs incorporated in oligonucleotides influence the immunostimulatory activity of CpG oligos, resulting in an improvement of host side effects. This study provides a basis for future development of synthetic immunostimulatory oligonucleotides as cancer therapeutic agents used alone or in combination with conventional therapies. (Supported by NIH/NCI grant CA 80698.)

#4812 Combined reovirus and 1,3-bis (2-chloroethyl)-1-nitrosourea therapy against murine hematopoietic tumors. Timothy A. Steele. *Des Moines University-Osteopathic Medical Center, Des Moines, IA.*

Reovirus therapy of tumors is an emerging area of study as evidenced by recent investigations (Coffey, et al., *Science* 282:1332-1334, 1998 and Steele and Cox, *Cancer Biother.* 10:307-315, 1995). In our laboratory, EL-4 or L1210 tumor-bearing B6D2F1 mice treated at day 4 (following intraperitoneal tumor injection) with 9 mg/kg 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU) followed at day 6 with 1×10^9 plaque-forming units of reovirus type 3 (Dearing) resulted in 65-80% of the mice being cured of tumor. Tumor-bearing mice treated with reovirus or BCNU alone typically yielded 0% and 20% survival, respectively. Challenge of cured mice with homologous tumor yielded 0% survival, whereas challenge with heterologous tumor produced 0% survival, suggesting an immune-mediated tumor elimination mechanism. That the mechanism may proceed via an immune-mediated mechanism was strengthened by the finding that daily *in vivo* administration of cyclosporine, an immunosuppressive drug, to therapy-treated animals abrogated the survival effect. Flow cytometric analysis revealed an increase in natural killer cells, macrophages, and CD8+ lymphocytes in the mesenteric nodes of therapy-treated mice compared to untreated or tumor-bearing control mice. In addition, an increase in the number of spleen macrophages was also observed in therapy-treated mice compared to untreated or tumor-bearing control mice. Since reovirus is a potent inducer of α/β interferons in mice, we investigated whether murine recombinant interferon- α (IFN- α) could replace reovirus in the therapy. Mice treated with IFN- α , varying between 500 - 10,000 units, were able to produce significantly ($p < 0.05$) above control mice. However, only the 10,000 unit dose could produce cures equivalent to reovirus. Challenge of cured mice with reovirus-treated mice with EL-4 tumor yielded 100% survival, whereas challenge with heterologous tumor (L1210) produced 0% survival, suggesting that tumor elimination proceeded via an immune-mediated mechanism. Intraperitoneal injection of anti-IFN- α antibodies did not diminish the effect of the therapy, suggesting the presence of redundant cytokines. Further studies are warranted to investigate the potential of combined reovirus and chemotherapy on hematopoietic tumors.

#4813 IL-6 and interferon- γ levels following chemotherapy in breast cancer. Andrea M. Mastro, Nancy I. Williams, Jennifer L. Guener, Elizabeth Orsega-Smith, William J. Kraemer, Aaron M. Goldstein, Richard H. Dixon, Judy Underwood, Mary Miles, and Kate Wagner. *University of Connecticut, University Park, PA, University of Connecticut, Storrs, CT, University of Connecticut, State College, PA, CMSA, State College, PA, and Montclair State University, Bozeman, MT.*

In a study designed to follow the effects of chemotherapy on CD4+ T lymphocytes following chemotherapy for breast cancer, we investigated if there were changes in interferon gamma (IFN γ), a Th1 cytokine, and interleukin 6 (IL6), a Th2 cytokine. These cytokines were measured in the plasma of women at the following times: prechemotherapy, postchemotherapy, postradiation and 3 and 6 months