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13. ABSTRACT *(Maximum 200 words)*

Targeting of adenovirus vectors, encoding for therapeutic genes, to tumor-specific receptors on breast cancer cells should result in specific killing of these cells. Targeting is necessary to prevent gene transfer in normal tissues resulting from the infection of normal cells by adenovirus. We have previously reported the use of an anti-knob antibody fragment (Fab), which prevents Ad infection, conjugated to folate to target adenovirus to folate receptor positive cells. In this report, the Fab has been conjugated on a large scale to an anti-EGFR antibody (425) and an anti-erbB-2R antibody (Herceptin) to yield Fab-425 and Fab-Herceptin conjugates, respectively. These conjugates were used to target adenovirus specifically to EGF and erbB-2 receptors on BT-474, MDA-MB-468, MDA-MB-134, MDA-MB-231, MDA-MB-453, and SK-BR-3 breast cancer cells. In addition, the conjugates could specifically target adenovirus to the receptors in a hetergeneous cell population. Preliminary *in vivo* studies were conducted which will be valuable for future evaluation of the conjugates. Also, studies using an adenovirus encoding for a therapeutic gene were evaluated. These results are significant in that they demonstrate that adenovirus vectors can be specifically delivered to breast cancer cells in a heterogeneous cell population. This will be significant for treating disseminated breast cancer with adenovirus vectors.

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Introduction

This research involves the targeting of adenviral vectors to breast cancer for gene therapy applications through immunological methods. Adenoviral vectors have been widely used for cancer gene therapy applications because of their ability to accomplish efficient *in vivo* gene transfer. However, adenoviral vectors also infect a wide variety of normal tissues, mostly due to the widespread expression of the coxsackie-adenovirus receptor (CAR) which binds the adenovirus knob. Therefore, preventing the knob-CAR interaction while simultaneously introducing a novel ligand on the adenovirus for targeting breast cancer cells will result in an adenoviral vector which can accomplish breast cancer-specific gene transfer. I have previously described the use of the Fab fragment of an anti-knob antibody (1D6.14) conjugated to various ligands for targeting to cancer cells *in vitro* and *in vivo*. The purpose of this proposal is to conjugate breast cancer ligands to the Fab fragment and evaluate the resulting conjugates ability to target adenoviral vectors *in vitro* and *in vivo*. This report will focus on the *in vitro* experiments performed since the last annual report and the initial *in vivo* experiments.

The previous report described the characterization of the breast cancer cell lines used, the synthesis of Fab-FGF2, Fab-EGF, and Fab-anti-erbB-2 antibody, and the ability of these conjugates to target an adenoviral vector encoding the luciferase reporter gene (AdCMVLuc) to the breast cancer cells *in vitro*. Due to the complexities of working with FGF2 in the previous report, this report describes work focusing on targeting adenovirus to the EGF receptor and the erbB-2 receptor. In addition, in preparing for *in vivo* studies, it was decided that using an anti-EGFR antibody (425) would be better than using the EGF ligand. Both of these represent changes from the statement of work that were necessary to focus this effort and accomplish the *in vivo* studies. This report describes the *in vitro* evaluation of two conjugates (Fab-425 and Fab-Herceptin; same as Fab-anti-erbB-2 described in previous progress report) that will ultimately be used for *in vivo* studies and preliminary non-targeted *in vivo* studies.

Body

Conjugation of 425 and Herceptin to Fab and evaluation of these conjugates in vitro. The murine monloclonal antibody, 425, to human EGFR and the humanized monoclonal antibody, Herceptin, to erbB-2R were conjugated to the anti-knob Fab as described in the previous progress report. As noted above, it was decided to use the 425 antibody for targeting to EGFR as opposed to the EGF ligand. This was based upon other studies in our laboratory which showed that the Fab-425 conjugate could efficiently target adenovirus to EGFR expressed on human gliomas (C.R. Miller, et al., *Cancer Res.* **58**, 5738 (1998)). These conjugations were performed using 10 mg of

Fab and 10 mg of 425/Herceptin to yield 2 mg of the final conjugates. This scale was necessary to perform the *in vivo* studies proposed for year 3.

The Fab-425 and Fab-Herceptin were evaluated *in vitro* using SKBR-3, BT-474, and MDA-MB-468 breast cancer cells. As reported previously, the SK-BR-3 cells are positive for EGFR and erbB-2R, the BT-474 cells are EGFR negative and erbB-2 positive, and the MDA-MB-468 cells are EGFR positive and erbB-2 negative. Twenty-four hours prior to infection, the cells were seeded in twenty-four well plates at a density of 1.2×10^4 cells/well. The cells were then infected at 100 pfu/cell with AdCMVLuc that had been premixed with various concentrations of the Fab-Herceptin or Fab-425 conjugates. Infections were performed in the presence or absence of a saturating amount of purified Ad5 knob to inhibit adenoviral infection. Twenty-four hours after infection, the cells were harvested and assayed for luciferase expression (**Figures 1,2**). Figures 1 and 2 show that the optimal ratio of Fab-Herceptin or Fab-425 to AdCMVLuc to achieve CAR independent gene transfer is 30 ng Fab-Herceptin or Fab-425:1.2×10^6 pfu of AdCMVLuc. This is the ratio that was used for all subsequent experiments. It also demonstrates the need for scaling up the synthesis described earlier. If 1×10^9 pfu is necessary to inject per animal for an *in vivo* experiment, this would require approximately 30 μg of conjugate/animal.

Specifically targeting AdCMVLuc to breast cancer cells in vitro. This is directly related to Specific Aim #2 of the application in order to confirm that the new conjugates made (Fab-425 and Fab-Herceptin) could specifically target AdCMVLuc to breast cancer cells *in vitro*. The statement of work said that these assays would be performed during the second year of funding. Although they were performed in the first year with the original conjugates, it was necessary to repeat these assays with the conjugates that will be used *in vivo*. Twenty-four hours prior to infection, the cells (MDA-MB-134, MDA-MB-231, MDA-MB-453, MDA-MB-468, BT-474, and SK-BR-3) were seeded in twenty-four well plates at a density of 1.2×10^4 cells/well. The cells were then infected at 100 pfu/cell with AdCMVLuc that had been premixed with the optimal concentration of Fab-Herceptin or Fab-425 determined in the titrations (30 ng/1.2×10^6 pfu). Infections were performed in the presence or absence of a saturating amount of Herceptin or 425 to inhibit adenoviral infection. Twenty-four hours after infection, the cells were harvested and assayed for luciferase expression (**Figure 3**). Figure 3 shows that the Fab-425 conjugate specifically targeted AdCMVLuc to the MDA-MB-468, MDA-MB-231, and SK-BR-3 cells. Targeting of AdCMVLuc to MDA-MB-468 with Fab-425 cells had the best targeting index (Ti; ratio of targeted luciferase expression to non-targeted luciferase expression) of ~5.8. The Fab-Herceptin conjugate targeted AdCMVLuc to BT-474, SK-BR-3 and MDA-MB-453 cells. The targeting index was ~1.1 for the

BT-474 cells and ~1.8 for the SK-BR-3 cells. The MDA-MB-134 cells did not demonstrate specific targeting of AdCMVLuc with either conjugate. Thus, these conjugates were shown to specifically target AdCMVLuc to breast cancer cells that express EGFR, erbB-2R or both. The level of receptor expression on these cells was discussed in the previous annual report.

Targeting adenovirus in a heterogenous mixture of cells. The previous progress report stated that "cocktails" of targeted adenoviral vectors may have applications for breast cancer cells of differing phenotypes that respond to one particular targeting conjugate. These assays demonstrate that adenovirus can be targeted to a particular cell type in a heterogeneous mixture of cells. For these studies, the BT-474 (erbB-2R+; EGFR-) and MDA-MB-468 (erbB-2R-; EGFR+) cells were used based on the results described above. The assays were performed using flow cytometry and an adenovirus encoding for green fluorescent protein (AdCMVGFP) as the reporter. The cells were seeded in six-well plates at a density of 2 x 10^5 cells/well and twenty-four hours later were infected with various doses of AdCMVGFP (0, 1, 5, 10, 20, 50, 100, 200, 500 pfu/cell). Twenty-four hours after infection, the cells were typsinized and resuspended in Hank's Balanced Salt Solution (containing 0.1% NaN_3 and 1% BSA) for FACS analysis. The results show that > 90% of the cells express GFP when infected at 10 pfu/cell or greater (**Figure 4**). Thus, 10-20 pfu/cell was used for targeting experiments in a mixture of the two cell lines.

The ability of Fab-425 and Fab-Herceptin to target AdCMVGFP was then evaluated in a mixed population of MDA-MB-468 and BT-474 cells. The cells were seeded at 2 x 10^5 cells/well in a six-well plate in a 20:80 ratio of MDA-MB-468:BT-474 to obtain ~30:70 ratio after 48 hours of growth. The cells were then infected twenty-four hours later at 10 pfu/cell with AdCMVGFP alone or AdCMVGFP that had been premixed with the optimal concentration of Fab-Herceptin or Fab-425 (30 ng/1.2 x 10^6 pfu). After incubating for an hour, the conjugate/virus mixture was replaced with fresh media and harvested twenty four hours later for FACS analysis as described above. EGFR expression and erbB2R expression was determined by incubating the cells with a mixture of 425 and Herceptin. The cells were then incubated with a mixture of goat anti-mouse and goat anti-human secondary antibodies that bind to 425 and Herceptin, respectively without cross-reactivity. The goat anti-mouse antibody was conjugated with allophycocyanin (APC), while the goat anti-human antibody was conjugated with phycoerythrin (PE). The cells were sorted by their APC and PE emissions by FACS and then analyzed for GFP expression. These results are summarized in **Figure 5**. The data are presented as the % positive gated cells. This shows that ~25% of the cells were EGFR positive (MDA-MB-468) and ~75% were erbB-2R positive (BT-474). In the 25% of cells that were EGFR positive, ~60% were positive for GFP

when infected with ADCMVGFP alone. This increased to ~90% when targeted with Fab-425 and decreased to ~30% when targeted with Fab-Herceptin. This is in agreement with the luciferase results discussed earlier. The erbB-2R positive cells were ~90% positive for GFP when infected with AdCMVGFP alone, this decreased to ~35% when infected targeted with Fab-425 and returned to ~90% when targeted with Fab-Herceptin. Thus, the conjugates could still specifically target adenovirus to their appropriate receptor in the presence of another cell line not expressing that receptor.

In vivo tumor growth and adenoviral infection. This is directly related to Specific Aim #3, which is to be completed in the third year of funding according to the statement of work. We chose to evaluate the *in vivo* growth of the MDA-MB-468 and BT-474 cells for our targeting experiments. The MDA-MB-468 cells (2×10^7) were injected subcutaneously in each rear flank of athymic nude mice. Tumors were measurable after three weeks (5 mm x 5 mm). An oestrogen pellet was implanted subcutaneously on the back of athymic nude mice one day prior to implantation of the BT-474 cells (2×10^7) subcutaneously in each rear flank. The tumors were ~ 4 mm x 4 mm after two weeks of growth.

A preliminary study was conducted to evaluate the level of tumor transduction after adenoviral infection. The statement of work indicated that adenovirus vectors containing the luciferase gene (AdCMVLuc) or β-galactosidase gene (AdCMVLacZ) would be used for this. However, an adenovirus encoding for the human somatostatin receptor subtype 2 (AdCMVhSSTr2) was used because of our experience with this vector (B.E. Rogers, et al., *Clin Cancer Res.* **5**, 383 (1999)). We have shown that AdCMVhSSTr2 can be used to evaluate gene transfer by localization of a radiolabeled peptide that binds to this receptor after expression in infected cells. In this preliminary study, AdCMVhSSTr2 was injected (1×10^9 pfu) intratumorally into the MDA-MB-468 tumors on one flank of the mice. The other tumor was not injected as a control. Forty-eight hours after adenoviral injection, a ^{99m}Tc-labeled peptide which binds with high affinity was injected i.v. into the mice. ^{99m}Tc is a gamma-emitting radionuclide commonly used for radiolabeling proteins and peptides. Four hours after injection of the ^{99m}Tc-labeled peptide, the animals were sacrificed, and the tissues harvested and counted in a gamma counter. The data in **Figure 6** are presented as the median percent injected dose/gram (% ID/g of tissue) of tissue for three animals. Results showed increased uptake in tumors injected with AdCMVhSSTr2 compared to tumors that received no virus (5.2 vs. 0.7 % ID/g). This experiment will be repeated with mice bearing BT-474 tumors and with the Fab-425 and Fab-Herceptin conjugates as described in the statement of work.

In vitro infection of breast cancer cells with an adenovirus encoding a therapeutic gene. These studies were not described in the statement of work, but were conducted to move the reporter gene experiments in animals described above into experiments using a therapeutic gene. We chose an enzyme-prodrug strategy using the bacterial cytosine deaminase (CD) enzyme, which converts the non-toxic drug 5-fluorouracil (5-FU) into the toxic 5-fluorocytosine (5-FC), due to our experience with this system (L.C. Pederson, et al., *Cancer Res.* **57**, 4325 (1997)). In a preliminary study, we demonstrated that MDA-MB-468 and BT-474 cells could be infected with an adenovirus encoding CD (AdCMVCD) and convert 5-FU to 5-FC. The cells were infected with 0, 10, or 100 pfu/cell of AdCMVCD and harvested and lysed forty-eight hours later. The lysates were centrifuged and 10 μl of supernatant were incubated with [^{3}H]-5-FC for six hours at 37°C. The samples were then spotted on a silica gel thin layer chromatography plate and developed in butanol:water (86:14). The plates were then cut and counted in a beta-counter to determine the percent radioactivity associated with 5-FC and the percent radioactivity associated with 5-FU (as determined by comparison to standards) (**Figure 7**). This shows that both MDA-MB-468 and BT-474 cells showed little conversion from 5-FC to 5-FU (< 8%) when uninfected. Infection of MDA-MB-468 cells at 10 pfu/cell showed 56% conversion of 5-FC to 5-FU which increased to 97% upon infection at 100 pfu/cell. Similarly, the BT-474 cells showed 57% when infected at 10 pfu/cell and 91% at 100 pfu/cell. Thus, these cells can convert 5-FC to 5-FU when infected with AdCMVCD. Future studies will demonstrate that the 5-FU produced is toxic to these cells. This system can than be used to evaluate the therapeutic response in mice bearing MDA-MB-468 or BT-474 cells with or without targeting of AdCMVCD using Fab-425 or Fab-Herceptin.

Summary

Most of the work performed in this funding period were preliminary studies for the *in vivo* experiments that will be conducted in the final year of funding, as described in the statement of work. Synthesis of the immunological conjugates (Fab-425 and Fab-Herceptin) were scaled up for the *in vivo* experiments to be performed in year 3. These conjugates were validated using the breast cancer cells and shown to specifically target adenovirus to the EGFR or erbB-2R on these cells. In addition, the conjugates could target adenovirus to EGFR or erbB-2R in a heterogeneous population of breast cancer cells. As a first step towards accomplishing the goals of year 3, it was established that the MDA-MB-468 cells would form tumors in athymic nude mice. The MDA-MB-468 tumors were infected with a reporter adenovirus to confirm *in vivo* transduction of these cells. Evaluation of a potential therapeutic strategy for treating breast cancer was also started.

Figure Legend

Figure 1. EGFR targeted AdCMVLuc gene transfer to SK-BR-3 (A) or MDA-MB-468 (B) cells was dependent on Fab-425 dose. The cells (1.2×10^4) were incubated in the presence (closed circles) or absence (open circles) of recombinant Ad5 knob and then infected with AdCMVLuc (100 pfu/cell) preincubated with varying concentrations of Fab-425 (ng conjugate per 1.2×10^6 pfu AdCMVLuc). Luciferase expression was assessed at 24 h post-infection and is shown as the mean ± s.d. of the relative light units (n=6).

Figure 2. erbB-2R targeted AdCMVLuc gene transfer to SK-BR-3 (A) or BT-474 (B) cells was dependent on Fab-Herceptin dose. The cells (1.2×10^4) were incubated in the presence (closed circles) or absence (open circles) of recombinant Ad5 knob and then infected with AdCMVLuc (100 pfu/cell) preincubated with varying concentrations of Fab-Herceptin (ng conjugate per 1.2×10^6 pfu). Luciferase expression was assessed at 24 h post-infection and is shown as the mean ± s.d. of the relative light units (n=6).

Figure 3. Targeting of AdCMVLuc to BT-474, MDA-MB-468, MDA-MB-134, MDA-MB-231, MDA-MB-453, and SK-BR-3 cells using the Fab-425 or Fab-Herceptin conjugate. The cells (1.2×10^4) were infected at 100 pfu/cell with AdCMVLuc in the presence or absence of Fab-425 or Fab-Herceptin (30 ng per 1.2×10^6 pfu). Cells were blocked with an excess of 425 or Herceptin for 1 h at 4°C prior to addition of AdCMVLuc alone or the AdCMVLuc conjugate. Luciferase expression was assessed at 24 h post-infection and is shown as the mean ± s.d. of the relative light units (n=6).

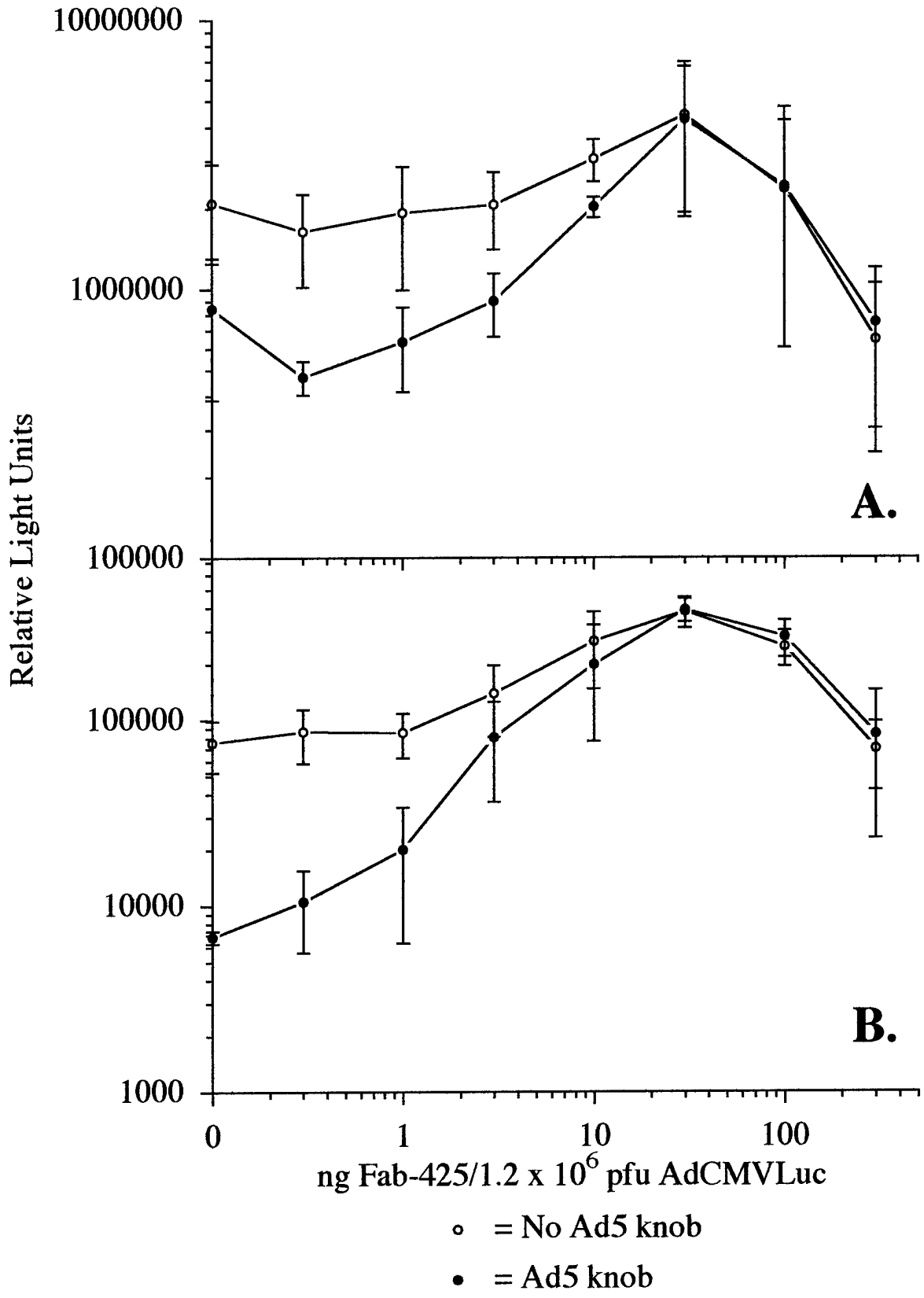
Figure 4. Expression of GFP in MDA-MB-468 (open circles) and BT-474 (closed circles) cells infected with various doses of AdCMVGFP was analyzed by indirect immunofluorescence. The data are expressed as the percentage of positively gated cells.

Figure 5. Expression of GFP in a mixed population of MDA-MB-468 and BT-474 cells was analyzed by indirect immunofluorescence. The cells were seeded at a 20:80 ratio 468:474 and infected with AdCMVGFP alone, AdCMVGFP premixed with Fab-425 or AdCMVGFP premixed with Fab-Herceptin. Twenty-four hours later the cells were harvested for analysis. The cells were first sorted to determine which expressed EGFR (EGFR+) or erbB-2R (erbB2+). These

populations were then sorted to determine the level of GFP expression (GFP in EGFR+ and GFP in erbB2+, respectively). The data are expressed as the percentage of positively gated cells.

Figure 6. Biodistribution of ^{99m}Tc-labeled peptide in nude mice bearing two subcutaneous MDA-MB-468 tumors. Three weeks after tumor cell inoculation, AdCMVhSSTr2 or no virus was injected intratumorally into each tumor, followed by intravenous administration of ^{99m}Tc-labeled peptide two days later. Four hours after ^{99m}Tc-labeled peptide injection, the mice were killed and the organs harvested and counted in a gamma counter. BL = blood; LI = liver; SP = spleen; KI = kidney; BO = bone; UT = uterus; PA = pancreas; TU1 = tumor with AdCMVhSSTr2 injection; TU2 = tumor with no viral injection. Each bar represents the median value from three animals.

Figure 7. Conversion of 5-FC to 5-FU in MDA-MB-468 and BT-474 cells infected with AdCMVCD. The cells were infected with AdCMVCD at 0, 10, or 100 pfu/cell and harvested and lysed 48 h later. The lysates were then incubated with [^{3}H]-5-FC for 6 h at 37°C. The samples were spotted on a silica gel thin layer chromatography plate and developed. The plates were cut and counted on a beta counter to determine the percentage of radioactivity associated with 5-FU. The data is plotted as the percentage of radioactivity associated with 5-FU.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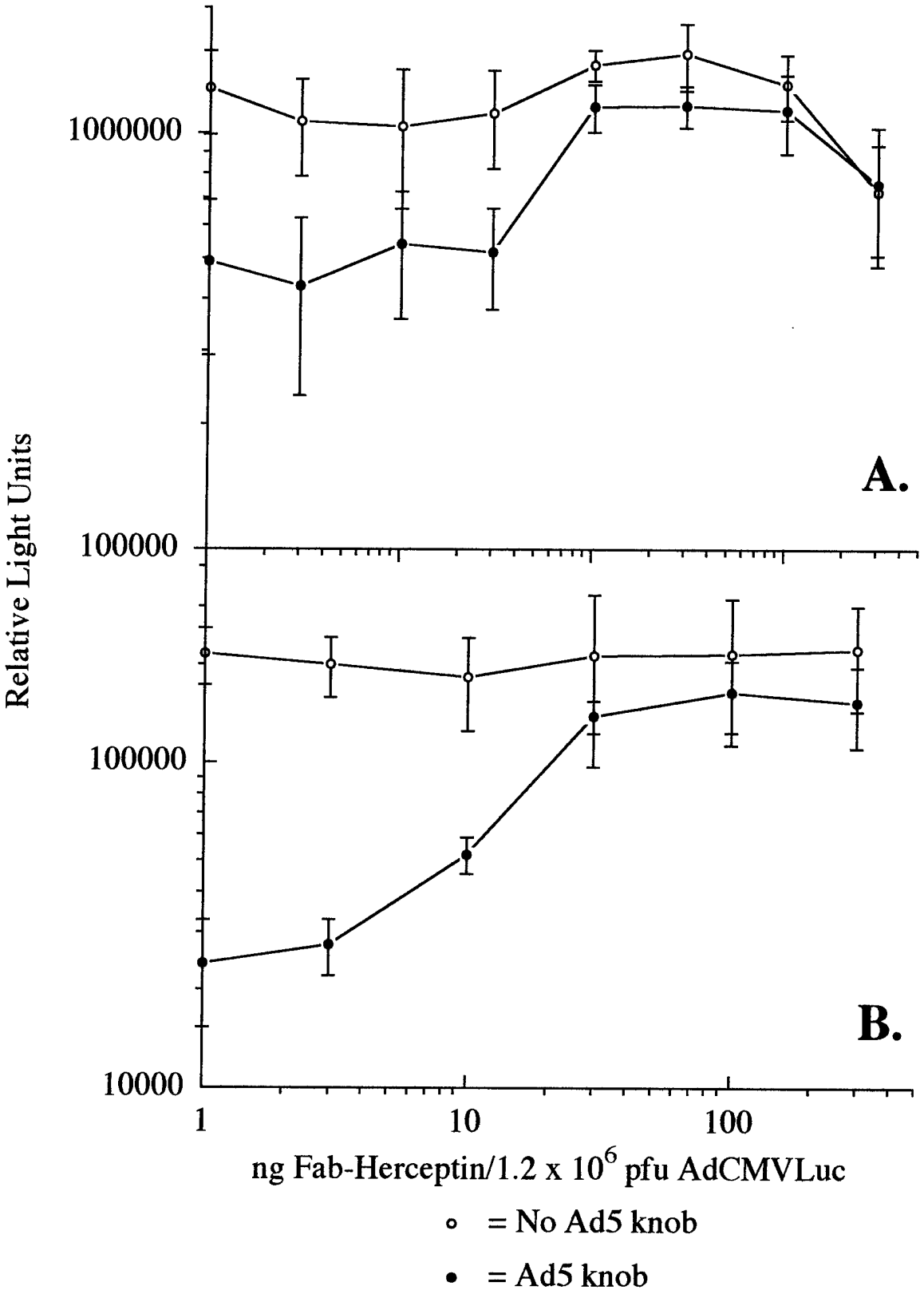
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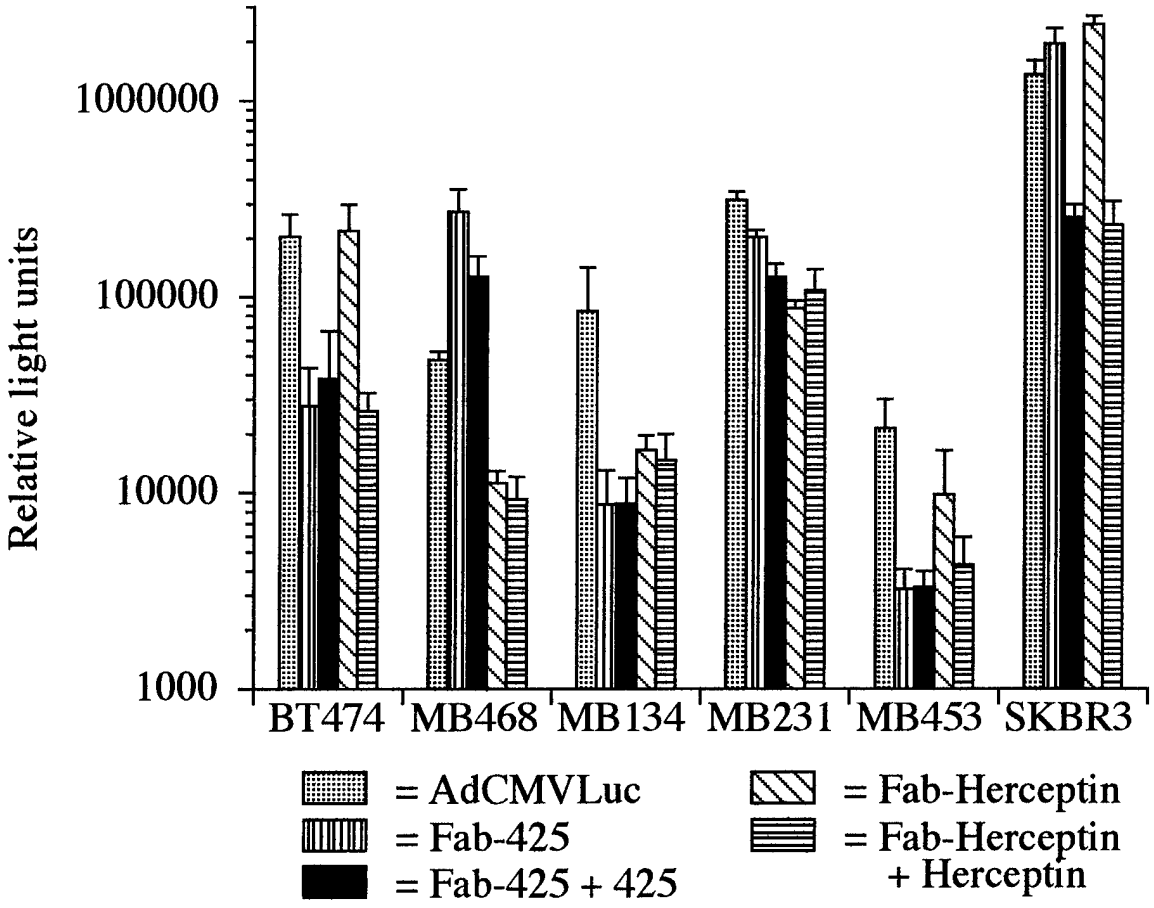
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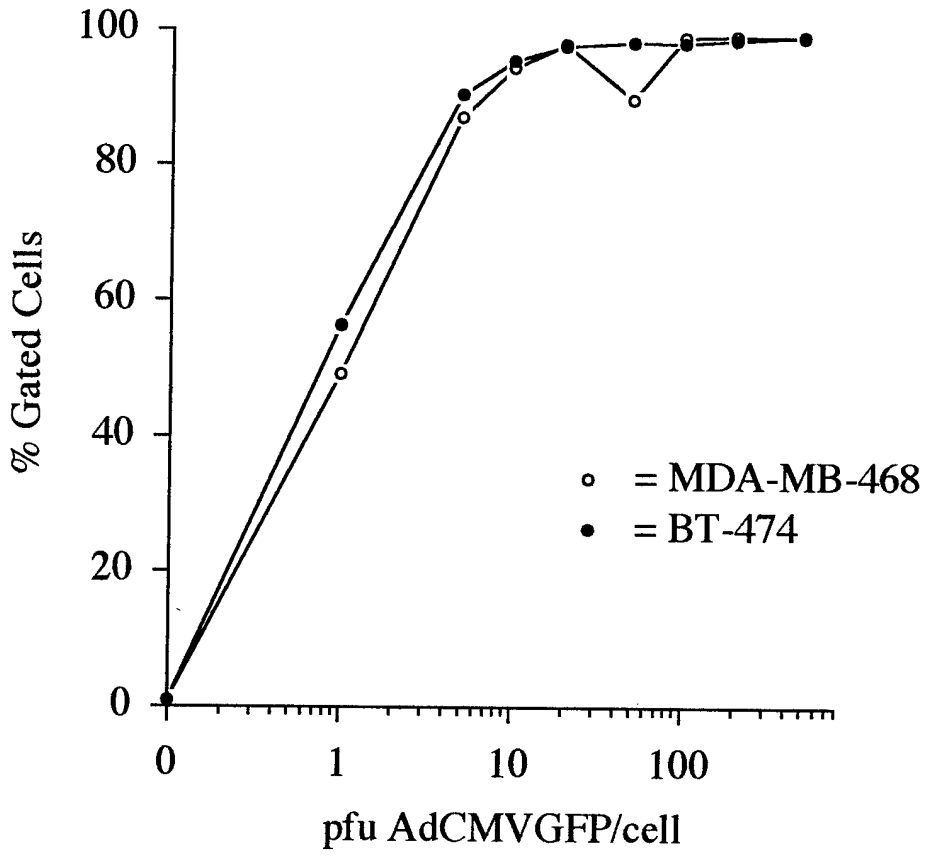
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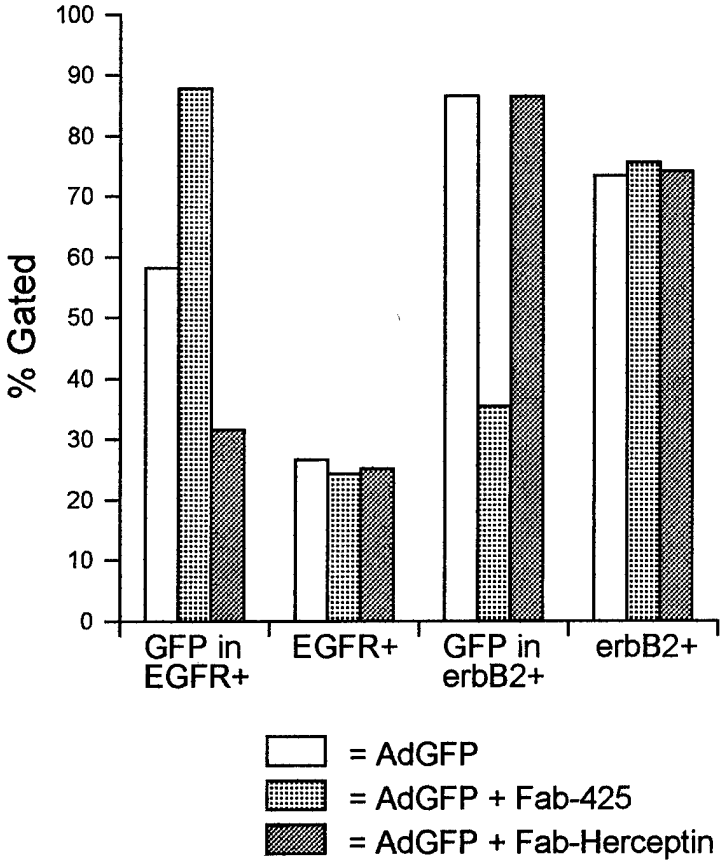
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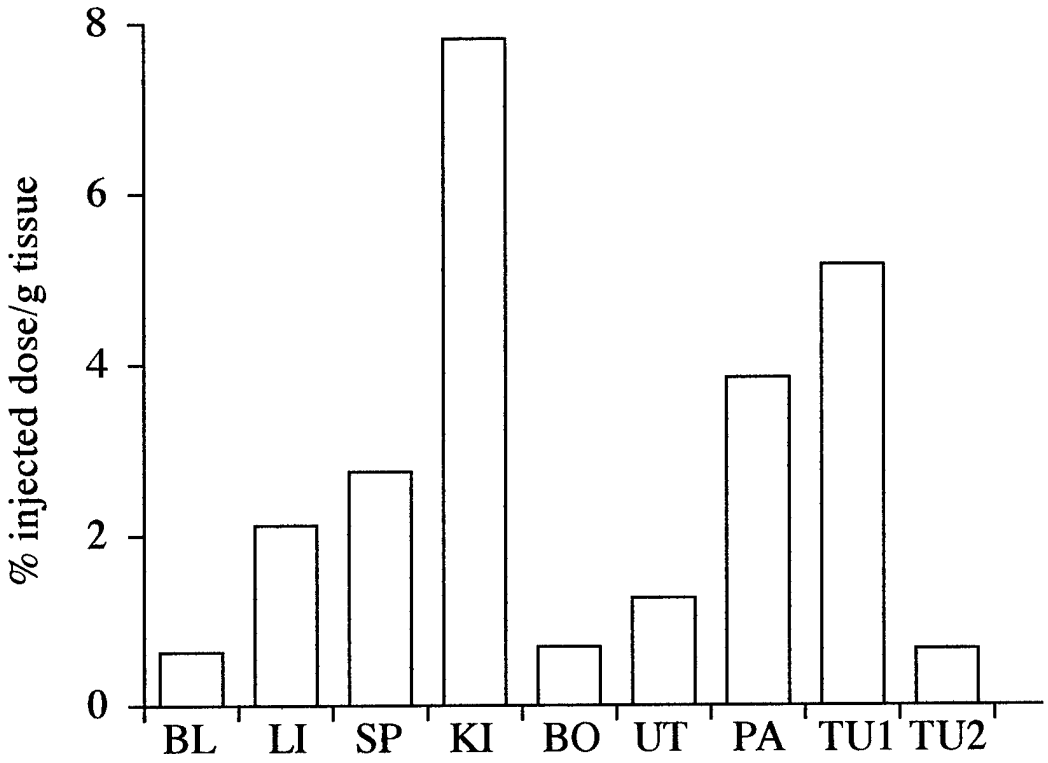
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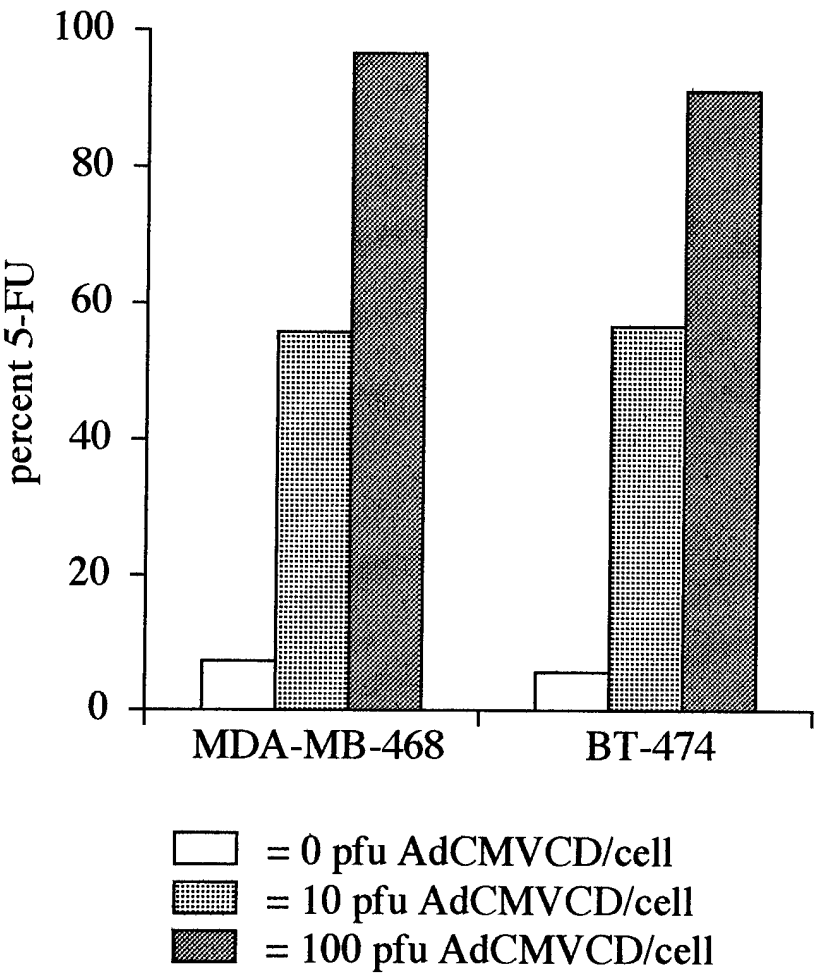
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percent 5-FU
MDA-MB-468
BT-474
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= 10 pfu AdCMVCD/cell
= 100 pfu AdCMVCD/cell

Key Research Accomplishments

- Large scale synthesis of immunological targeting reagents (Fab-425 and Fab-Herceptin) for *in vivo* targeting of adenovirus.
- Targeting of adenovirus to a heterogeneous population of breast cancer cells using Fab-425 and Fab-Herceptin.
- Establishment of the *in vivo* models of breast cancer to be used in the final year of funding.
- Evaluation of a possible therapeutic strategy in human breast cancer cells using targeted adenovirus.

Reportable Outcomes

- Abstract presentation at the American Society for Gene Therapy 2nd Annual Meeting, Washington D.C., June 9-13, 1999.

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Abstract Receipt Deadline: March 2, 1999

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- ☐ 3. Hybrid Vectors

B. Nonviral Vector Development
- ☐ 1. Naked DNA Gene Transfer
- ☐ 2. Lipid Mediated Gene Transfer
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C. Animal Disease Models and Clinical Applications
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IMMUNOLOGICAL TARGETING OF ADENOVIRUS TO THE HER-2/neu RECEPTOR OVEREXPRESSED ON BREAST CANCER CELLS

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Adenovirus (Ad) vectors have been widely investigated for cancer gene therapy applications because of their ability to accomplish in vivo gene transfer with high efficiency. However, the broad tropism of Ad also causes the transduction of normal tissues in vivo. We have previously described an immunological method for specifically targeting Ad to tumor associated receptors on cancer cells. This method involves the chemical conjugation of a receptor specific ligand to the Fab fragment of a monoclonal antibody (1D6.14) which neutralizes Ad infection through binding to the Ad fiber knob, preventing binding of Ad to its cellular receptor. The aim of this study was to determine if this strategy can be used to specifically target Ad to the HER-2/neu receptor overexpressed on breast cancer cells. Herceptin is a humanized antibody that binds to the HER-2/neu receptor and is currently being used in clinical trials to treat patients with breast cancer overexpressing HER-2/neu. To determine the level of HER-2/neu expression on BT-474, SK-BR-3, MDA-MB-453, and MDA-MB-231 human breast cancer cells, binding assays with ^{125}I-labeled Herceptin were performed. This showed that 45%, 47%, 21%, and 4% of the total radioactivity added specifically bound to BT-474, SK-BR-3, MDA-MB-453, and MDA-MB-231 cells, respectively. Infection of these cells with an Ad vector encoding the firefly luciferase gene (AdLuc) showed luciferase expression of 1.6 x 10^5, 3.7 x 10^6, 2.3 x 10^4, and 2.8 x 10^5 relative light units, respectively. This was inhibited >95% in all cell lines by adding an excess of recombinant Ad5 fiber knob to the cells. Herceptin was conjugated to the anti-fiber knob Fab (Fab-Herceptin) and used to target AdLuc to HER-2/neu receptors on breast cancer cells in vitro. The HER-2/neu positive BT-474, SK-BR-3, and MDA-MB-453 cells had Fab-Herceptin targeted AdLuc to non-targeted AdLuc luciferase expression ratios of 1.0, 1.6, and 0.6, respectively. Targeting AdLuc with Fab-Herceptin to these cell lines was inhibited >70% by incubating the cells with an excess of Herceptin prior to infection. The HER-2/neu negative MDA-MB-231 cells had a targeted AdLuc to non-targeted AdLuc luciferase expression ratio of 0.1, demonstrating the selectivity of the Fab-Herceptin conjugate for targeting HER-2/neu positive cells only. These studies show that Ad can be specifically targeted to the HER-2/neu receptor expressed on breast cancer cells. This strategy should be applicable for selectively treating metastatic breast cancer with Ad vectors carrying therapeutic genes.

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