Targeting HER-2/neu with Antirat Neu Virosomes for Cancer Therapy

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ABSTRACT

HER-2/neu (p185HER2) oncogene represents an attractive target for antibody-mediated immunotherapy. The major problem of combining chemotherapy and immunotherapy is the severe side effects that limit the use of doxorubicin (Doxo) as a cytotoxic drug. We have used virosomes (Vir; reconstituted fusion-active viral envelopes) as a new drug delivery system and have shown that Vir are capable of binding and penetrating into tumor cells, delivering cytotoxic drugs. We have additionally demonstrated that conjugating Fab\(^{-}\) fragments of an antirat Neu (anti-rNeu) monoclonal antibody to Vir selectively and efficiently inhibits tumor progression of established rNeu-overexpressing breast tumors. Fab\(^{-}\)Doxo-Vir combine the antiproliferative properties of the monoclonal antibody and the cytotoxic effect of Doxo in vitro. Furthermore, Fab\(^{-}\)-Doxo-Vir significantly inhibit tumor formation at a tumor load representing metastatic spread. These results indicate that Vir conjugated with an antibody against a tumor antigen are a promising new selective drug delivery system for the treatment of tumors expressing a specific tumor antigen.

INTRODUCTION

Among various antigens specific for tumor cells, HER-2/neu, a member of the epidermal growth factor receptor family encoding a transmembrane growth factor receptor (1), is an attractive target antigen. The overexpression of HER-2/neu protein has been observed in various tumors, especially in 20–30% of breast cancers (2–4). Moreover, HER-2/neu overexpression occurs in the primary tumor as well as in metastatic sites (5). 4D5, a murine mAb\(^{4}\) directed against the extracellular domain of human HER-2/neu protein, has been shown to elicit receptor internalization and ultimately to inhibit proliferation of HER-2/neu-overexpressing breast cancer cells in vitro and in breast cancer xenografts (6–9). Another anti-HER-2/neu mAb (clone 7.16.4), initially raised against the ectodomain of rNeu, was shown to share an epitope with 4D5 and to inhibit tumor formation of HER-2/neu-overexpressing tumor cells (10–14).

On the basis of observations of a synergistic effect between antibody-mediated immunotherapy and the use of doxorubicin (Doxo) as a cytotoxic drug, we have investigated whether Vir could be used as a drug carrier system into cells. The advantage of using Vir is the intrinsic capability of influenza virus to enter any mammalian cells triggered by its HA. This effect is conserved if the native HA is inserted into the virosomal lipid bilayer (22, 23). Moreover, anti-rNeu-Fab\(^{-}\) fragments were conjugated to the virosomal surface by a linker with a long PEG spacer arm, thus enabling Vir to target rNeu-expressing cancer cells. We analyzed the binding and internalization of Fab\(^{-}\)-Vir to rNeu\(^{+}\) tumor cells and compared it with unconjugated Vir. We demonstrated the cytotoxic effect of Fab\(^{-}\)-Vir in vitro. Furthermore, we have shown a significant inhibition of tumor progression in mice with established tumors treated with Doxo encapsulated in Fab\(^{-}\)-Vir (Fab\(^{-}\)-Doxo-Vir). The same virosomal construct was capable of successfully inhibiting tumor formation in mice through early treatment after tumor inoculation. Our results demonstrate that Vir conjugated with a tumor-specific antibody are a new and efficient drug delivery system with tumor-specific targeting.

MATERIALS AND METHODS

Chemicals. C\(_{12}\)-E\(_{x}\), PC solution, 5(6)-carboxyfluorescein N-succinimidyl ester, and DoxorHCl were purchased from Fluka (Buchs, Switzerland). PE was purchased from Avanti Polar-Lipids (Alabaster, AL). Bio-Beads SM-2 were purchased from Bio-Rad (Richmond, CA). NHS-PEG-MAL (M, 2000) was purchased from Shearwater Polymers (Huntsville, AL).

Animals and Cell Lines. Mouse mammary tumor virus (MMTV)/rNeu: FVB mice transgenic for the rNeu protein (rNeu-TG) were purchased from Charles River (Sulzfeld, Germany). Animal care was in accordance with institutional guidelines. The rNeu\(^{+}\) breast cancer cell line NF 9006, derived from a rNeu-TG mouse, and the rNeu\(^{+}\) breast cancer cell line M/BB 659, derived from a c-myc-TG mouse, have been described previously (24).

Production of Anti-rNeu mAb and Fab\(^{-}\) Fragments. Hybridoma cells producing the mAb 7.16.4 were kindly provided by M. Greene (University of Pennsylvania, Philadelphia, PA; Ref. 25). The mAb 7.16.4 was purified through a protein G column. Fab\(^{-}\) was produced using partial digestion with pepsin and further reduced to Fab\(^{-}\) fragments in 30 mM cysteine, 100 mM Tris (pH 7.6) for 20 min at 37°C as described previously (26).

Synthesis of PE-PEG-Fab\(^{-}\). To perform a site-directed conjugation of Fab\(^{-}\) fragments to keep the antigen binding site available, the Fab\(^{-}\) fragments were conjugated to the linker NHS-PEG-MAL containing a long PEG spacer arm. Thus, 100 mg of NHS-PEG-MAL were dissolved in 3 ml of anhydrous methanol containing 10 µl of triethylamine. Then, 45 mg of diolcoxy phosphatidylethanolamine dissolved in 4 ml of chloroform and methanol (1:3, v/v) were added to this solution. The mixture was triggered under nitrogen for 3 h at room temperature. Methanol/chloroform was removed under decreasing pressure, and the products were redissolved in chloroform. The solution was extracted with 1% NaCl to remove unreacted material and water-soluble byproducts. The PE-PEG-MAL was further purified by silic acid chromatography.
Preparation of Vir and Fab'-Vir. HA from the A/Singapore/6/86 strain of influenza virus was isolated as described previously (23, 28). Supernatant containing solubilized HA trimer (2.5 mg/ml) in 0.01 M C₁₂E₈ was used for the production of Vir. PC (38 mg) in chloroform was added to a round-bottomed flask, and the chloroform was evaporated by a rotary evaporator. The supernatant containing 10 mg of HA and 3.6 ml of PE-PEG-Fab' (containing 4 ng of Fab' fragments) were added to this flask. Under gentle shaking, the PC film covering the glass wall of the flask was solubilized by the C₁₂E₈-containing mixture. The detergent of the resulting solution was removed by extraction with sterile Biobeads SM-2. After this procedure, a slightly translucent solution, 10 mg of PC and 0.18 mg of Fab' fragments) were loaded into Vir. PE-PEG-Fab' were prepared by the same method, with the exception that carboxylfluorescein-N-succinimidyl ester coupled to PE was built into the lipid bilayer. The procedure for the production of Vir was the same without PE-PEG-Fab'.

Encapsulation of Doxo into Vir and Fab'-Vir. Doxo was loaded into Vir through a proton gradient generated by Vir-entrapped ammonium sulfate as described (29). In brief, to load Vir with ammonium sulfate, an ammonium sulfate solution (4.17 g/ml) was added to the Vir solution, sonicated for 1 min, and dialyzed (Spectra/Por 2.1; Biotech DispoDialyzers, molecular weight cutoff: 15,000; Spectrum Medical Industries, Houston, TX) against 1 liter of PBS containing 5% glucose for 24 h at 4 °C. To prepare the Doxo loading solution, 10 mg of Doxo were dissolved in 3 ml of water and sterilized (0.2 μm filter); then 750 μl of sterile 5× concentrated PBS and 5% glucose were added. Afterward, the Vir solution and Doxo loading solution (2:1, v/v) were incubated for 10 h at 33 °C and further incubated overnight at 28 °C. Nonencapsulated Doxo was separated from the Vir by gel filtration on a High Load Superdex 200 column (Pharmacia, Uppsala, Sweden). The void volume fractions containing Fab'-Vir with encapsulated Doxo were eluted with 5% glucose in PBS and collected. The Doxo concentration was determined by absorbance at 480 nm. Vir preparations contained 150 μg/ml Doxo.

The mean diameter of the Vir was determined by photon-correlation spectroscopy with a Coulter N4Plus Sub-Micron-Particle Size Analyzer (Miami, FL). The proper expression of viral fusogenic activity of the Vir was measured as described previously (30).

Immunofluorescent Labeling. For binding, rNeu⁺/rNeu breast cancer cell lines (NF 9006 and M/BB 659) were incubated at 4 °C for 30 min with FITC-conjugated anti-rNeu-Vir (Fab'-Vir) and FITC-Vir. The fluorescence was analyzed by flow cytometry on a FACScan (Becton Dickinson, Heidelberg, Germany).

For internalization, cells in suspension were incubated with 1 μg/ml FITC-conjugated Fab'-Vir or Vir for 30 min at 4 °C and then washed in PBS. Internalization was performed in 1 ml of complete medium for 1 h at 37°C. Control samples were incubated at 4°C for 30 min with anti-Neu mAb Ab-4 (Oncogene Science, Tarzana, CA) and then washed with PBS, followed by incubation with FITC-conjugated goat antimouse IgG (Southern Biotechnology, Birmingham, AL). Internalization was monitored for 1 h at 37°C in medium. Samples were washed and fixed in 3% paraformaldehyde. Fixed cells were permeabilized in 0.2% Triton X-100 for 15 min and stained for F-actin with rhodamine-phalloidin (1:100; Molecular Probes, Leiden, the Netherlands). After staining, the cells were centrifuged on slides, and preparation mounted in PBS:glycerol (2:1; Calbiochem, Lucerne, Switzerland).

For CSLM, a Microdiance system from Bio-Rad combined with an inverted Nikon microscope (Eclipse TE3000) was used (Lasers, Ghe/Ne 543 nm and Ar 488 nm). Optical sections at intervals of 0.3 μm were taken with a ×100/1.4 Plan-Apochromat objective. Image processing was done on a Silicon Graphics workstation using IMARIS, a three-dimensional multichannel image processing software (Bitplane AG, Zurich, Switzerland).

Colorimetric Cell Cytotoxicity Study. Cytotoxic activities of the conjugates were tested by an XTT assay for measuring cell proliferation (31). Briefly, cells (10,000) of both cell lines were seeded in 96-well plates overnight in DMEM with 10% FCS. Cells were then cultured in fresh medium with various concentrations of anti-rNeu antibody (7.16.4). Fab'-Vir and empty FITC-Vir for 48 h. XTT solution was added according to the manufacturer’s directions (Roche Diagnostics, Rotkreuz, Switzerland). After 4 h of incubation at 37 °C, the absorbance was measured using an ELISA reader. Each value represents mean ± SE of three samples.
Treatment of Established Tumors. Tumor implantation and therapeutic treatment were performed after anesthesia with i.p. injection of medetomidine hydrochloride (Domitor; Orion, Espoo, Finland; 500 μg/kg body weight), cliazolamum (Climasol; Graub, Bern, Switzerland; 5 mg/kg), and fentanyl citrate (Fentanyl-Janssen; Janssen-Cilag, Baar, Switzerland; 50 μg/kg). Mice were shaved at the injection sites, and rNeuNF9006 and rNeuM/BB659 cells at a concentration of $2 \times 10^6$ were injected s.c. Treatment was started when palpable tumors of at least 5 mm in diameter had formed. Injections into the tail vein of 200 μl of Doxo-Vir (Doxo, 150 μg/ml), Fab'-Doxo-Vir (same Doxo concentration; Fab' at 182 μg/ml), Fab'-Vir (Fab' at 182 μg/ml), and free Doxo at a concentration of 150 μg/ml were performed three times/week for the whole observation period. Tumors were measured every 3–4 days, the length and width of each tumor were measured with Vernier calipers. Tumor volume was calculated using the formula: $\frac{1}{2} \times \text{largest diameter} \times (\text{smallest diameter})^2$.

Treatment of Recently Implanted Tumors. For the micrometastatic stage of tumor formation, 0.2 $\times 10^6$ tumor cells were injected s.c., and treatment was started 3–5 days later. Again, different Vir combinations were compared with mice injected with free Doxo and controls. After nine injections (over 3 weeks), the treatment was stopped in all groups. Tumor formation was assessed by palpation, followed by measurement of tumor size as described above. Tumor formation was defined as tumor size beyond possible regression (90 mm$^3$). Mice were sacrificed when tumors exceeded institutional guidelines.

Histological Analysis. Mice were shaved at the injection sites, and cell suspensions from rNeuNF9006/rNeuM/BB659 breast tumors ($2 \times 10^6$ in 100 μl of PBS) were injected s.c. in the back. Mice were sacrificed at the indicated time points, and tissues were collected for histological analysis. Deparaffinized slides were stained with H&E.

RESULTS

Anti-rNeu Fab'-Vir Bind to Murine Breast Cancer Cells and Are Efficiently Internalized. The produced Vir (reconstituted fusion-active viral envelopes), composed of a single phospholipid bilayer and densely covered with HA spikes, were relatively homogeneous in size, ranging from 80 to 200 nm. The PE-PEG-MAL
cross-linker was used to conjugate anti-rNeu Fab' fragments by their free thiol group to the maleimido group. The long PEG spacer arm of the cross-linker allowed an extended and site-directed binding of Fab' molecules and thus prevented blockage of the antigen binding sites by the neighboring HA trimers. The chosen ratio of total phospholipid: PE-PEG-Fab' resulted in 100–150 Fab' fragments/Vir.

To be effective as a drug delivery system, Vir have to bind to tumor targets. FITC-labeled, unconjugated Vir and FITC-labeled Vir conjugated with Fab' fragments of an anti-rNeu mAb (Fab'-Vir) were analyzed for their binding capacity to breast cancer cell lines. As depicted in the FACS histograms in Fig. 1A, the NF9006 (rNeu') breast cancer cell line expressed significant levels of rNeu on the cell surface in comparison with the negative rNeu expression of M/BB 659 breast cancer cells (Fig. 1C) when the specific anti-rNeu mAb (clone 7.16.4) was used. In contrast to the specific binding with anti-rNeu mAb, Vir showed an increased binding to breast tumor cells independent of their expression of rNeu on the cell surface. We observed that cells overexpressing rNeu had an augmented binding of Fab'-Vir compared with the unconjugated FITC-labeled Vir (Fig. 1B). Although FITC-Vir and Fab'-Vir showed a strong binding to rNeu' breast cancer cells, there was no difference between Fab'-Vir and FITC-Vir (Fig. 1D). These results indicated that Vir were capable of binding to the tumor cell membranes and that Fab'-Vir showed an increased binding to rNeu' breast cancer cell lines through the specific binding of anti-rNeu Fab' fragments.

CLSM was used to study the internalization and distribution of FITC-conjugated virosomes in tumor cells after binding. NF9006 and M/BB 659 breast cancer cells were incubated at 37°C for 1 h with FITC-conjugated Fab'-Vir and Vir. CLSM with a three-dimensional reconstruction showed that in both cell lines, bound Vir were efficiently internalized, as evidenced by the large aggregates of FITC fluorescence observed within the breast cancer cell line (Fig. 2, B and D). There was no visible difference in internalization between Fab'-Vir and Vir (data not shown). By contrast, in NF9006 cells, FITC-conjugated anti-rNeu mAb (clone 7.16.4) was predominantly localized in the membrane or in its close proximity (Fig. 2A), whereas no FITC fluorescence was observed in M/BB 659 tumor cells (Fig. 2C). These results suggest that Vir may represent a novel carrier system to deliver encapsulated drugs into the cytosol of solid tumors.

**In Vitro Cytotoxicity Studies of Fab'-Vir.** To clarify whether Vir containing anchored Fab' fragments of an anti-rNeu mAb might have an antiproliferative activity, we investigated the effect of empty Vir, Fab'-Vir, and anti-rNeu mAb on breast cancer cells in vitro. Cells were cultured in the presence of increasing concentrations of anti-rNeu mAb, Fab'-Vir, and Vir, and proliferation of the cells was assessed by the colorimetric XTT assay (10). As shown in Fig. 3, neither rNeu' nor rNeu' breast cancer cells were affected in their proliferation, where different concentrations of empty Vir were added to the cultures. In contrast, mAb 7.16.4 was capable of specifically inhibiting proliferation of rNeu' breast cancer cells in a dose-dependent manner, whereas the rNeu' breast cancer cells were only marginally affected in their proliferation. Monovalent Fab' fragments are known to be much less effective in the inhibition of proliferation (15); however, the conjugation of monovalent anti-rNeu Fab' fragments to the surface of Vir showed an important antiproliferative effect on rNeu' breast cancer cells. Whereas the addition of 10 μg/ml of intact anti-rNeu mAb induced >90% growth inhibition, the addition of 50 μg/ml of Fab'-Vir was necessary to induce a 50% proliferation inhibition. The antiproliferative effect of Fab'-Vir was specific for rNeu' cells because no inhibitory effect was seen in cultures with rNeu' breast cancer cells. Because Vir showed no inhibitory or toxic effect on breast cancer cells in vitro and Fab'-Vir exhibited a dose-dependent inhibition of proliferation of rNeu' cells, we conclude that the virosomal lipid envelope with inserted HA was not cytotoxic to breast cancer cells.

**Treatment of Established Tumors.** In a first set of experiments, we wanted to clarify whether the observed in vitro binding and internalization of Vir into tumor cells also corresponded to an enhanced delivery of encapsulated cytotoxic drugs in vivo. The therapeutic effect on breast tumor implants of i.v.-injected free Doxo was compared with Doxo-encapsulated in Vir (Doxo-Vir). In a second set of experiments, the specific drug targeting with immunovirosomes (Fab'-Doxo-Vir) was tested for an increased therapeutic effect. Mice were inoculated s.c. with 2 × 10^6 rNeu' and rNeu' tumor cells. After tumor formation (5-mm diameter), treatment was started with i.v. injections of either free Doxo, Doxo-Vir, Fab'-Doxo-Vir, or Fab'-Vir every 3–4 days. Control groups received no treatment. As shown in Fig. 4A, there was a marginally significant decrease in tumor progression over time in mice treated with Doxo-Vir compared with the control groups. However, there was no significant difference in tumor progression in mice treated with free Doxo or Doxo-Vir. In contrast, as shown in Fig. 4, B and C, tumor progression was almost completely inhibited in the group of mice treated with the Fab'-Doxo-Vir, which was significantly more effective than Doxo-Vir. To determine whether the effect of Fab'-Doxo-Vir was dependent on targeting Doxo to the tumor cells or on the antibody blocking effect of the Fab' fragments, mice with established tumors were also treated with Fab'-Vir. There was a significant inhibition on tumor progression when Fab'-Doxo-Vir were used for treatment compared with the group of mice where only Fab'-Vir was injected, demonstrating an additive effect of targeting Doxo to the tumor cells by Fab' fragments on Vir (Fig. 4C).

The specificity of rNeu targeting was further assessed by treating mice with established rNeu' (M/BB 659) tumors with the different Vir compounds. As shown in Fig. 4D, the treatment of mice with Doxo-Vir, Fab'-Vir, and Fab'-Doxo-Vir had no significant effect on rNeu' tumor progression over time as compared with untreated control groups. These results demonstrated that in all experiments, rNeu' tumor growth was specifically and efficiently suppressed in mice treated with Fab'-Doxo-Vir as compared with mice treated with free Doxo, Doxo-Vir, and Fab'-Vir. Although these results presented a superiority of Fab'-Doxo-Vir in suppressing tumor growth of rNeu' tumors, these immunovirosomes were unable to completely eradicate established tumors.
Cytotoxicity of Anti-rNeu Immunovirosomes Containing Doxo.

To gain additional insight into the histological pattern induced by the different Vir treatments on inoculated tumors, histopathological sections were prepared at different time points after tumor injection. At days 5–7 after rNeu tumor cell implantation, tumors were excised and analyzed. In untreated mice, the border of the tumor was well demarcated from the neighboring s.c. normal tissue (Fig. 5A). Tumor cells appeared uniform with a large, slightly granular cytoplasm, and some nuclei showed mitosis, but no inflammatory infiltrates were detected. In mice treated with Doxo-Vir, again the tumor border was well delineated from the underlying normal tissue, and there was hardly any inflammatory infiltrate in the surrounding s.c. tissue nor any necrotic tumor cells visible (Fig. 5B). In contrast, in mice treated with Fab'-Vir, a large amount of necrotic cells was found predominantly in the center of the tumors (Fig. 5C). The surviving tumor cells appeared in contiguous groups surrounded by a significant infiltrate of granulocytic cells also infiltrating the underlying s.c. tissue. In mice treated with Fab'-Doxo-Vir, moreover, most of the tumor cells were necrotic and replaced by an inflammatory infiltrate composed mainly of granulocytes and eosinophils. There was also an impressive granulocytic infiltrate in the vicinity of surviving tumor conglomerates (Fig. 5D). In contrast, mice injected with the rNeu tumor cell line (M/BB 659) showed no necrosis in any of the Vir-treated animals, and some inflammatory infiltration surrounding the tumors was mainly seen in Fab'-Vir- and Fab'-Doxo-Vir-treated mice (data not shown). These results confirmed the effect seen in vivo by Fab'-Doxo-Vir-treated mice on established rNeu tumors.

Treatment of Recently Implanted Tumors. As an alternative evaluation of the efficacy of our virosomal carrier system, we investigated the long-time protection from tumor formation in animals with recently implanted tumors. Therefore, treatment was started 3–5 days after s.c. injection of $2 \times 10^5$ Neu$^+$ breast cancer cells into mice. All
mice were treated with nine injections of virosomal compounds for 3 weeks, and tumor formation was monitored in the following weeks. Within 4 weeks after tumor cell inoculation, all mice of the control group and groups treated with free Doxo and Doxo-Vir developed tumors and had to be sacrificed because of excessive tumor load (Fig. 6). The median time to tumor formation in mice treated with Doxo-Vir was 20 days and did not significantly differ from mice treated with free Doxo (20 days) or the control group (17 days; \( P > 0.4–0.8 \)). Whereas mice treated with Fab’-Vir had no significant \( (P > 0.3) \) difference in the median time to tumor formation compared with the control group, we noticed that 20% of mice did not develop tumors during the observation period of >90 days. Mice treated with Fab’-Doxo-Vir showed a significant increase \( (P < 0.005) \) in time to tumor formation with 90% of mice having no tumor at >90 days after tumor cell inoculation. These data suggest that Fab’-Doxo-Vir are highly efficient in delivering cytotoxic drugs to tumor cells and preventing tumor formation in recently implanted tumors.

**DISCUSSION**

The data presented here demonstrate that Vir can be used as a new drug carrier system and be selectively targeted to breast tumors to deliver cytotoxic drugs. Anti-rNeu Vir (Fab’-Vir) had the advantage to bind to the ubiquitous sialic acid residues on the cell surface by HA and retained the binding capacity to rNeu receptors by Fab’ fragment of the mAb (clone 7.16.4). Our data of FACS analysis demonstrated that site-directed conjugation of Fab’ fragments to a cross-linker with a long PEG spacer arm resulted in specific binding of Fab’-Vir to rNeu-expressing cancer cells. By CLSM, we demonstrated that Viro bound to tumor cells became rapidly internalized. In contrast, internalization of targeted liposomes (immunoliposomes) were shown previously to occur slowly, dependent upon the cell surface density of HER-2/neu and the internalization rate of receptor after cross-linking with anti-HER-2/neu mAb (19–21). The Fab’ fragments of the anti-rNeu mAb retained the antiproliferative effect when coupled to Vir and were linked to the ability to cause receptor internalization (10). Thus far, evidence for an in vivo cytotoxic effect of Doxo-containing liposomes was mostly based on the release of drugs from liposomes into the extracellular space (32, 33). Internalizing epitopes, such as HER-2/neu, are thought to be more efficient at increasing the intracellular drug concentration because entry of the drug into the cells is not dependent upon passive diffusion alone. However, the appropriate selection of mAb and the targeted antigen are crucial for the success.
of intracellular delivery (15, 21). Vir, in contrast, are rapidly and efficiently internalized through receptor-mediated endocytosis and are trapped in the endosomes, where a pH change from 5 to 6 triggers the fusion of the virosomal membrane with the endosomal membrane (22, 34). As a consequence, the encapsulated Doxo is delivered into the cytoplasm. The in vivo efficacy of Vir loaded with Doxo (Fab‘-Doxo-Vir) demonstrated a significant decrease of tumor progression in large established tumors compared with tumor-bearing mice receiving free Doxo or Doxo-Vir (drug-loaded, nontargeted Vir). Although our study confirms the specificity and the increased therapeutic index of Fab‘-Doxo-Vir in vivo on rNeu established tumors, it is important to emphasize that both in our work and in previous studies with immunoliposomes, the established tumors were not eradicated completely (35, 36). The inability of immunoliposomes to obliterate established tumors was attributed in part to the specific binding to cell surface receptors at the periphery of the tumor and the ineffectiveness of liposomes to penetrate tumor cells (18, 37). We showed that viruses have a higher capacity to enter tumor cells; however, the large amount of cells in an established tumor may not be completely attainable for Vir. In contrast, the treatment with targeted Vir of small poorly vascularized tumors prevented the formation of established tumors, thus suggesting that treatment with Vir may be effective in early metastatic spread or combined with other tumor-reducing modalities, such as surgery, radiotherapy, and chemotherapy.

This study also provided some impact with respect to animal models for studying anticancer therapeutics. Thus far, experiments mainly in SCID mice were used for testing anti-HER-2/neu-coated liposomal compounds (15). A considerable difference exists between the tolerated dose of Doxo between SCID mice (2–3 mg/kg) and normal mice (6 mg/kg; Ref. 38). Because SCID mice have a defect in DNA repair mechanisms, these models may not be the appropriate mouse models to test immunotherapeutic strategies. We have used an immune competent mouse strain and used syngeneic breast tumor cell lines derived from the same transgenic mouse (24). In our experiments, mice were injected with 7.5 mg/kg virosomal Doxo, a dose required for successful therapeutic outcome.

Vir may have therapeutic potential, and this report has established a specific targeting by coupling Fab’ fragments to Vir. Our results provide evidence that internalization of Vir occurred, and the encapsulated drug was subsequently delivered to the targeted tumor cells.

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