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A Single Treatment of Yttrium-90-labeled CHX-A″–C6.5 Diabody Inhibits the Growth of Established Human Tumor Xenografts in Immunodeficient Mice

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ABSTRACT

Antitumor diabody molecules are noncovalent single-chain Fv dimers that recapitulate the divalent binding properties of native IgG antibodies. Diabodies are capable of substantial accumulation in tumor xenografts expressing relevant antigens in immunodeficient mouse models. With a M_r of approximately 55,000, diabodies are rapidly cleared from the circulation, resulting in tumor-to-blood ratios that significantly exceed those achieved early after the administration of monoclonal antibodies. We have evaluated the therapeutic potential of the β-emitting isotope yttrium-90 ($t_{1/2}$, 64 hours) conjugated to the C6.5K-A diabody that specifically targets the HER2/*neu* human tumor-associated antigen. We have found that a single intravenous dose of 150 μCi (200 μg) ^{90}Y-CHX-A″–C6.5K-A diabody substantially inhibits the growth rates of established MDA-361/DYT2 human breast tumor xenografts in athymic nude mice. In contrast, 300 μCi (300 μg) ^{90}Y-CHX-A″–C6.5K-A diabody resulted in only a minor delay in the growth of SK-OV-3 human ovarian cancer xenografts. The maximum tolerated dose was also dependent on the tumor xenograft model used. These studies indicate that genetically engineered antitumor diabody molecules can be used as effective vehicles for radioimmunotherapy.

INTRODUCTION

Although major advances have been made in the treatment of diffuse malignant diseases with radioimmunotherapy (RAIT; refs. 1, 2), similar progress has not been made in the treatment of solid tumors. A number of factors are responsible for the limited progress in RAIT of solid tumors. These include the large size of intact monoclonal antibodies (mAbs), their prolonged circulation, poor targeting specificity, and disparate half-lives for the mAb and the radioisotope. To address these limitations, we have focused on altering the size, affinity, and valence of small antibody-based proteins to increase the rate of tumor uptake and enhance the specificity of targeting. We have found that M_r 55,000 diabody molecules exhibit a unique combination of highly specific tumor localization and relatively rapid pharmacokinetics (3). Diabodies are noncovalent single-chain Fv (scFv) dimers formed by producing scFv with short (5 AA) linkers between their variable light (V_L) and variable heavy (V_H) chains (4). This prevents the V_H and V_L chains in a single molecule from associating with each other into a scFv orientation. Instead, the V_L from one molecule associates with the V_H from a second molecule, and *vice versa*, to form a dimer that binds antigen divalently. We have produced the human C6.5 diabody that is specific for the extracellular domain of HER2/*neu* and that exhibits a 40-fold increase in affinity over the affinity observed with C6.5 scFv. The C6.5 diabody displays an exceptional combination of quantitative and selective tumor targeting in *scid* mice. At 24 hours postinjection, 6% injected dose per gram (ID/g) of radio-iodinated C6.5 diabody was retained in SK-OV-3 tumor xenografts in mice, and tumor-to-blood ratios of 10:1 were observed (3). The human nature of the C6.5 diabody should allow it to be administered multiple times to patients without eliciting an immune response, making it amenable to the delivery of fractionated RAIT dosage strategies that have a much higher likelihood for success in the treatment of solid tumors. Diabodies, thus, represent an improved strategy for selective tumor targeting as compared with scFv, Fab, or IgG molecules. Furthermore, decreasing the size of the molecule increases both its diffusion rate into tumor (5) and its rate of elimination from circulation. This enhances both the degree of penetration and the specificity of retention in the tumor.

We have previously reported that the *in vivo* tumor targeting of the C6.5 diabody does not occur rapidly enough for it to mediate effective RAIT of solid tumors when an extremely short-lived α-emitting radionuclide (bismuth-213) is used (6). In the studies described here, we have examined the potential of the C6.5 diabody molecule to serve as a vehicle for the delivery of longer-lived β-emitting radioisotopes to solid tumors. We have found that diabody-based RAIT can be an effective form of therapy.

MATERIALS AND METHODS

Antibodies and Cell Lines. The C6.5K-A (C6.5K100gA)-modified C6.5 diabody molecule was used in these studies. It contains an alanine in place of the lysine at position 100 in the heavy chain CDR3 (7, 8). This substitution allowed the use of chelate conjugation strategies that target amino groups without inactivating the antigen-binding site (6). The C6.5K-A scFv and C6.5 scFv molecules both have similar affinities (8). Diabodies formed from these molecules also have similar affinities and biodistributions when labeled with ^{125}I by the Chloramine-T method (data not shown). The C6.5K-A diabody was produced by periplasmic expression from *Escherichia coli* in shake flasks and purified by immobilized metal affinity chromatography and subsequent size exclusion chromatography, as described previously (7, 8). The cell lines used in these studies were the human ovarian carcinoma line SK-OV-3 (HTB 77, American Type Culture Collection, Manassas, VA) and the human breast carcinoma line MDA-361/DYT2 which was subcloned to select for the ability to form tumors in immunodeficient mice (a generous gift of Dr. Dajung Yang of Georgetown University, Washington, DC; ref. 9).

Flow Cytometry. The HER2/*neu* expression levels on the SK-OV-3 and MDA-361/DYT2 cell lines were determined by flow cytometry with techniques described elsewhere (3). Briefly, 5×10^6 cells were incubated with the anti-HER2 mAb, 520C9 (a kind gift of Chiron Corp., Emeryville, CA), or a negative control mAb MOPC-21 (Sigma Chemical Company, St. Louis, MO) for 30 min at 4°C. The cells were washed with fluorescence-activated cell sorting (FACS) buffer [0.154 mol/L sodium chloride, 10 mmol/L sodium phosphate, 1% bovine serum albumin, 0.1% sodium azide (pH 7.2)] before the addition of fluorochrome-conjugated goat antimouse mAb (ICN Immunobiologicals, Costa Mesa, CA). The cells were then fixed in 1% paraformaldehyde, and the degree of fluorescence was determined using a FACScan flow cytom-

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eter (Becton-Dickinson, San Jose, CA) and was analyzed using the CELLQuest software (Becton-Dickinson, San Jose, CA).

Conjugation. The bifunctional chelating agent CHX-A″ DTPA (10) was conjugated to the C6.5K-A diabody as previously described (11). The final antibody concentration was measured spectrophotometrically using an extinction coefficient of 210,000 m^{-1} cm^{-1} for the diabody molecule, and the chelate-to-protein ratio (C/P) was determined as described in the literature (12).

Radiolabeling. CHX-A″-conjugated C6.5K-A diabody molecules were labeled with indium-111 or yttrium-90, as described previously (13). Briefly, 5.0 mCi of $^{111}InCl_3$ (NEZ304, NEN Life Sciences Products, Boston, MA) or 24.0 mCi of $^{90}YCl_3$ (NEZ306C, NEN Life Sciences Products, Boston, MA) was neutralized to pH 6.5 with 2.5 mol/L NH_4OAc, which was rendered metal free by passage through a Chelex-100 column (Bio-Rad, Richmond, CA). Chelated diabody (8.0 mg in 1.5 mL) was placed in a clear acid-washed metal free 2.0-mL Eppendorf tube, and the radioisotope was added. The solution was gently mixed and allowed to incubate at room temperature for 60 minutes, after which 0.1 mol/L EDTA was added to the tube (50 μL) to scavenge free radiometal. After 5 minutes, an aliquot was removed to determine the ratio of bound-to-free radiometal in an instant thin-layer chromatography (ITLC) assay. Free radiometal was removed from the labeled protein by two sequential passages through a centrifuged Sephadex G50–80 (Sigma, St. Louis, MO) column (14), and aliquots were removed from the final preparation for immunoreactivity and ITLC assays. In the ITLC assay, 1 μL from each of the reaction mixtures and the final products was applied to silica ITLC strips (Biodex Medical Systems, Shirley, NY) and were allowed to migrate using normal saline as a mobile phase. The strips were cut at the midpoint and the two halves were counted in a gamma well counter (Gamma4000, Beckman, Irvine, CA). The immunoreactivity of the radiopharmaceutical was determined in a live-cell binding assay (14). Briefly, 10 ng of labeled diabody in 100 μL of potassium-buffered saline (PBS) were added in triplicate into 15-mL polypropylene centrifuge tubes containing 3×10^6 SK-OV-3 cells. The cells were allowed to incubate for 30 minutes at room temperature. One milliliter of PBS, 4°C, was added to each tube, and the tubes were centrifuged for 5 minutes at 500 × *g* at 4°C. Supernatants were separated from the cell pellets; both were transferred to 12 × 75-mm counting tubes and the percentage of radioactivity associated with the cell pellet was determined by counting in a gamma counter.

Biodistribution Studies. Four-to-six-week-old inbred C.B17/Icr-*scid* mice were obtained from the Fox Chase Cancer Center Laboratory Animal Facility. Three $\times 10^6$ SK-OV-3 cells or 5×10^6 MDA-361/DYT2 cells in 0.1 mL of PBS were injected subcutaneously into the abdomen of each mouse. Approximately 2 months after the implantation of the SK-OV-3 cells and 3 weeks after the implantation of the MDA-361/DYT2 cells, the tumors had achieved a size of about 100 mm^3, and the distribution studies were initiated.

Twenty micrograms of ^{111}In-CHX-A″–C6.5K-A diabody (5 μCi/μg) were administered to each mouse by tail vein injection. Total injected doses were determined by counting the mice on a Series 30 multichannel analyzer/probe system (probe model 2007, Canberra, Meridian, CT). Blood sample collection and whole body counts of the mice were performed immediately after injection and just before euthanization. Groups of five or six mice were euthanized at 4 and 24 hours after injection; tumor, organ, and blood retentions were determined, as described previously (14, 15). The mean and SEM for each group of data were calculated, and tumor-to-organ ratios (T/O) were determined. The significance between the results from each group were determined by unpaired *t* test (GraphPad Software, San Diego, CA).

Gamma Camera Imaging. The localization of ^{111}In-CHX-A″–C6.5K-A diabody in tumor and normal organs was visualized by gamma camera imaging. Briefly, 0.1 mCi (93 μg) of ^{111}In-CHX-A″-C6.5 diabody were administered by intravenous tail vein injection to *scid* mice bearing established subcutaneous MDA-361/DYT2 tumor xenografts on their abdomen. Twenty-four hr later, the mice were euthanized by CO_2 asphyxiation and imaged for 10 min on a Prism 2000 gamma camera (Picker 2000XP, Dual Head).

Tumor Implantations. Athymic nude mice were used in all therapy experiments in place of *scid* mice because the immunodeficient status of the scid mice is caused by an inability to repair DNA damage, making these mice overly sensitive to radiation. Male mice were used because their larger size was expected to diminish the normal tissue toxicity associated with the long-track-length β emissions in the RAIT studies described below. Eight-week-old male athymic nude mice were acquired from Taconic Labs (Germantown, NY). In the mice that were to receive the estrogen-dependent MDA-361/DYT2 tumors, β-estradiol pellets were first implanted subcutaneously in their backs; the pellets were 1.7 mg per pellet, 60-day release to maintain >900 pg/mL in blood (no. SE121, Innovative Research of America, Sarasota, FL). Two and a half million SK-OV-3 cells or 5.0 million MDA-361/DYT2 cells in a total volume of 0.1 mL of PBS then were implanted subcutaneously in the abdomen of the animals.

Therapy with Unconjugated Diabody. To determine whether unconjugated C6.5K-A diabody affected the growth of the tumor xenografts, a study was performed comparing the growth of established 25-day-old MDA-361/DYT2 tumors in nude mice treated with multiple doses of diabody with tumor growth in untreated mice. A cohort of eight mice (average tumor volumes, 558 ± 93 mm^3; average weight, 25.8 ± 0.7 g) were treated every 3 days, over a period of 21 days, with an intravenous injection of 200 μg (100 μL) of C6.5K-A diabody. A second cohort of six mice (average tumor volume, 759 ± 261 mm^3; average weight, 27.2 ± 0.5 g) was not treated. The tumors in both groups of mice were measured with calipers every 3 days. Tumor volumes were determined using the ellipsoidal formula length (mm) × width (mm) × height (mm) × 0.52 (derived from $\pi/6$; ref 16). All of the studies described in this article were terminated when tumor volumes exceeded 10% of the animal's body weight as per institutional guidelines.

Radioimmunotherapy Studies. Because the β emissions from ^{90}Y have a long track length, radioactivity localized in the tumor could contribute to the maximum tolerated dose (MTD). Additionally, the shedding of target antigen from a tumor can alter the pharmacokinetics of a radiolabeled antibody. Accordingly, the MTD of ^{90}Y-CHX-A″–C6.5K-A diabody was determined over the course of multiple studies in mice bearing subcutaneous SK-OV-3 tumors (average tumor size, 81.6 ± 8.6 mm^3; average mouse weight, 30.2 ± 0.4) and MDA-361/DYT2 tumors (average size, 314.4 ± 15.7 mm^3; average mouse weight, 24.7 ± 0.6 g). Cohorts of six to nine SK-OV-3 tumor-bearing mice were treated with a single intravenous bolus of 0, 50, 100, 200, 300, or 500 μCi of ^{90}Y-CHX-A″-labeled C6.5K-A diabody (1 mCi/mg diabody). Cohorts of 7–10 MDA-361/DYT2 tumor-bearing mice were treated with 0, 90, 150, 200, 250, 300, 350, or 400 μCi of ^{90}Y-CHX-A″-labeled C6.5K-A diabody (at a specific activity of 1 mCi/mg diabody). In the latter model, the 200-μCi- and 300-μCi-dose groups were examined in more than one study. Two to three times per week, the mice were observed for signs of weight loss and toxicity, and tumor volumes were measured with calipers. The LD_{10} was determined using the method described by DeNardo *et al.* (17). Briefly, the percentage lethal dose (LD) at each ^{90}Y dose level was calculated by adding the number of mice that died at that dose level and all lower dose levels (accumulated deaths), adding the number of survivors at that dose level and all higher dose levels (accumulated survivors), and then dividing the accumulated deaths by the sum of the accumulated deaths and accumulated survivors.

The therapeutic efficacy of ^{90}Y-CHX-A″–C6.5K-A diabody was then determined in athymic nude mice bearing established, 30-day-old, SK-OV-3 tumors or 20-day-old MDA-361/DYT2 tumors. In the SK-OV-3 model, a cohort of seven SK-OV-3 tumor-bearing mice (average tumor size, 99.4 ± 22.7 mm^3; average mouse weight, 31.3 ± 1.2 g) was treated with a single intravenous injection of 300 μCi (300 μg; approximately equal to the LD_{10} determined above) of ^{90}Y-CHX-A″–C6.5K-A diabody. A cohort of nine negative control mice (average tumor size, 120.2 ± 22.2 mm^3; average mouse weight, 31.7 ± 0.9 g) was treated with 200 μg of unconjugated C6.5K-A diabody. In the MDA-361/DYT2 model, cohorts of 10 MDA-361/DYT2 tumor-bearing athymic nude mice were either treated with a single intravenous injection of 150 μCi (200 μg; the LD_{10}) or 200 μCi (266 μg; the LD_{20}) of ^{90}Y-CHX-A″–C6.5K-A diabody or with 200 μg of unconjugated C6.5K-A diabody. The average tumor volumes at the time of treatment were 276.9 ± 54.4 mm^3, 279.3 ± 81.4 mm^3, and 338.8 ± 80.3 mm^3, for the control, 150-μCi-dose group, and 200-μCi-dose group, respectively. The average mouse weights for the three groups were 28.7 ± 0.5, 28.8 ± 0.5 and 30.7 ± 0.6 g, respectively. Tumor measurements and animal weights were acquired every 3–4 days and tumor volumes were calculated as described above. The significance of the difference in growth rates of the tumors in the treatment and control groups was determined using Wilcoxon one-sided tests.

Because the diabody is eliminated primarily through the kidneys, a possible outcome of diabody-based RAIT could be renal damage. Because this type of

damage would not become immediately apparent, a long-term study was initiated. A group of three 16-week-old non-tumor-bearing athymic nude mice were treated with 194 μCi of ^{90}Y-CHX-A″–C6.5K-A diabody. Blood samples were obtained by retro-orbital bleeding 1 year after the treatment. The samples were analyzed for serum creatinine levels using a colorimetric end point assay (Sigma Diagnostics). The assay was performed according to the manufacturer's protocols using scaled volumes appropriate for a microtiter plate-scanning spectrophotometer. The mice were then euthanized, their kidneys were fixed in formalin, and sections were obtained for histopathological examination. Sections were stained with H&E and were then examined by the Fox Chase Cancer Center Histopathology Facility for abnormalities.

RESULTS

HER2/*neu* Receptor Expression. Both the SK-OV-3 and the MDA-361/DYT2 cell lines were observed to overexpress the HER2/*neu* antigen by flow cytometry (Fig. 1). The SK-OV-3 cells exhibited mean fluorescence intensities of 71 and 843, respectively, with the negative control and anti-HER2/*neu* mAbs; and the MDA-361/DYT2 exhibited mean fluorescence intensities of 30 and 479, respectively, with the negative control and anti-HER2/*neu* mAbs.

Characterization of the Radiolabeled Diabody. After the conjugation of the CHX-A″ to the diabody, the chelate to protein (C/P) ratios were determined to be 0.5:1 and 1.4–1.5:1, respectively, for the preparations used in the biodistribution and RAIT studies. Quality control assays performed on the purified ^{111}In-CHX-A″–C6.5K-A diabody that was used in the biodistribution and imaging studies, revealed immunoreactivities of >64% and radiochemical purities of >97.5%. The immunoreactivity of the ^{90}Y-CHX-A″–C6.5K-A diabodies used in the MTD and RAIT studies averaged 66%, and the radiopharmaceutical purity averaged 97.8%.

Biodistribution Studies. The 4-hour and 24-hour biodistributions of the ^{111}In-labeled CHX-A″–C6.5K-A diabody were compared in mice bearing subcutaneous SK-OV-3 and MDA-361/DYT2 tumor xenografts. At 4 hours postinjection, the tumor retention of the ^{111}In-labeled C6.5K-A diabody in the SK-OV-3 tumors was significantly greater than that observed in the MDA-361/DYT2 tumors (7.8% ID/g *versus* 2.9% ID/g, respectively; $P < 0.005$; Table 1), likely reflecting the higher concentration of HER2/*neu* on the SK-OV-3 cells (Fig. 1). Over the next 20 hours, the quantity retained in both tumor types increased; and by 24 hours postinjection, the differences in tumor localization were no longer significant ($P = 0.0863$). This suggests that active tumor uptake of the diabody continued after the 4-hour time point, even though the content remaining in circulation had dropped below that which remained in the tumor (Table 1). The comparatively greater uptake by the MDA-361/DYT2 tumors between 4 and 24 hours postinjection is consistent with the rapid internalization of HER2/*neu* by this cell line.

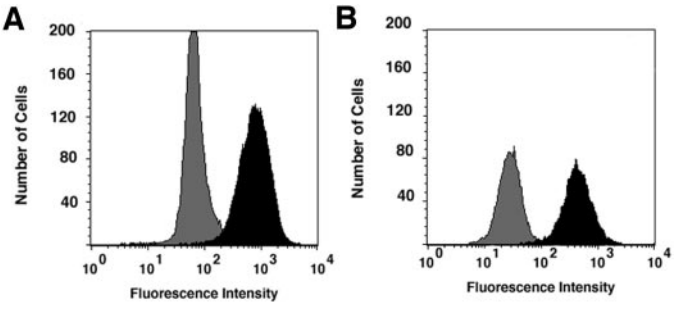

Fig. 1. Cell surface expression of HER2/*neu* target antigen. Expression of HER2/*neu* on both target cell lines was determined by flow cytometry. SK-OV-3 (*A*) or MDA-361/DYT2 (*B*) cells were stained with an irrelevant murine mAb (*gray*) or with the murine anti-HER2/*neu* mAb 520C9 (*black*), followed by goat antimouse-FITC.

Table 1 *Comparative biodistribution between ^{111}In-CHX-A″ C6.5K-A diabody at 4 and 24 hours postinjection in scid mice bearing subcutaneous SK-OV-3 or MDA-361/DYT2 tumors overexpressing the HER2/neu target*

	4 h		24 h	
	MDA-361/DYT2	SK-OV-3	MDA-361/DYT2	SK-OV-3
Tumor	2.9	7.8	8.3*	9.8*
Blood	2.1	5.8	0.4*	0.5*
Liver	1.8	5.27	2.5	3.6
Kidney	6.3	27.9	16.1†	21.0†
Spleen	1.2	7.3	2.3	6.3
Intestines	0.6	3.4	0.8	2.3
Lung	2.2	6.5	0.7	2.2
Bone	0.4	1.6	0.7	1.1
Muscle	0.4	1.3	0.4	1.3

NOTE. Cohorts of five to six mice were used in each group. Results are expressed as percentage of the injected dose localized per gram of tissue (% ID/g) or milliliter of blood (% ID/mL). SEM are ≤20% of the associated values. Unless indicated, the level of significance of the difference of localization in the tissues in the two models at a given time point was $P < 0.005$.

* Difference not significant.

† $P < 0.05$.

The normal tissue retention of the ^{111}In-labeled diabody also differed in each model, and seemed to reflect slower blood clearance in the mice bearing SK-OV-3 tumors (Table 1). As early as four hours after administration, there was significantly more ^{111}In-labeled CHX-A″–C6.5K-A diabody in every tissue of the mice bearing SK-OV-3 tumors as compared with those of the mice bearing MDA-361/DYT2 tumors ($P < 0.005$). By 24 hours postinjection, the quantity remaining in the blood of both models was similar, but the differences in normal tissue retention persisted.

Gamma Camera Imaging. Images acquired 24 hours after the administration of the ^{111}In-CHX-A″–C6.5K-A diabody to *scid* mice bearing subcutaneous MDA-361/DYT2 tumors revealed significant tumor localization (Fig. 2). However, as predicted from the biodistribution studies described above, the renal route of elimination of the immunoconjugates also led to substantial kidney retention.

Therapy with Unconjugated Diabody. Initial therapy studies were performed to determine whether unconjugated C6.5 diabody was capable of altering the *in vivo* growth of tumors that overexpress HER2/*neu*. Two groups of athymic nude mice bearing MDA-361/DYT2 human breast tumor xenografts were studied. The first group of eight mice was treated every 3 days, over a course of 21 days, with intravenous injections of 200 μg (100 μL) of C6.5K-A diabody, whereas the second group of 6 mice was left untreated. No significant differences were detected between the growth of the tumors in the treated and the control groups (Fig. 3). Thus, the C6.5 diabody alone was not capable of altering the growth properties of HER2/*neu*-overexpressing tumors.

Radioimmunotherapy Studies. Initially, RAIT studies were focused on determining the MTD of ^{90}Y-CHX-A″–C6.5K-A diabody in the SK-OV-3 and MDA-361/DYT2 tumor xenograft models. Cohorts of six to nine mice bearing established subcutaneous SK-OV-3 tumors were treated with 50, 100, 200, 300, or 500 μCi of ^{90}Y-CHX-A″-labeled C6.5K-A diabody (1 mCi/mg diabody), whereas cohorts of 7 to 10 MDA-361/DYT2 tumor-bearing mice were treated with 0, 90, 150, 200, 250, 300, 350, or 400 μCi of ^{90}Y-CHX-A″-labeled C6.5K-A diabody (1 mCi/mg diabody). In both groups, increasing doses were associated with increased weight loss and greater mortality. The dose of ^{90}Y-CHX-A″–C6.5K-A diabody associated with a 10% mortality rate (LD_{10}) was 9.5 μCi/g body weight in the SK-OV-3 xenograft model (Fig. 4*A*) and 5 μCi/g body weight in the MDA-361/DYT2 tumor xenograft model (Fig. 4*B*). At a dose of 200 μCi of ^{90}Y-CHX-A″–C6.5K-A diabody, mice in both tumor models exhibited average transient weight losses of approximately 7% of the initial body weight that resolved within 18 days (data not shown).

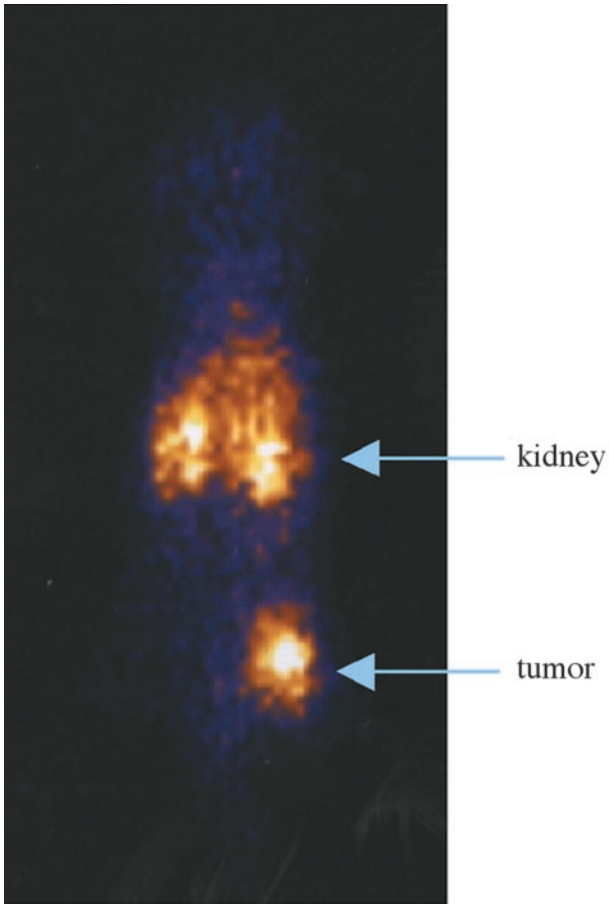

Fig. 2. Gamma camera image of ^{111}In-CHX-A″–C6.5K-A diabody 24 hours after intravenous administration to a *scid* mouse bearing a 395-mg subcutaneous MDA-361/DYT2 human breast carcinoma xenograft.

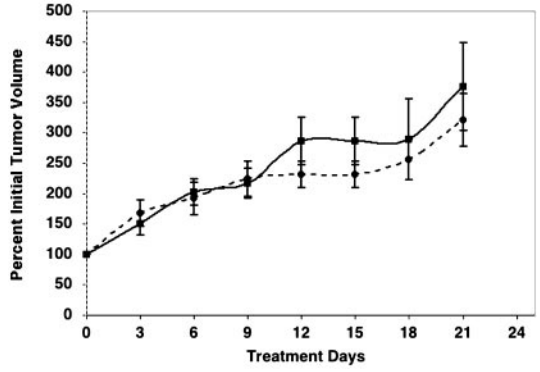

Fig. 3. Unconjugated C6.5K-A diabody does not effect the growth of established human tumor xenografts. Athymic nude mice bearing subcutaneous 25-day-old MDA-361/DYT2 tumors either were treated every 3 days with unconjugated C6.5 diabody over a period of 21 days (●) or were left untreated (■). Tumors were measured every 3 days; volumes were calculated as indicated in the Materials and Methods and were represented as a percentage of the initial volume at the time of first treatment. $n = 8$ mice in treatment group and 6 mice in control group. *Error bars*, SEM.

The efficacy of ^{90}Y-CHX-A″–C6.5K-A diabody RAIT was evaluated in both tumor models. In the SK-OV-3 model, a single dose of 300 μCi (approximately the LD_{10}) of ^{90}Y-CHX-A″–C6.5K-A was administered by intravenous tail vein injection to a cohort of seven mice bearing established tumors. An additional group of nine tumor-bearing mice were treated with unconjugated C6.5K-A diabody. In this model, treatment with ^{90}Y-CHX-A″–C6.5K-A led to an approximately 3-day delay in the doubling time of the volume of the tumors as compared with that observed in the group that received the unconjugated diabody (~10 days *versus* ~7 days, respectively; Fig. 5). In contrast to the SK-OV-3 model, lower-dose RAIT treatment of mice bearing faster growing MDA-361/DYT2 human breast cancer xenografts led to a substantial tumor growth delay. A single dose of 150 μCi (approximately the LD_{10}) of ^{90}Y-CHX-A″–C6.5K-A, administered by intravenous tail vein injection, to mice bearing established

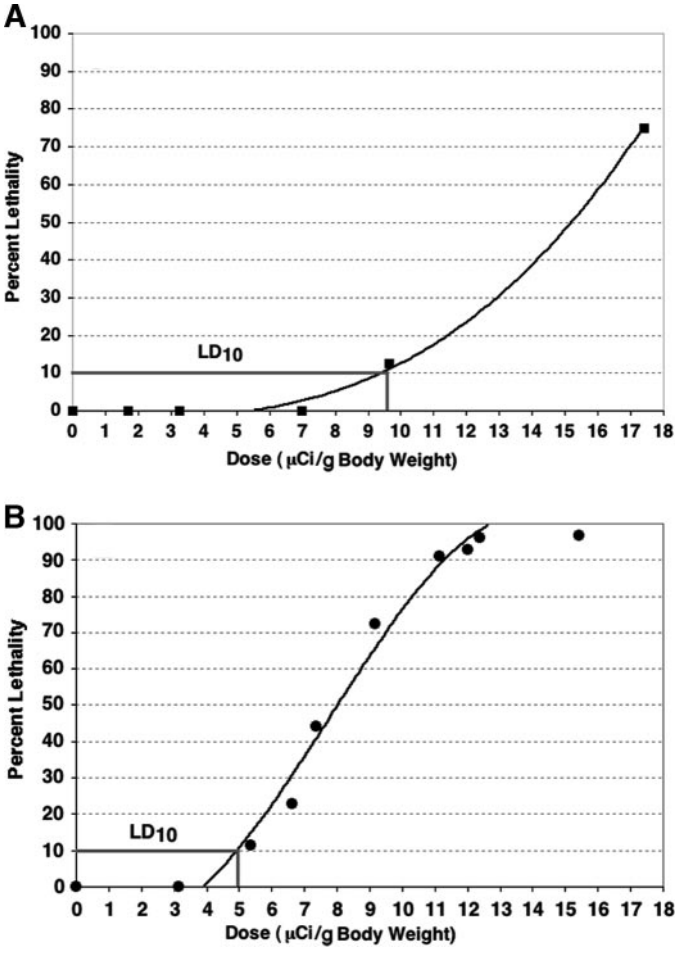

Fig. 4. Studies were performed to determine the MTD of ^{90}Y-CHX-A″–C6.5K-A diabody in athymic nude mice bearing established subcutaneous SK-OV-3 tumors (*A*) or MDA-361/DYT2 tumors (*B*). The MTD was determined as described in the Materials and Methods using the method developed by DeNardo *et al.* (17).

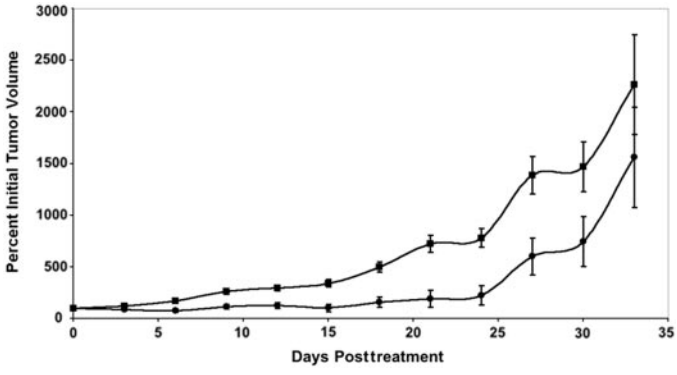

Fig. 5. RAIT of immunodeficient mice bearing established (111.9 ± 15.8 mm^3) subcutaneous SK-OV-3 tumors. A cohort of seven mice was treated with a single intravenous dose of 300 μCi (300 μg) of ^{90}Y-CHX-A″–C6.5K-A diabody (●), a second cohort of nine mice were treated with 200 μg of unconjugated C6.5K-A diabody (■). *Error bars*, SEM.

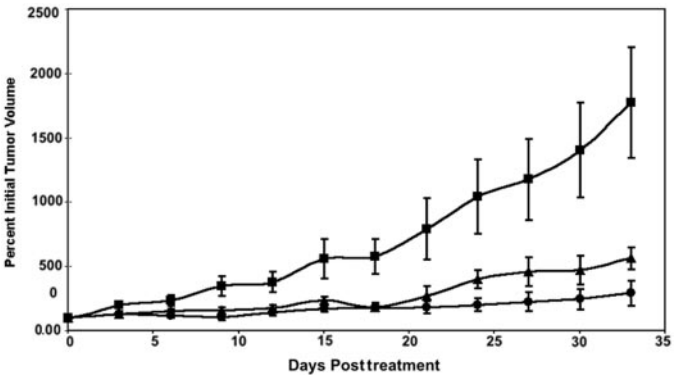

Fig. 6. RAIT of immunodeficient mice bearing established (298.3 ± 32.7 mm^3) subcutaneous MDA-361/DYT2 tumors. Cohorts of 10 mice were treated with a single intravenous dose of 150 μCi (200 μg; ▲) or 200 μCi (266 μg; ●) of ^{90}Y-CHX-A″–C6.5K-A diabody or 200 μg of unconjugated C6.5K-A diabody (■). SEM.

tumors, resulted in an average delay of nine days in the rate of tumor doubling (Fig. 5) as compared with that observed in the group that received the unconjugated diabody. Increasing the dose to 200 μCi (approximately the LD_{20}) resulted in a further enhancement in therapeutic effect in which two (25%) of the eight mice exhibited durable complete responses lasting more than 1 year (Fig. 6).

The high renal retention observed for this molecule in the biodistribution study above indicated that long-term renal damage could be a dose-limiting toxicity of radiometal-labeled C6.5 diabody RAIT. To address this possibility, we treated three non-tumor-bearing athymic nude mice with a therapeutic dose of 194 μCi of ^{90}Y-CHX-A″–C6.5K-A diabody. One-year later, serum samples were acquired by retro-orbital bleeding, the mice were euthanized, and their kidneys were examined by histopathology for signs of toxicity. All three mice exhibited increased serum creatinine levels (average, 6.0 ± 2.3 mg/dL) that were greater than the mean values found in the serum of untreated control mice (0.8 ± 0.1 mg/dL). The effect of RAIT on the kidneys was varied. There was no overt sign of renal damage in one mouse. The second mouse exhibited early-stage renal disease, including a limited degree of inflammation, sclerosis, and hyalin material in the glomeruli. The damage to the kidneys of the third mouse was severe, with substantial inflammation, the presence of Russell bodies, and hyalin material in all of the glomeruli (Fig. 7). Although these results are based on observations from a small number of mice, they suggest that long-term renal damage may be a complication of RAIT with small, engineered antibody fragments.

DISCUSSION

The studies presented here provide the first demonstration that diabody molecules can be effective targeting vehicles for the RAIT of solid tumors. Because of their small size, diabodies are rapidly eliminated from circulation (3, 18) and are expected to be associated with increased tumor penetration as compared with intact mAb molecules (5).

In the *scid* mouse model, the anti-HER2/*neu* C6.5 diabody has a $T_{1/2}\alpha$ (equilibration) of 0.7 hours and a $T_{1/2}\beta$ (elimination) of 6 hours after intravenous administration (3). Whereas the fraction of the injected dose of a diabody that is retained in tumor is about one half to one third of the quantity that would be localized by a corresponding mAb, the rapid systemic clearance of a diabody also leads to significantly greater tumor-targeting specificity. When antibody-based molecules are used as radioisotope vehicles for RAIT, the cumulative exposure [area under the curve (*AUC*)] of the tumor and normal tissues governs both the therapeutic efficacy and the toxicity. The potential advantage of diabodies over intact mAbs is suggested by the AUC ratios between tumor and blood, which are ~3.0:1 for the diabody and ~1:1 for IgG (3).

Diabodies have distinct advantages for use in RAIT over intact IgG molecules and their enzymatically prepared fragments. With a M_r of ~55,000, diabodies fall beneath the renal threshold for first-pass clearance (19). This leads to a relatively rapid systemic clearance that,

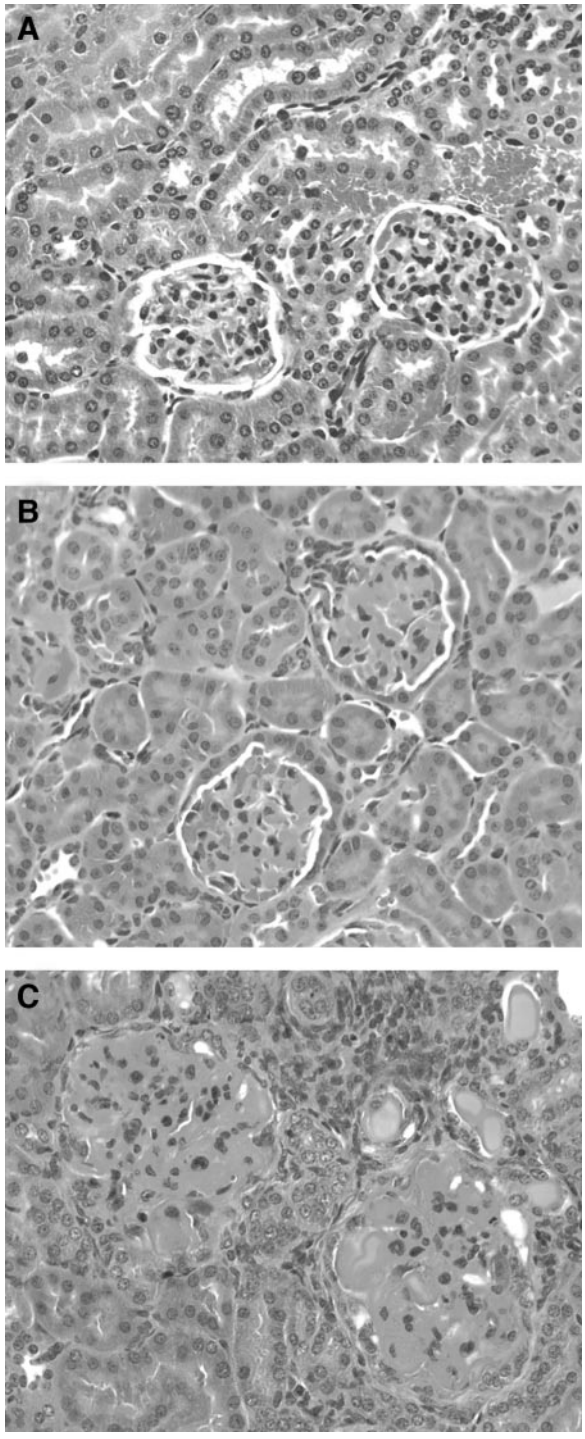

Fig. 7. Renal histopathology. The kidneys from three non-tumor-bearing mice that were treated with 194 μCi of ^{90}Y-CHX-A″–C6.5K-A diabody were compared with those from age-matched untreated control mice 1 year after treatment. Sections were H&E stained and were examined for signs of renal toxicity. *A*, a normal section from an untreated control mouse. One of the treated mice showed no signs of renal damage (not shown), the second mouse exhibited early-stage renal disease (*B*), and the third mouse had severe renal damage (*C*).

in turn, decreases the exposure of the bone marrow to nontargeted radiation and reduces myelotoxicity. On the basis of the studies published by Jain (5, 20), indicating that smaller antibody fragments are associated with faster tumor penetration, the small size of the diabody should lead to a greater degree of tumor penetration than is possible with intact IgG molecules. Although their size is very similar to that of an enzymatically produced Fab fragment, divalent scFv-based molecules are associated with a greater binding avidity that can lead to prolonged retention in tumor (14) and increased internalization. In contrast, enzymatically produced $F(ab')_2$ fragments exceed the renal threshold for first-pass clearance and exhibit a longer retention in circulation.

In the biodistribution studies, we observed substantially greater uptake in the SK-OV-3 tumors than in the MDA-361/DYT2 tumors at 4 hours postinjection; however, by 24 hours postinjection, the quantity of ^{111}In-labeled C6.5K-A diabody retained in both types of tumors was not substantially different. It is our opinion that the slower uptake by the MDA-361/DYT2 tumors reflects their lower HER2/*neu* antigen density and that the more rapid HER2/*neu* internalization rate by this cell line was responsible for the equalization in the tumor retentions by 24 hours after the injection. The MDA-361/DYT2 tumor-bearing mice also exhibited a more rapid systemic elimination of the ^{111}In-labeled C6.5K-A diabody, as evidenced by the lower overall normal tissue and blood retentions at 4 hours postinjection as compared with the animals bearing SK-OV-3 tumors.

Although prolonged tumor retention associated with radiometals enhances the cytotoxic effect, renal retention can be a serious concern. A number of groups have administered cationic amino acids (*e.g.*, lysine) to reduce the amount of radiometal retained in the kidneys (19). We have achieved significant reductions (~40–50%) in the renal retention of the ^{111}In-labeled diabody in biodistribution and gamma-camera-imaging studies by administering L-lysine to the mice before the radiolabeled diabody (data not shown). Although this is effective in animal models, high doses of lysine may be toxic in a clinical setting. Alternatively, the use of radioactive halogens (*e.g.*, ^{131}I) instead of radiometals leads to lower renal retention because of rapid de-iodination on internalization in the kidney (21). Other groups are investigating the use of cleavable linkers between the antibody and the chelate to alter the processing and retention of the radiometal (22)

Because high doses of cationic amino acids may be associated with complicating unacceptable toxicities, we chose to perform the therapy studies described here without using blocking strategies. This allowed us to assess the efficacy of diabody-based RAIT in the setting of maximum toxicity and to determine the long-term effect of this therapy on renal function. It is unclear why the mice with the SK-OV-3 tumors exhibited a higher MTD and greater tolerance to therapy than did the mice bearing MDA-361/DYT2 tumors when the former exhibited a slower systemic clearance of the radiometal-labeled diabody. This could suggest that differences in the degree of HER2/*neu* antigen shedding in the two tumor models could account for the differences in MTDs. This is an underexamined factor that could have a great impact on the success of antibody-based delivery of therapeutic agents. However, it is also possible that the differences in MTD resulted from differences in the retention of the radiometal in the two tumor models after the period of time covered by the biodistribution studies. The long track length of ^{90}Y β-emissions can lead to substantial normal tissue exposures from radioisotopes that are localized in subcutaneous tumors in a small-animal model such as a mouse. Similarly, the long track length of these β-emissions makes it particularly difficult to evaluate ^{90}Y-based RAIT agents in small-animal models such as the mouse because the higher levels of toxicity or therapeutic efficacy could occur that would not be present in human patients, in which substantially greater distances are involved.

In the mouse models used here, diabody-based RAIT was very effective in the treatment of established MDA-361/DYT2 tumors. Single doses of ^{90}Y-CHX-A″–C6.5K-A administered at the LD_{10} (150 μCi) and LD_{20} (200 μCi) resulted in significant delays in tumor growth. In contrast, a single dose of ^{90}Y-CHX-A″–C6.5K-A administered at the LD_{10} (300 μCi) exhibited significantly less efficacy in the treatment of established SK-OV-3 tumors.

The disparate responses of the two different tumor models to RAIT with ^{90}Y-CHX-A″–C6.5K-A diabody could be due to a number of differences between the tumor cell lines. These include the rate of internalization of HER2/*neu* after diabody binding, radiation sensitivity, tumor size, and, finally, p53 status, which is involved in the initiation of apoptosis in response to ionizing radiation. The SK-OV-3 cell line internalizes the C6.5 diabody relatively slowly (10% in 3 hours), whereas internalization by the MDA-361/DYT2 line is rapid (~50% in 1 hour; data not shown). Although the sensitivity of the two cell lines to β-radiation is not known, we have analyzed their sensitivity to γ-radiation using a cesium-137 irradiator. We determined in clonogenic studies that the MDA-361/DYT2 cell line is ~7-fold more radiation sensitive than the SK-OV-3 cell line (LD_{50}, ~0.5 Gy *versus* ~3.5 Gy, respectively; data not shown). The long track of the β emissions from Y-90 could result in decreased therapeutic efficacy when treating tumors of smaller volumes because a greater percentage of the β emissions could exert their therapeutic effect outside of the tumor. In the study reported here, the MDA-361/DYT2 tumors were ~2.6 times the size of the less responsive SK-OV-3 tumors. However, the average size of the SK-OV-3 tumors (112 mg) at the time of treatment would be expected to be large enough to attenuate this effect.

The SK-OV-3 cell line is p53 null (23) whereas the MDA-361 cell line, the parent line of the MDA-361/DYT2 subclone used in these studies, has wild-type p53 (24), likely making the latter cell line more sensitive to ionizing radiation. Any of these factors could be responsible for the differences in response to RAIT. Because the same range of properties could be present in patients treated with this and other RAIT agents targeting HER2/*neu*, it will be important to identify which of these factors is responsible, so that this treatment would be preferentially provided to those patients who would benefit the most.

The high degree of renal retention that we observed with ^{111}In-conjugated diabody suggests that the dose-limiting organ for this therapeutic approach may be the kidneys. Accordingly, we performed a preliminary evaluation of the impact of ^{90}Y-CHX-A″–C6.5K-A diabody therapy in non-tumor-bearing nude mice. In this study, we observed that 1 year after the receipt of a therapeutic dose of ^{90}Y-CHX-A″–C6.5K-A diabody, serum creatinine levels were elevated above the range detected in untreated control mice. Histopathological examination of the kidneys revealed that some of the mice experienced renal damage. Additional studies with larger cohorts of mice will be required to clearly elucidate the impact of ^{90}Y-diabody RAIT on the kidneys.

Because of its association with a poor prognosis in metastatic breast cancer (25, 26), HER2/*neu* is an attractive target for antibody-based intervention. The Food and Drug Administration approval of trastuzumab represented a major advance for the field of antibody-based therapeutics because it is the first licensed mAb for the treatment of solid tumors. Although a number of patients treated with trastuzumab have experienced significant antitumor effects, 84% fail to respond (27). This occurs even in the setting of high (3+) HER2/*neu* expression on their tumors. Trastuzumab-mediated tumor cell killing is believed to be due both to antibody-dependent cellular cytotoxicity (28) and to its direct action on the tumor cells (*e.g.*, down-regulation of HER2/*neu*, cell cycle arrest, and inhibition of constitutive HER2/*neu* cleavage and shedding; reviewed in ref. 29). Accordingly, the use

of a cytotoxic payload in HER2/*neu* targeting strategies would introduce additional cytotoxicity mechanisms and could, therefore, improve therapeutic outcomes. In fact, Tsai *et al.* (30) reported that a single dose of ^{90}Y-4D5 (the murine mAb from which trastuzumab was derived) led to significant tumor growth delays in mice bearing established MCF7/HER2/*neu* tumors, whereas treatment with the same dose of unlabeled 4D5 was ineffective.

Although in the studies described here, we observed significant antitumor effects with a single dose of ^{90}Y-conjugated anti-HER2/*neu* C6.5K-A diabody, a single dose is unlikely to be as effective in the clinical setting. Because the C6.5 diabody was derived from a human scFv phage display library, it is not expected to be immunogenic in patients, potentially allowing for its use in repetitive treatment strategies.

In conclusion, we have shown that diabody molecules can used as vehicles for RAIT of established solid tumors. Although the present studies demonstrated that ^{90}Y-conjugated anti-HER2/*neu* C6.5 diabody effectively reduced the growth rate of human breast tumor xenografts, greater therapeutic efficacy could be achieved by using radioisotopes with physical half-lives that are closer to the biological half-life of the C6.5 diabody. These studies also indicate that antigen expression is not the only factor that predicts the efficacy of RAIT. Developing a clear understanding of these factors will be critical to developing RAIT strategies that can succeed in the clinical setting.

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