A Novel Reduced Immunogenicity Bispecific Targeted Toxin Simultaneously Recognizing Human Epidermal Growth Factor and Interleukin-4 Receptors in a Mouse Model of Metastatic Breast Carcinoma

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Abstract

Purpose: To develop a targeted biological drug that when systemically injected can penetrate to metastatic breast cancer tumors, one needs a drug of high potency and reduced immunogenicity. Thus, we bioengineered a novel bispecific ligand–directed toxin (BLT) targeted by dual high-affinity cytokines with a PE38KDEL COOH terminus. Our purpose was to reduce toxin immunogenicity using mutagenesis, measure the ability of mutated drug to elicit B-cell antitoxin antibody responses, and show that mutated drug was effective against systemic breast cancer in vivo.

Experimental Design: A new BLT was created in which both human epidermal growth factor (EGF) and interleukin 4 cytokines were cloned onto the same single-chain molecule with truncated Pseudomonas exotoxin (PE38). Site-specific mutagenesis was used to mutate amino acids in seven key epitopic toxin regions that dictate B-cell generation of neutralizing antitoxin antibodies. Bioassays were used to determine whether mutation reduced potency, and ELISA studies were done to determine whether antitoxin antibodies were reduced. Finally, a genetically altered luciferase xenograft model was used; this model could be imaged in real time to determine the effect on the systemic malignant human breast cancer MDA-MB-231.

Results: EGF4KDEL 7mut was significantly effective against established systemic human breast cancer and prevented metastatic spread. Mutagenesis reduced immunogenicity by ~90% with no apparent loss in in vitro or in vivo activity.

Conclusions: Because EGF4KDEL 7mut was highly effective even when we waited 26 days to begin therapy and because immunogenicity was significantly reduced, we can now give multiple drug treatments for chemotherapy-refractory breast cancer in clinical trials. (Clin Cancer Res 2009;15(19):6137–47)

Targeted catalytic toxins are under investigation as potential anticancer agents because they are perhaps the most potent anticancer drugs, killing tumor cells in picomolar concentrations. Their value as alternative drugs is validated by an impressive 60% complete response rate in phase 2 trials for hairy cell leukemia (1). Another advantage is their unique mechanism of action that renders them even more effective when combined with chemotherapy (2). Immunogenicity is clearly their most significant problem against solid tumors because effective therapy requires multiple treatments, which result in the generation of neutralizing antibodies, mostly against the toxin (3). However, if targeted toxins are to achieve success against solid tumors, a solution must be found. We have made use of the ability to genetically modify biological targeted toxins to address immunogenicity and other problems that limit their usefulness for carcinoma therapy.

Onda and Pastan recently showed that there are only seven major epitopes in the Pseudomonas toxin (PE) recognized by B cells and it is possible to remove or replace the hydrophilic amino acids at these B-cell epitopes to create a low immunogenic form of PE that will limit the formation of neutralizing antibodies in mice (4, 5). Therefore, we used these to mutate a truncated form of the PE toxin selected due to previous research describing a series of internal frame deletion mutations that established the best location for genetic fusion of PE to targeting...
Materials and Methods

Construction of EGF4KDEL. Synthesis and assembly of hybrid genes encoding the single chain EGF4KDEL were accomplished using DNA shuffling and DNA cloning techniques. The fully assembled fusion gene (from 5′ end to 3′ end) consisted of an Ncol restriction site, an ATG initiation codon, the genes for human EGF and circularly permuted human IL-4 (ppl4) linked by a 20-amino-acid segment of human muscle aldolase (hma), the 7-amino-acid EASGGPE linker, the 362 amino acids of PE38 with the COOH terminus replaced with the ER retention sequence KDEL, and a NotI restriction site at the end of 3′. The gene encoding cpl4 was generously provided by Drs. RJ Kreitman and I Pastan (NIH, Bethesda, MD). The resultant 1,752-bp Ncol/NotI fragment gene was spliced into the pE2T124 bacteria expression vector under control of an isopropyl-β-D-thiogalactopyranoside-inducible T7 promoter (Fig. 1). DNA sequencing analysis (Biomedical Genomics Center, University of Minnesota) was used to verify that the gene was correct in sequence and cloned in frame. To create a mutated EGF4KDEL molecule (EGF4KDEL 7mut) with decreased immunogenicity, 8 amino acids representing the seven major epitopes on PE38 (5) were mutated using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) and were confirmed by DNA sequencing. The following amino acids were altered: R490A, R513A, R467A, E548S, K590S, R432G, Q332S, and R313A (5). Genes for monospecific cytotoxins splicing mutated PE38 (KDEL) to human EGF and IL-4 were created using the same techniques. An additional anti-B-cell BLT containing the PE38 KDEL fragment was created as a specificity control. 2219ARLKDEL was produced by joining two scFvs specific for human anti-CD22 and anti-CD19 to PE(KDEL). Bic3, recognizing human T cells, was synthesized by fusing two repeating scFvs recognizing human CD3 to DT390 (17). Control anti-B-cell DT2219ARL was previously reported (14).

Isolation of inclusion bodies, refolding, and purification. Proteins were produced as previously described with some minor modifications (13). Dithioerythritol, at 10 mg/mL, was included in refolding buffer to decrease aggregation. Additionally, refolded protein was diluted instead of being dialyzed before loading onto ion exchange column. Finally, the purity of the protein isolated from the ion exchange column was further enhanced using fast protein liquid chromatography and Supra
dex 1000 size exclusion column (Sigma). This modified protocol resulted in a yield of 5 to 10 mg of protein per liter of culture.

Antibodies and cell lines. For blocking studies, anti-human EGF and anti–IL-4 antibodies were obtained from R&D Systems. Anti-Lys2, a rat IgG2a from clone A20-1.7, provided by Dr. Uli Hammerling (Sloan Kettering Cancer Research Center, New York, NY), was used as a negative control because it recognizes mouse CD45, an allotypic hematopoietic cell surface marker not expressed on human cells. The EGFRIIL4R human breast cancer carcinoma MDA-MB-231 was originally derived from pleural effusion of patients with stage III breast carcinoma (18). MDA-MB-231 was genetically altered by transfection with a dual reporter gene encoding both firefly luciferase and green fluorescent protein, creating the MDA-MB-231-luc cell line for imaging. The line was sub
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Bioassays. To determine the effect of EGF4KDEL on MDA-MB-231 cells, protein synthesis assays measuring [3H]leucine incorporation were used (13). Cells (10⁴ per well) were plated in 96-well flat-bottomed plates and incubated overnight at 37°C at 5% CO₂. Cytotoxins in varying concentrations were added to wells in triplicate. Incubation continued for 72 h and [methyl-3H]leucine (GE Healthcare, UK) was added (1 μCi per well) for the final 24 h of incubation. Plates were frozen to detach cells, and then cells were harvested onto a glass fiber filter, washed, dried, and counted using standard scintillation methods. In some assays, proliferation was analyzed by measuring [3H]thymidine

Translational Relevance

Targeted toxins represent a biological drug class that can be used as an alternative for failed chemotherapy. New bispecific ligand–directed toxins are reported that are superior to their monospecific counterparts and dependent on having dual cytokine ligands on the same single-chain toxin. Further genetic alterations are used to enhance potency with the addition of an ER retention sequence and mutations that reduce the immunogenicity of the toxin by ~90%. The EGF4KDEL 7mut drug was studied in a powerful bioluminescence luciferase reporter gene model in which tumors grew aggressively, metastasized, and imaging could be done in real time. EGF4KDEL 7mut was highly effective even when we waited 26 days to begin therapy. Because immunogenicity was significantly reduced, these studies show that these alterations will permit us to give multiple drug treatments required for antitumor effects and opens the door for clinical studies of chemotherapy-refractory breast cancer.

ligands (6). PE contains the A fragment of native PE that catalyzes ADP ribosylation of elongation factor 2, leading to irreversible inhibition of protein synthesis and cell death with as few as 1,000 molecules (7). Also, PE was further modified to include Lys-Asp-Glu-Leu (KDEL) as a COOH-terminal signal that prevents secretion of luminal endoplasmic reticulum (ER) proteins, resulting to ER accumulation and enhanced potency (8).

This study also addresses the unique dual use of human epidermal growth factor (EGF) and interleukin 4 (IL-4) as bispecific ligands to create bispecific ligand–directed toxins (BLT) superior to their monospecific counterparts that work best when combined on the same single-chain molecule. The two discoveries were combined, and EGF and human IL-4 DNA fragments were genetically spliced to mutated low immunogenicity PE DNA to create the hybrid protein EGF4KDEL 7mut, which has powerful anti–breast cancer effects against established malignant MDA-MB-231 tumors. EGF4KDEL 7mut has remarkable anti–breast cancer effects due to its recognition of EGF receptor (EGFR) and IL-4 receptor (IL-4R), which are overexpressed on breast cancer cells and are known to be expressed on some normal tissues (9, 10). Our laboratory had discovered other BLTs that work for different types of cancers as well (11–15). According to the American Cancer Society statistics, an estimated 178,480 new cases of invasive breast cancer will be diagnosed among women in 2007 and 40,460 are expected to die of drug-refractory disease (16). Thus, alternative drugs are urgently needed.

This study set out to determine whether a set of genetic modifications could be used to create a new, more effective low immunogenicity anticancer BLT that would control the growth of an aggressive metastatic breast cancer tumor. This novel protein was studied in a powerful bioluminescence luciferase reporter gene model in which tumors grew aggressively and metastasized and imaging could be done in real time.
incorporation. Assays measuring \[^3H\]thymidine uptake differed from \[^3H\]leucine assays only in that leucine assays were done in leucine-free medium. Data are reported as percentage of control counts. MDA-MB-231 assays were measured with \[^3H\]leucine incorporation (Fig. 1A and B). HPB-MLT cells were measured with \[^3H\]thymidine incorporation (Fig. 1C).

For blocking assays, the anti-EGF or anti IL-4 antibody was added to leucine-free medium containing 0.01 nmol/L EGF4KDEL at a final concentration of 50 μg/mL. Resulting mixtures were added to wells containing MDA-MB-231 cells, and protein synthesis was measured. Ly 5.2 was included as a negative blocking control.

**In vivo efficacy studies.** Male nu/nu mice were purchased from the National Cancer Institute, Frederick, Cancer Research and Development Center, Animal Production Area, and housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)–accredited specific pathogen-free facility under the care of the Department of Research Animal Resources, University of Minnesota. Animal research protocols were approved by the University of Minnesota Institutional Animal Care and Use Committee.

To test the efficacy of EGF4KDEL against metastatic breast cancer, MDA-MB-231-luc cells were injected directly into the spleen of anesthetized mice, resulting in systemic metastatic breast cancer. Mice given intrasplenic (i.s.) tumor cells on day 0 were treated with multiple i.p. injections of BLT. Mice were imaged in real time, and images were captured using the Xenogen Ivis imaging system (Xenogen Corporation) and analyzed using the IGOR Pro 4.09a software (WaveMetrics, Inc.). Before imaging, mice were anesthetized using isoflurane gas. All mice received 100 μL of a 30 mg/mL d-luciferin aqueous solution (GoldBio-technology) as a substrate for luciferase 10 min before imaging. All images represent a 5-min exposure time, and all regions of interest are expressed in units of photons/s/cm²/sr. For treatment, mice were given 10 courses. A course is defined as four consecutive i.p. injections, one injection given each day (MTWTh). Thus, mice received 40 injections over the 10 wk of the experiment. Treatments were begun either on day 5 or day 26 post tumor inoculation.

Detection of antitoxin antibody by ELISA assay. To detect IgG antitoxin antibodies, immunocompetent normal BALB/c mice were immunized with weekly injections of 0.25 μg nonmutated EGF4KDEL or mutated EGF4KDEL 7mut. After five injections, serum was collected 4 d after the final injection. A standard ELISA assay was used in which recombinant PE38KDEL was adhered to the plate. The test serum from
the immunized mice was then added followed by the detection antibody, anti-mouse IgG peroxidase (Sigma). Plates were developed with o-phenylenediamine dihydrochloride (Pierce Biotechnology) for 15 min at room temperature. The reaction was stopped with the addition of 2.5 mol/L H₂SO₄. Absorbance was read at 490 nm and the final concentration was determined from a standard curve using highly purified anti-PE₃₈KDEL. All samples and standards were tested in triplicate.

**Statistical analyses.** All statistical analyses of in vivo data were done using Prism 4 (Graphpad, Inc.). Groupwise comparisons of mean data were made by Student’s t test. P values <0.05 were considered significant.

**Results**

**Potency and specificity of EGF4KDEL.** Following purification, EGF4KDEL (Fig. 1, center) was of the expected molecular weight (63.6 kDa) with a purity of 95% (not shown). EGF4KDEL was tested against the EGFR+ and IL-4R+ breast cancer cell line MDA-MB-231. This line was chosen because of its aggressive growth in nude mice and the fact that i.s. injection always resulted in liver metastases. Figure 1A and B shows that against MDA-MB-231 cells, bispecific EGF4KDEL was able to kill with an IC₅₀ of 10⁻¹⁰ to 10⁻⁷ nmol/L. Monospecific EGFKDEL was at least 1,000-fold less effective with an IC₅₀ of 0.0001 nmol/L. Monospecific IL4KDEL was 10,000- to 100,000-fold less effective than EGF4KDEL with an IC₅₀ of 0.0009 nmol/L. In Fig. 1B, the BLT EGF4KDEL was at least 1,000-fold more effective than an equimolar mixture of monospecific EGFKDEL and cpIL4KDEL. Irrelevant control Bic3, 2219KDEL, and DT2219ARL were minimally inhibitory. Together, these findings indicated that bispecific EGF4KDEL had superior potency to its monospecific counterparts and that this superior effect was dependent on positioning both ligands on the same single-chain molecule.

In Fig. 1C, EGF4KDEL showed no activity against the EGFR-, IL-4R+ human T-cell leukemia line HPB-MLT, whereas control Bic3, a previously reported anti-IL-4 B-cell immunotoxin, was highly effective. To confirm that the EGF and IL-4 ligands were both active on the EGF4KDEL molecule, anti-EGF or anti-IL-4 antibodies were used to block the killing of MDA-MB-231 cells by EGF4KDEL (Fig. 1D). When added to 0.01 nmol/L EGF4KDEL, both antibodies were capable of blocking ~70% to 95% of the cytotoxic effect, but neither of the antibodies completely blocked, likely because when only one ligand was blocked, the other ligand remained active. The addition of the anti-mouse Ly5.2 monoclonal antibody had no blocking effect. Together, these findings indicated that the tripartite assembly of EGF4KDEL did not sterically compromise the binding ability of the EGF and IL-4 ligands and that both were intact.

**Efficacy in an aggressive intrasplenic breast cancer model.** To determine whether EGF4KDEL could mediate a systemic anti-breast cancer effect, a model was developed in which MBA-MB-231 cells transfected with a luciferase reporter gene were injected into the spleen of nude mice. Figure 2A shows the aggressiveness of the model 60 days after injection with 5 x 10⁵ cells; organs were removed and imaged for tumor presence. High tumor levels were detected in the liver, spleen, pancreas, and intestines. Low levels were measured in the heart, lung, and kidneys. Based on liver imaging data in Fig. 2A, 2.6 x 10⁸ photons/s/cm²/sr roughly correlates with a tumor size of 0.126 cm³.

The maximum tolerated dose (MTD) was established (n = 5/group) at 4 μg/injection (or 160 μg/kg) given four consecutive injections per week (MTWTh; data not shown), which is referred to as one course of treatment and could be repeated each week without weight loss. Animals lost weight and some died when given 10 μg/injection.

In experiment 1 (Fig. 2B), animals were injected i.s. with 10⁶ MDA-MB-231 cells to initiate tumors. Treatment was begun on day 5 and mice were given 10 courses of EGF4KDEL i.p. on days 5, 12, 19, 26, 33, 40, 47, 54, 61, and 68. Individual data from all animals M6-M11 are shown. A marked reduction in tumor over time, as determined by a reduction in total bioluminescent activity, was observed in five of six treated mice. Animal 11 died without weight loss and was killed by a cage-mate. In contrast, untreated controls (M1-M5) progressed in an aggressive manner. Two control mice (M12 and M13) are shown that were given an irrelevant anti-B-cell control immunotoxin, 2219ARLKDEL, not reactive with MBA-MD-231 cells. Tumors in these mice did not regress. The mean total bioluminescent activity for each group was pooled, averaged, and is also shown. The two curves were significantly different on day 26 (P < 0.05). Three of six (50%) EGF4KDEL-treated mice were long-term tumor-free survivors at day 145. Histologic studies of liver, kidney, spleen, and pancreas revealed that mouse M6 and M9 were tumor-free on day 147. M8 was studied on day 201 and was tumor-free. A third group of mice were given the identical dose and injection schedule of EGF4 bioengineered without toxin, and results verified that EGF4 alone did not have an anti-tumor affect (shown in Fig. 3B).

**Antitumor effect of EGF4KDEL given 26 days post tumor.** EGF4KDEL was highly effective in preventing tumor growth of newly established tumors; thus, in a different experiment (experiment 2), a group of animals were given i.s. tumors and then treatment was begun 26 days later. Figure 3A shows the EGF4KDEL group treated in the same manner as mice in experiment 1. These mice were given 10 courses of EGF4KDEL i.p., which began on day 5 (days 5, 12, 19, 26, 33, 40, 47, 54, 61, and 68). Treated mice showed complete tumor responses in all cases (M14-M18) just as in Fig. 2. The last image shown is day 82 but all animals were tumor-free at day 103, at which time two mice, M15 and M18, were taken for histology, which verified their tumor-free status (not shown). Two of the animals, M16 and M17, were imaged at day 201 and remained tumor-free. In mice M19 to M22, treatment was delayed until day 26. Mice were given 10 courses on days 26, 33, 40, 47, 54, 61, 68, 75, 82, and 89. Two of four mice showed antitumor response and the rest showed no response. The two responders, M19 and M20, had smaller, late developing tumors, whereas M21 and M22 were larger, which may explain the outcome. Long-term histology studies were done on day 201 on M19 to confirm that the tumor grew no further. These studies confirmed the tumor-free status of M20. Figure 4A and B shows the pooled data of experiments 1 and 2 and tumor growth for all of the individual animals. Tumor growth in nontreated mice steadily progressed. Nontreated mice died between days 82 and 95 (data not shown). Treatment with EGF4KDEL resulted in a sharp decline in tumor growth for all treated animals. All but one mouse were complete responders. The curves significantly differed on day 33 (P < 0.01). M23 to M27 were injected with equimolar mixtures of monospecific EGFKDEL and cpIL4KDEL to compare with bispecific EGF4KDEL. All of the
Fig. 2. The selective efficacy of EGF4KDEL against MDA-MB-231-luc in vivo. A, to determine the metastatic ability of the MBA-MB-231-luc tumor, a representative tumor-injected mouse was organ-imaged on day 60 following i.s. tumor injection on day 0. Two images are shown for each organ. Left, organ without bioluminescence imaging; right, the same organ with bioluminescence imaging. Bioluminescence intensity is shown as a function of photons/s/sr/cm² and is listed for each image.

B, bioluminescence imaging for experiment 1 in which nude mice were given an i.s. injection of \(10^6\) MBA-MB-231 cells on day 0. Mice M1 to M5 were untreated, whereas mice M6 to M11 were treated i.p. with 10 courses (40 injections) of EGF4KDEL beginning on day 5. Mice M12 and M13 received treatment with the identical dose and schedule, but with anti–B-cell 2219ARLKDEL instead. Also shown is a graph of the average total photon tumor activity from the mice in this experiment. Averaged data are compared by Student’s t test (\(P < 0.01\)).
Fig. 3. Antitumor effect of EGF4KDEL given 26 d post tumor. A, bioluminescence imaging from experiment 2 in which nude mice were given an i.s. injection of 10^6 MBA-MB-231 cells on day 0. Mice M14 to M18 were treated i.p. with 10 courses (40 injections) of EGF4KDEL beginning on day 5. In contrast, mice M19 to M22 were treated with 10 courses of EGF4KDEL beginning on day 26. M23 to M27 were injected an equimolar mixture of monospecific EGFKDEL + IL4KDEL with the same schedule as with EGF4KDEL. Bioluminescence intensity is shown as a function of photons/s/sr/cm^2. B, mice M28 to M31 were treated with EGF4 devoid of toxin as part of experiment 1. C, mice M32 to M36 were part of experiment 3 whereby mice given i.s. injection of 2 x 10^6 MBA-MB-231 cells on day 0 were treated with mutated EGF4KDEL 7mut. Mice were treated i.p. with 10 courses (40 injections) of EGF4KDEL 7mut beginning on day 5.
animals receiving the mixture died before day 12 after their second injection of mixture, indicating that the mixture was more toxic than the BLT.

Together, these data show that multiple course treatment with EGF4KDEL is highly effective even when treatment is begun as late as 26 days post tumor inoculation. The effect was definitely dependent on multiple course therapy because treatment with only three courses on days 3, 10, and 18 shown in the representative mice in Fig. 4C resulted in tumor relapse. The effect of EGF4KDEL is selective and related to the EGF4 ligand because the control BLT was not as effective.

Reduced immunogenicity EGF4KDEL 7mut. To be able to give multiple treatments to sustain an antitumor effect, the same EGF4KDEL used in Figs. 1 to 3 was mutated in the toxin region to create EGF4KDEL 7mut. Evaluation of EGF4KDEL 7mut proceeded in three steps: (a) measuring its ability to induce antitoxin antibodies in normal mice compared with parental nonmutated EGF4KDEL, (b) determining whether it had lost any activity compared with parental EGF4KDEL, and (c) determining whether EGF4KDEL 7mut had the ability to prevent breast cancer metastasis in vivo. Normal immunocompetent mice were immunized with weekly doses of EGF4KDEL 7mut and then blood serum was collected 4 days after the final immunization. Using an ELISA assay that measures the amount of anti-PE antibody using a standard curve of highly purified mouse anti-PE, Fig. 5A shows a significant decline in the level of anti-PE antibody in mice immunized with EGF4KDEL 7mut compared with mice immunized with parental EGF4KDEL (n = 4-5 per group). Mutagenesis resulted in a reduction of antibody production of at least 90%. Interestingly, results were similar when either PE38KDEL or EGF4KDEL was adhered to the plate, indicating that the EGF4 portion of our hybrid molecule was less immunogenic (data not shown). Perhaps this is attributed to conservation of sequence and/or structure. Figure 5B shows that mutagenesis resulted in only a slight reduction in the in vitro ability of EGF4KDEL 7mut to kill the EGFR+IL4R+ breast cancer cell line BT-474 compared with parental EGF4KDEL. Thus, this experiment also shows that the drug works against more than one breast cancer cell line.

For in vivo efficacy studies, 2×10⁶ MDA-MB-231-luc cells were inoculated into mice that were treated with EGF4KDEL 7mut 5 days later. Mice were treated with 10 courses of EGF4KDEL i.p. given on day 5, 12, 19, 26, 33, 40, 47, 54, 61, and 68. Four of five (80%) treated mice were complete responders and long-term survivors (Fig. 3C). Figure 5D is the averaged data for the treated mice (minus the one relapsed mouse) and shows a significant difference between tumor progressions in the untreated group and the significant precipitous decline in tumor growth in the treated group (P < 0.01). Figure 6 shows that treatment did not cause the animals to lose weight because all of the treated mice had gained at least 2 g in weight over the course of 40 injections. The histology section in Fig. 6 shows kidney from M8, a 201-day survivor that received 10 courses of EGF4KDEL. The kidney appeared normal and healthy with intact glomeruli and tubules. Liver was also normal and tumor-free (not shown). Interestingly, toxicity studies in mice show that bispecific EGF4KDEL (MTD = 160 μg/kg) is tolerated 10 times better than monospecific EGF4KDEL (MTD = 10 μg/kg) and that hepatotoxicity is dose limiting (20).

Together, these data show that the mutations resulting in EGF4KDEL 7mut produces a reduced immunogenicity variant.
that shows little loss of in vitro activity. Despite mutagenesis, mutated or nonmutated EGF4KDEL retains its powerful in vivo anti-breast cancer ability at dosages that are not toxic although animals are treated multiple times.

Discussion

The original contributions of this article was the discovery of a BLT that was highly potent and impressively effective against a highly aggressive metastatic human breast cancer. Moreover, toxin immunogenicity was reduced ~90% by mutation. Drug was given systemically and was even effective when given several weeks after tumor establishment. Importantly, although therapy was stopped on day 92, long-term tumor-free survivors were observed more than 100 days later.

If targeted toxins are to be effective for treatment of solid tumors, then a means must be found to reduce their immunogenicity so that multiple treatments can be given to sustain high enough serum levels of drug to penetrate the tumor (21). In a recent clinical trial in which PE-based cytotoxins were administered to glioma patients, 73% of the patients developed IgG antibody to PE (22). The produced antibody was specific to the PE portion of the molecule and not to the ligand portion of the cytotoxin, probably due to PE being a highly immunogenic foreign protein. Recently, Onda and Pastan used a large library of anti-PE (Pseudomonas exotoxin A) monoclonal antibodies to map the immunogenic epitopes of PE toxin and identified seven major epitopes on the toxin recognized by B cells (4). Fortunately, the immunogenic portions of the molecule clustered into discrete regions and were not widely distributed throughout the molecule; widespread mutation would more likely alter the three-dimensional steric molecular configuration. Using a variant of a PE-based immunotoxin currently under phase 2 investigation for hairy cell leukemia, critical amino acids were identified and removed in all seven regions without compromising the activity of the immunotoxin (5). Importantly, mutation resulted in a significant reduction in the production of anti-PE antibodies in normal mice without reducing the activity of the molecule. Taking advantage of this advance, these mutations were incorporated into EGF4KDEL and we report for the first time a new variant EGF4KDEL...
7mut with reduced immunogenicity and an impressive ability to systemically eliminate a highly aggressive metastatic human breast carcinoma.

The reduction in immunogenicity was measured by immunizing normal immunocompetent mice. Importantly, Onda studied serum from patients given multiple injections of a PE-based immunotoxin and found that antibodies from these patients bind to some of the same epitopes as recognized by mice supporting the contention that mice are an acceptable model for human immunogenicity (5). In future studies, it will be important to determine which of these are missing from the serum of mice immunized with EGF4KDEL 7mut. Studies also addressed the possibility that the mutations might alter T-cell epitopes rather than B-cell epitopes by performing immunogenicity studies with three strains of mice with different MHC class II molecules (A/J for haplotype a; C57BL/6 for b; BALB/c for d; ref. 23). Because these class II molecules presented different peptides to T cells and immunogenicity was still reduced in all three strains, it was far more likely that mutation altered B-cell recognizing epitopes rather than T-cell epitopes.

Another original contribution of these studies is the finding that EGFR and IL-4 can successfully serve as highly effective BLT. BLTs are novel single-chain biologicals synthesized by linking a truncated toxin to two well-established targeting ligands, with the goal of increasing targeting capability. Our criteria for a successful BLT selection is that the final construct must result in better antitumor activity than its monospecific counterparts and a mixture of the two proteins is shifted so as to not obstruct ligand-receptor binding with the new terminus placed at amino acids 37-38. The peptide GGNGG was used to join the native carboxyl terminus and the native amino terminus of IL-4.

Another important aspect of these studies is the use of a luciferase reporter gene model that permitted the assessment of systemic tumor development in real time. This permitted optimization of multiple dose treatment schedules that showed remarkable antitumor potency of the EGF4KDEL 7mut; antitumor effects in long-term survivors were corroborated using their histology. Simultaneously, weight loss, a function of BLT toxicity (11–13), was monitored; it indicated that the regimen was nontoxic. Bioluminescence imaging was used to confirm that of what constitutes a positive cell, but EGFR is considered a rational target. Activation of the EGFR promotes processes responsible for tumor growth and progression, including proliferation and maturation, angiogenesis, invasion, metastasis, and inhibition of apoptosis. In addition, EGFR expression has been detected to varying degrees in a wide range of other solid tumors. Studies indicate that the IL-4 receptor is also expressed on breast cancer cells (10) and that that IL-4R is internalized at high levels after binding to IL-4 (25, 26). IL-4R is an excellent target because its normal expression is mostly limited to hematopoietic targets. Breast cancer cell lines and primary cell cultures were extremely sensitive to the cytotoxic effect of IL-4-PE, but IL4-PE showed a limited antitumor activity in MDA-MB-231 breast tumor xenografts in nude mice (27), the same model that we have modified and are using in the studies described in this article.

In these studies, we used circular permutation as described by Kreitman et al. (28). The fusion of ligands such as cytokines to other proteins can reduce the affinity of the ligand for its receptor because the new amino or carboxyl terminus is too close to the site at which ligand binds receptor. This is the case in constructing IL-4 cytotoxins, so a circularly permuted form, IL437-38, was used in which the attachment point between the two proteins was shifted so as to not obstruct ligand-receptor binding with the new terminus placed at amino acids 37-38. The peptide GGNGG was used to join the native carboxyl terminus and the native amino terminus of IL-4.

Another important aspect of these studies is the use of a luciferase reporter gene model that permitted the assessment of systemic tumor development in real time. This permitted optimization of multiple dose treatment schedules that showed remarkable antitumor potency of the EGF4KDEL 7mut; antitumor effects in long-term survivors were corroborated using their histology. Simultaneously, weight loss, a function of BLT toxicity (11–13), was monitored; it indicated that the regimen was nontoxic. Bioluminescence imaging was used to confirm that

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**Fig. 6.** The toxicity of mutated and nonmutated EGF4KDEL. A, average body weight is plotted for the EGF4KDEL 7mut mice shown in Fig. 5C and D. No significant differences were observed with the EGF4KDEL 7mut-treated mice compared with the 2219ARLKDEL 7mut and nontreatment controls. B, kidney section prepared by H&E staining and magnified 200×, taken from 202-day survivor receiving multiple injections of BLT.
the i.s. injection in this model always results in liver metastases. This model will be useful in further studies that measure therapeutic index and pharmacokinetics and determine if other means of delivery such as pumps are superior. Recently, we reported that convection enhanced delivery of BLT by pump are highly effective (15).

BLT themselves may represent an important advance in the field because only certain combinations of ligands can enhance activity as BLT. This observation does not seem to be limited to cytokines, because dual scFv targeting prominent cancer-associated markers can also be used. For solid tumors, anti-EpCam in combination with anti-Her2/neu scFvs shows promise as a BLT (12). For hematopoietic tumor targeting, scFvs targeting CD22 combined with scFv targeting CD19 shows potential for leukemia/lymphoma therapy (14) and a phase 1 study is under way. The reason that certain combinations of ligands show superiority over others is not yet clear. If we target EGFR and IL-4R simultaneously with EGF4KDEL 7mut, then the total density of antigens bound will be the sum of the EGFR and IL-4R densities, which would be greater than the EGFR density alone or the IL-4R density alone. Studies indicate that it is not strictly attributed to binding; therefore, the advantage may be at the level of cellular internalization or perhaps subcellular compartmentalization.

Although toxicity studies were not the focus of this article, an important issue is whether toxicity studies in mice will predict human toxicities. This is unknown, but we have shown that s.c. injection of EGF-containing BLT result in dermato logic injury (29), a finding that is in agreement with the known reactivity of EGF with EGFR in skin (30). Furthermore, monospecific EGFKDEL is highly toxic to mice, indicating that the 70% homology of human and mouse EGF is sufficient for reactivity rendering the mouse at least a partial on target model. Still, studies indicate that EGF4KDEL 7mut is ~16-fold less toxic compared with EGFKDEL (21), but high doses of EGFKDEL result in damage to the liver, predicting that hepatotoxicity might be dose limiting, a finding common to other targeted toxins (1). More complete toxicity studies are under way.

In summary, these findings show that EGF4KDEL represents an exciting new class of biological drug and an alternative strategy for breast cancer therapy that even at picomolar concentrations is capable of killing MDA-MB-231 and BT-474 cell lines. The BLT has superior activity compared with its monospecific counterparts. Because the BLT has greater activity than an equimolar mixture of the monomeric forms, its superior activity is dependent on having both ligands on the same single-chain molecule. Mutated EGF4KDEL addresses a major problem in the field—the immunogenicity of the toxin. Mutated drug retained its functional activity in its entirety and had 90% reduced capacity to generate antitoxin antibodies in normal mice, indicating that we may be able to give multiple treatments of anticarcinoma BLT with diminished immunogenicity in humans. These studies, using a highly sensitive imaging model, show that EGF4KDEL is effective in preventing the spread of aggressive metastatic solid tumors, making it a desirable alternative drug that can be used when chemotherapy fails because of its toxicity or resistance.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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A Novel Reduced Immunogenicity Bispecific Targeted Toxin Simultaneously Recognizing Human Epidermal Growth Factor and Interleukin-4 Receptors in a Mouse Model of Metastatic Breast Carcinoma

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