Research Article

Molecular Cancer Therapeutics

Bioengineering a Unique Deimmunized Bispecific Targeted Toxin That Simultaneously Recognizes Human CD22 and CD19 Receptors in a Mouse Model of B-Cell Metastases

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Abstract

A drug of high potency and reduced immunogenicity is needed to develop a targeted biological drug that when injected systemically can penetrate to malignant B cells. Therefore, a novel deimmunized bispecific ligand-directed toxin targeted by dual high-affinity single-chain Fvs (scFv) spliced to PE38 with a KDEL COOH-terminus was genetically engineered. The aims were to reduce toxin immunogenicity using mutagenesis, measure the ability of mutated drug to elicit antitoxin antibody responses, and show that mutated drug was effective against systemic B-cell lymphoma *in vivo*. Both human anti-CD22 scFv and anti-CD19 scFv were cloned onto the same single-chain molecule with truncated pseudomonas exotoxin (PE38) to create the drug. 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 ELISAs were done to determine whether antitoxin antibodies were reduced. Finally, a powerful genetically altered luciferase xenograft model was used that could be imaged in real time to determine the effect on systemic malignant human B-cell lymphoma, Raji-luc. Patient B-lineage acute lymphoblastic leukemia, B-cell chronic lymphocytic leukemia, and B lymphoma were high in CD22 and CD19 expression. 2219KDEL7mut was significantly effective against systemic Raji-luc in mice and prevented metastatic spread. Mutagenesis reduced neutralizing antitoxin antibodies by ~80% with no apparent loss in *in vitro* or *in vivo* activity. Because 2219KDEL7mut immunogenicity was significantly reduced and the drug was highly effective *in vivo*, we can now give multiple drug treatments with targeted toxins in future clinical trials. *Mol Cancer Ther; 9(6); 1872–83. ©2010 AACR.*

Introduction

Acute leukemia represents 30% of all cancer in American children under the age of 15 years and 12% of cancer cases in those ages 15 to 19 years and is the most common childhood malignancy. Eighty percent of these are B-lineage acute lymphoblastic leukemia (B-ALL). Chemotherapy resistance is a frequent cause of treatment failure in relapsed patients (1), and alternative therapies are urgently needed.

One alternative is targeted toxins, which are synthesized by coupling an antibody or antibody fragment to a potent, catalytic toxin, such as pseudomonas exotoxin, capable of inhibiting protein synthesis (2). Investigators have continued to develop targeted toxins because these agents are potent, catalytic, and irreversible in their action. Our research group has specialized in enhancing potency even further with the development of certain bispecific ligand-directed toxins (BLT) designed to promote activity (3–7). Despite successes in potency enhancement, immunogenicity is the major problem because effective therapy requires multiple treatments that result in the generation of neutralizing antibodies, mostly against the toxin (8). In the past 20 years, despite great efforts, no solution has been found for this problem. We have made use of the ability to genetically modify biological targeted toxins to address immunogenicity and other problems such as potency and toxicity (6, 7) that limit their usefulness for therapy.

Onda et al. (9) recently showed that there are only seven major epitopes in the Pseudomonas exotoxin A (PE) recognized by B cells. Furthermore, it is possible to remove or replace 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 (10). Therefore, we used these to mutate a truncated form of PE toxin (PE38) selected based on previous research describing a series of internal frame deletion mutations that established the best location for genetic fusion of

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PE to targeting ligands (11). PE38 contains the A fragment of native PE that catalyzes ADP ribosylation of elongation factor-2 (EF-2), leading to irreversible inhibition of protein synthesis and cell death with as few as 1,000 molecules (12). We further modified PE to include Lys-Asp-Glu-Leu (KDEL) as a COOH-terminal transport signal that enhances EF-2 access and potency (13).

For CD19 targeting, investigators using conventional biochemically linked anti-CD19 immunotoxin have reported anticancer affects (14–18). However, these have not reached the mainstream because of varied degrees of effectiveness. The 95-kDa CD19 membrane glycoprotein is broadly expressed on B-cell leukemia/lymphoma (19), including B-ALL. CD22 is also expressed on B cells and anti-CD22 immunotoxins have proven successful in the treatment of rare hairy cell leukemia (20). To broaden our toxin delivery and our antileukemia effect, we previously cloned a new molecule by fusing two repeating sFv subunits recognizing human CD19 and human CD22 spliced downstream of truncated DT_{390} (3) and subsequently used genetic engineering to improve it (21).

DT2219 had better antitumor activity than its monospecific counterparts or a mixture of the two, thus indicating an advantage of including both ligands on the same single-chain molecule and fulfilling our criteria for a successful BLT. Phase 1 studies are currently under way, but we do anticipate immunogenicity problems with the drug because DT is highly immunogenic, a problem in clinical trials with DT-based targeted toxins (22).

This study represents an important improvement in the creation of a new anticancer biological combining two advancements: "reduced immunogenicity" toxin and the "enhanced potency" BLT. In this study, the recently optimized anti-CD22 and anti-CD19 single-chain Fvs (scFv) were genetically spliced to mutated "low immunogenicity" PE DNA to create a hybrid protein 2219KDEL7mut that had powerful antilymphoma effects and produced long-term disease-free survivors. The anticancer benefit was shown in a powerful bioluminescence luciferase reporter gene model that permitted real-time imaging.

Materials and Methods

Construction of 2219KDEL7mut

The hybrid gene was synthesized using assembly PCR. In its final configuration, the 2219ARLKDEL fusion gene (from 5′ end to 3′ end) consisted of an *Eco*RI restriction site and then the anti-CD22 scFv gene (Fig. 1A). This anti-CD22 scFv gene was oriented with the V_L domain preceding the V_H domain and was conjoined by a fragment encoding the ARL linker (GSTSGSGKPGSGEGSTKG; ref. 23). Next, a G_4S linker (GGGGS) followed by anti-CD19 scFv (in the same V_L/V_H orientation and same ARL linker) was cloned and then a downstream seven–amino acid EASGGPE linker. The linker was followed by PE38 (360 amino acids) with its COOH-terminal REDLK replaced with the endoplasmic reticulum (ER) retention sequence KDEL and, finally, a *Not*I restriction site at the 3′ end. The resultant 2,650-bp *Eco*RI/*Not*I fragment gene was spliced into the pET21d bacteria expression vector under control of an isopropyl-L-thio-B-D-galactopyranoside (IPTG)–inducible T7 promoter. 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 2219ARLKDEL molecule (2219ARLKDEL7mut) with decreased immunogenicity, eight amino acids representing the seven major epitopes on PE38 KDEL were mutated using the QuikChange Multi Site-Directed Mutagenesis kit (Stratagene) and confirmed by DNA sequencing. The following immunogenic, hydrophilic amino acids were altered: R490A, R513A, R467A, E548S, K590S, R432G, Q332S, and R313A (Fig. 1B; refs. 9, 10). An irrelevant control Bic3, recognizing human T cells, was synthesized by fusing two repeating scFvs recognizing human CD3ε to DT_{390} (24). CD3CD3KDEL was another control immunotoxin in which the same anti-CD3 scFvs were partially humanized and then fused to PE38. Both of these killed the $CD3^+$ HPBMLT T leukemia cell line and not the $CD3^-$ Raji line so they were used interchangeably. Control anti–B-cell DT2219ARL was previously reported and contains the exact same 2219 targeting ligands (21).

Inclusion body isolation, refolding, and purification

Plasmid was transformed into the *Escherichia coli* strain BL21(DE3) (EMD). Bacteria were grown in 800-mL Luria broth supplemented with 100 μg/mL carbenicillin in a 2-L flask at 37°C with shaking. Expression of the hybrid gene was induced by the addition of IPTG (FisherBiotech). Two hours after induction, the bacteria were harvested by centrifugation. The cell pellets were suspended and homogenized using a polytron homogenizer. After sonication and centrifugation, the pellets were extracted with 0.3% sodium deoxycholate, 5% Triton X-100, 10% glycerin, 50 mmol/L Tris, 50 mmol/L NaCl, and 5 mmol/L EDTA (pH 8.0) and washed.

The proteins were refolded using a sodium *N*-lauroylsarcosine air oxidation method modified from a previously reported procedure for isolating sFv (24). Refolded 2219KDEL7mut was purified by fast protein liquid chromatography ion exchange chromatography (Q Sepharose Fast Flow, Sigma) using step gradient of 0.3 mol/L NaCl in 20 mmol/L Tris-HCl (pH 9.0) over four column volumes followed by gel filtration chromatography (Superdex 200, GE Healthcare).

Antibodies and cells

The anti-CD19 monoclonal antibody (mAb) hybridoma HD37 that secretes mouse IgG1κ has been previously described by Dorken et al. (25) and has been studied as a targeted toxin conjugated to ricin toxin A chain (15). RFB4 (anti-CD22) was generously provided by Dr. Ellen Vitetta (University of Texas Southwestern Medical Center, Dallas, TX). The scFv used for 2219KDEL was derived from these mAbs. Anti-Ly5.2,

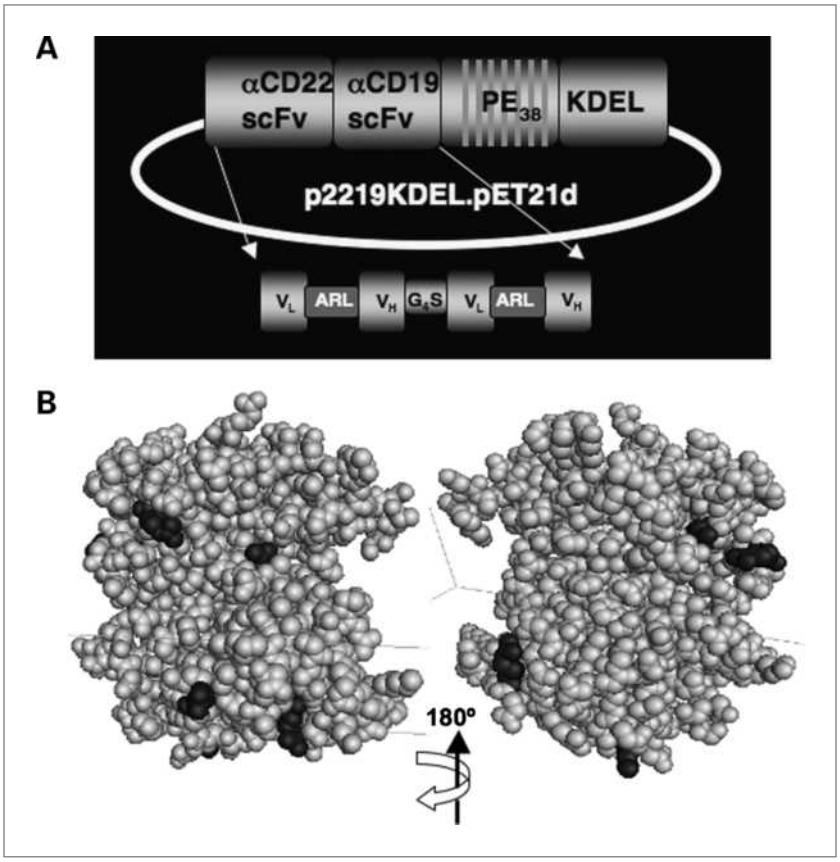

Figure 1. Construction of the plasmid containing the 2219KDEL7mut gene. The gene is described in Materials and Methods. The PyMol graphic was generated by downloading the Protein Data Bank (http://www.rcsb.org/pdb/home/) X-ray crystallographic structure of PE (file identification number: 1KLT) into the PyMol three-dimensional molecular modeling program (http://www.pymol.org/). Shown is a frontal view of PE38KDEL and a 180° reverse orientation of the molecule. The eight surface immunogenic amino acids that were mutated are darkened for enhanced visualization.

a rat IgG2a from clone A20-1.7, was generously provided by Dr. Uli Hammerling (Sloan Kettering Cancer Research Center, New York, NY). Anti-Ly5.2 was used as a control because it recognized mouse CD45.1, a hematopoietic cell surface marker not expressed on human cells.

Human cell lines included the $CD19^{-}CD22^{-}$ T-cell leukemia HPBMLT (26) and the $CD22^{+}19^{+}$ Burkitt's lymphoma Raji (27). Raji was stably transfected with a vector containing the firefly luciferase and green fluorescent protein genes, as well as a blastocidin resistance gene (Clontech Laboratories). Transfection was done with Lipofectamine reagent (Invitrogen), and stable clones were established using a FACSDiva flow cytometer (University of Minnesota Flow Cytometry Core Facility of the Masonic Cancer Center) to seed individual green fluorescent protein–positive cells into a 96-well plate. A subclone was chosen that retained identical morphologic and biological properties to the specific parental cell line. The $CD22^{+}19^{+}$ Burkitt's lymphoma cell line Daudi and the REH B-ALL cell line were also used (28). Lines were obtained from the American Type Culture Collection. Patient-enriched peripheral blood mononuclear cells were obtained under Institutional Review Board approval without identifiers and according to Health Insurance Portability and Accountability Act rules to protect privacy of personal health information.

Bioassays

To determine the effect of 2219KDEL7mut on normal B and malignant B-cell function, Raji, Daudi, and REH were used. Flow cytometry shows that they are >90% positive for both CD19 expression and CD22 expression (data not shown). Cells (2×10^4) were plated in a 96-well flat-bottom plate in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. Immunotoxin in varying concentrations was added to triplicate wells containing cells. The plates were incubated at 37°C and 5% CO_2 for 72 hours. Cells were then incubated with 1 μCi [methyl-^{3}H]thymidine (GE Healthcare) per well for 8 hours and harvested onto glass fiber filters, washed, dried and counted for 10 minutes in a standard scintillation counter. Data were analyzed using Prism 4 (GraphPad Software, Inc.) and presented as "percent control response" calculated by dividing the cpm of untreated cells by the cpm of the immunotoxin-treated cells (×100).

Blocking studies were conducted to test specificity. Briefly, 0.05, 0.5, 5, or 50 nmol/L RFB4 or HD37 was added to media containing 1 nmol/L 2219KDEL7mut. Resulting mixtures were added to wells containing Daudi cells, and proliferation was measured by [^{3}H]thymidine uptake as described. The mouse-specific anti-Ly5.2 was studied as a negative control. Data were presented as percent control response.

Flow cytometry

BLTs or mAbs were conjugated to FITC for direct immunofluorescence studies using flow cytometry as previously reported (4). Cells were incubated with FITC-labeled mAbs, washed, and then resuspended for analysis. Irrelevant control 3A1e-FITC was used to determine the degree of background binding. Flow cytometry was done on a FACSCalibur (Becton Dickinson). A minimum of 10,000 viable cells was analyzed.

In vivo efficacy studies

Male severe combined immunodeficient (SCID) mice were purchased from the National Cancer Institute, Frederick Cancer Research and Development Center, Animal Production Area and housed in an Association for the Assessment and Accreditation of Laboratory Animal Care–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 2219KDEL7mut against metastatic B-cell cancer, Raji-luc cells were injected i.v. (caudal vein), resulting in systemic metastatic cancer. Mice given Raji-luc cells on day 0 were treated with multiple i.p. injections of BLT. Mice were imaged in real time, and images were captured using Xenogen IVIS imaging system (Xenogen Corp.) and analyzed by 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 (Gold Biotechnology) as a substrate for luciferase 10 minutes before imaging. All images represent a 5-minute exposure time, and all regions of interest are expressed in units of photons/s/cm^2/sr.

Mice were given four weekly cycles of i.p. treatment. A single cycle was three i.p. injections every other day (Monday, Wednesday, and Friday). Cycles of treatment were begun on days 3, 17, 31, and 45 after Raji-luc inoculation. Thus, mice received a total of 12 injections.

Detection of serum IgG antitoxin content using ELISA assay

Our assay to detect IgG antitoxin antibodies was previously reported (7). Briefly, immunocompetent normal C57BL/6 mice (National Cancer Institute) were immunized with weekly injections of 0.25 μg nonmutated 2219KDEL or mutated 2219KDEL7mut. After five injections, serum was collected 4 days after the final injection. A standard ELISA assay was used in which recombinant PE38KDEL was adhered to the plate. 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 minutes at room temperature. The reaction was stopped with the addition of 2.5 mol/L H_2SO_4. 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.

To show neutralization, test serum samples were evaluated for their ability to block the killing of Raji cells by 0.2 nmol/L 2219KDEL. BLT was added to test serum, and then the mixture was added to the cells and incubated 72 hours. Thymidine uptake was determined as described above.

Statistical analyses

All statistical analyses of *in vivo* data were done using Prism 4 (GraphPad). Groupwise comparisons of mean data were made by Student's *t* test. Probability (*P*) values of <0.05 were considered significant.

Results

2219KDEL7mut

Figure 1B shows 2219KDEL7mut construction and a PyMol depiction of X-ray crystallographic structure of PE38KDEL in both front and back (180°) positions. The eight mutated amino acids are darkened so their surface position on the molecule can be readily visualized. Figure 2A shows the final SDS-PAGE gel analysis of 95-kDa 2219KDEL7mut with a purity of >95% as determined by Coomassie blue staining (photo is grayscale). The procedure was previously reported (4). Molecular weight size of 2219KDEL is estimated at 97.9 kDa from molecular weight standards.

In vitro killing of the Raji cancer cell line

Raji, Daudi, and REH were selected as target cell lines in these studies because flow cytometry studies showed high levels of both CD19 and CD22 expression and the cell lines showed aggressive metastatic growth in SCID mice. To determine the ability of BLT to kill Raji, the drugs were tested in proliferation assays and a representative experiment is shown in Fig. 2B. Bispecific 2219KDEL was able to kill with an IC_{50} of 0.04 nmol/L and was 150-fold more effective than CD19KDEL and 7.5-fold more effective than CD22KDEL. Irrelevant control CD3CD3KDEL was minimally inhibitory. Next, proliferation experiments were done in which increasing amounts of blocking antibody were added to a constant inhibitory concentration of 1 nmol/L 2219KDEL7mut, which inhibited Daudi cell proliferation in Fig. 2C. Increasing concentrations of RFB4 or HD37 reversed the inhibition of 2219KDEL7mut in a dose-dependent manner. The addition of an irrelevant control antibody, anti-Ly5.2, had no effect. Neither antibody blocked 100% of the activity because blocking one ligand would not necessarily fully block the other. In Fig. 2C, the killing capability of 2219KDEL and the ability of DT2219ARL were compared in proliferation assays against a third cell line, REH leukemia. This is because DT2219ARL is identical to 2219KDEL but contains DT_{390} instead of PE and is currently in phase 1 trials (21). When two different batch lots

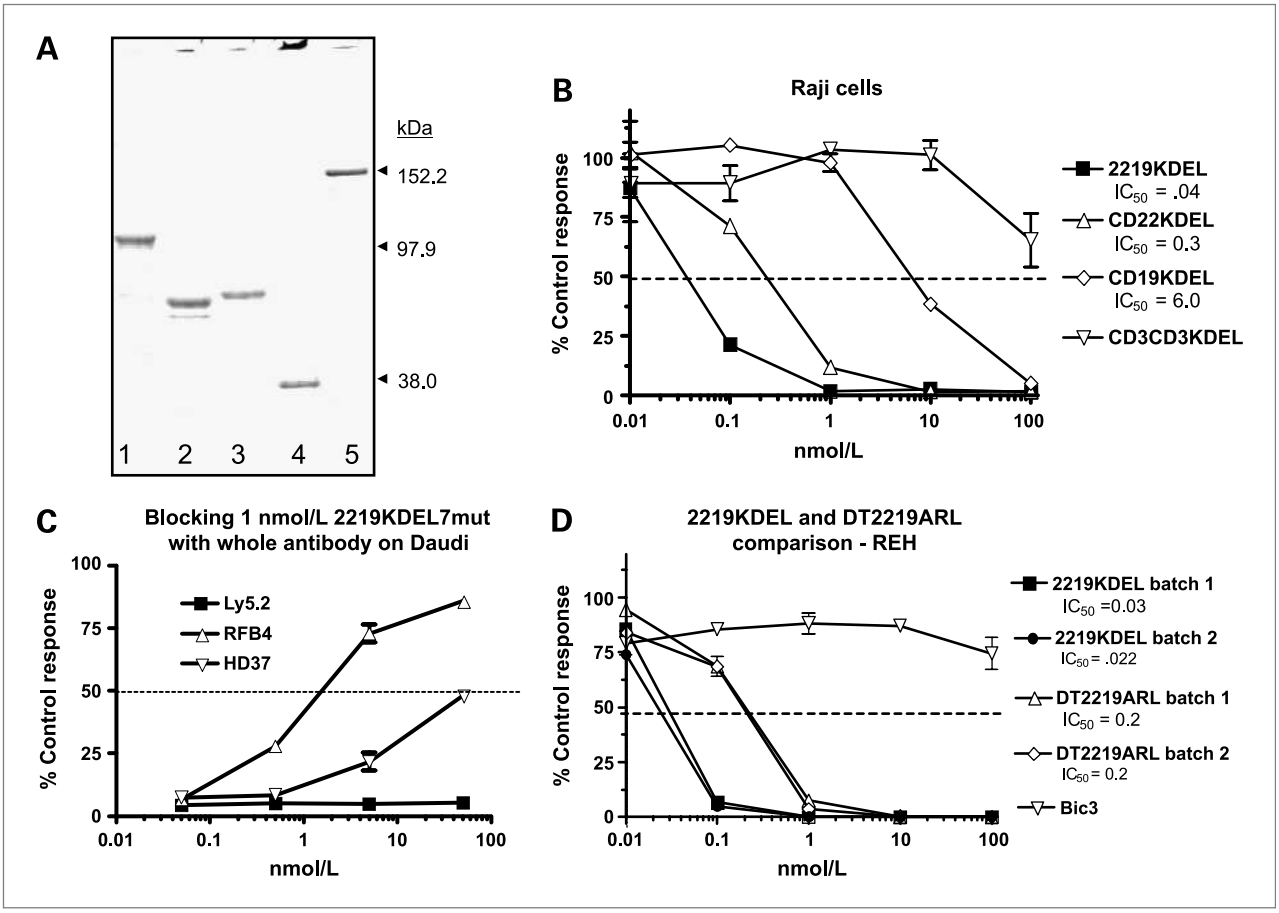

Figure 2. 2219 BLT. A, SDS-PAGE gel analysis. Lane 1, bispecific 2219KDEL; lane 2, monospecific 22KDEL; lane 3, monospecific 19KDEL; lane 4, PE38; lane 5, HD37 mAb. The gel was stained using Coomassie blue. Photo is grayscale. B, Raji cells were cultured with the BLT and its monomeric counterparts and proliferation were measured by uptake of tritiated thymidine. Data are percentage of control response where control response is untreated cells. Points, mean; bars, SD. The mean values of untreated cells were 82,048 ± 13,192 cpm/20,000 cells. C, to determine the intactness of the anti-CD19 sFv and the anti-CD22 sFv ligands, proliferation studies were done in which Daudi cells were treated with a constant concentration of 1 nmol/L 2219KDEL7mut and then blocked with increasing concentrations of HD37 mAb, RFB4 mAb, or nonreactive control Ly5.2 antibody. Thymidine uptake was then measured. Points, mean of triplicate determinations; bars, SD. Activity was calculated in comparison with the unblocked control and then graphed. Counts for untreated Daudi cells were 111,802 ± 13,786 cpm/20,000 cells. D, to determine the activity of different batches of 2219KDEL in comparison with two different batches of DT2219ARL, proliferation studies were done in which the various batches were incubated with REH B leukemia cells. Control counts = 65,368 ± 6,621 cpm/20,000 cells.

of 2219KDEL were compared with two different batch lots of DT2219ARL, the 2219KDEL was about a log more potent (Fig. 2D). Again, control Bic3 was minimally inhibitory. Trypan blue viability assays were done in addition to proliferation assays and as an additional check to verify that drug was indeed killing and not simply inhibiting cell proliferation (data not shown).

Together, these findings indicated that bispecific 2219KDEL had superior potency to its monospecific counterparts and killing was selective. Importantly, 2219KDEL was more potent than DT2219ARL and the drug was highly effective against two different B-cell lymphoma lines, Raji and Daudi, and one B-cell leukemia line, REH.

Studies of patient cells

To determine whether 2219KDEL reacted against patient cells, peripheral blood blasts were collected from a B-cell ALL patient and then reacted directly with 100 nmol/L FITC-labeled BLT. 2219KDEL-FITC showed 84.3 positive cells, whereas control Bic3-FITC showed a background of 19.8% positive cells, indicating that patient cells were indeed positive for the drug (data not shown).

To study CD22 and CD19 expression on patients with various B-cell malignancies, patient cells were stained with either anti-CD22 (RFB4-FITC) or anti-CD19 (HD37-FITC). Table 1 shows that T-ALL and acute myelogenous leukemia (AML) cases were mostly negative, whereas B-cell malignancies including B-ALL, B-cell chronic lymphocytic leukemia (B-CLL), and lymphoma were positive. Because peripheral blood B cells are in 6% to 8% range, our control cases stained appropriately at an average of 6.3% CD19 and 7.4% anti-CD22. Importantly, all B-cell cases stained for both markers and would likely benefit from treatment with our BLT. In

addition, CD19 expression was usually higher than CD22 expression.

Reduced immunogenicity 2219KDEL7mut

To be able to give multiple treatments to sustain an antitumor effect, PE38KDEL was mutated as shown in Fig. 1. Evaluation of 2219KDEL7mut must proceed in three steps: (*a*) determining whether mutated 2219KDEL had lost any activity compared with parental 2219KDEL, (*b*) measuring the ability of 2219KDEL7mut compared with nonmutated parental 2219KDEL to induce antitoxin antibodies in immunocompetent mice, and (*c*) determining whether 2219KDEL7mut had the ability to prevent cancer metastasis *in vivo*.

When three different mutated batches of 2219KDEL7-mut were compared with nonmutated 2219KDEL, Fig. 3A shows that mutagenesis resulted in no reduction in the *in vitro* killing of Daudi cells. Normal immunocompetent mice were immunized with weekly doses of 2219KDEL7mut, and blood serum was collected 4 days after each immunization. Using an ELISA assay that measures the amount of anti-PE antibody based on a standard curve of highly purified mouse anti-PE, Fig. 3B shows a significant ($P < 0.05$) decline in the level of anti-PE antibody in mice immunized with 2219KDEL7mut compared with mice immunized with parental 2219KDEL ($n = 5$ per group). Mutagenesis resulted in a reduction of antibody production of ~80% on day 76 after nine immunizations. To determine whether the antitoxin antibodies were neutralizing, serum from the 2219KDEL and 2219KDEL7mut immunized mice was incubated with a constant inhibitory concentration of 0.2 nmol/L 2219KDEL in proliferation assays. Figure 3C shows that serum taken from three individual mice immunized with 2219KDEL7mut on day 118 did not block 2219KDEL

Table 1. CD22 and CD19 expression on patient cells with various hematologic malignancies

Normal peripheral blood (6 volunteers)		CLL (6 patients)		AML (6 patients)	
CD19	**CD22**	**CD19**	**CD22**	**CD19**	**CD22**
4.2	7.2	76.2	38.5	3.8	5.0
6.6	8.7	89.1	90.9	0.6	0.4
7.3	7.3	66.6	58.1	0.4	1.0
6.4	7.2	38.9	27.5	1.8	3.6
9.2	9.3	87.7	78.7	1.6	9.9
4.1	4.4	80.5	75.7	0.4	0.4
Average					
6.3	7.4	73.2	61.6	1.4	3.4
B-ALL (5 patients)		**Lymphoma (12 patients)**		**T-ALL (2 patients)**	
CD19	**CD22**	**CD19**	**CD22**	**CD19**	**CD22**
27.1	20.6	16.1	13.0	3.7	4.2
88.4	76.5	81.3	50.2	3.5	3.6
100.0	64.2	18.4	19.0		
86.5	49.0	23.2	21.0		
93.2	97.1	23.7	23.0		
		46.4	35.8		
		98.7	83.0		
		68.3	70.0		
		37.4	6.9		
		89.1	13.9		
		69.6	46.6		
		52.5	50.3		
Average					
79.0	61.4	52.1	36.1	3.6	3.9

NOTE: Cell surface expression of either anti-CD22 or CD19 was determined by standard immunofluorescence with either RFB4-FITC or HD37-FITC. Cells were obtained from B-CLL, B-ALL, B lymphoma, control T-ALL, or control AML patients with Institutional Review Board approval as described in Materials and Methods. Data are calculated as percent positive cells relative to a known negative control. Normal enriched peripheral blood cells were obtained from healthy volunteers. Data points were averaged for each group.

activity. Sera from two mice immunized with 2219KDEL displayed high titers of antitoxin antibody neutralized 2219KDEL activity. Together, data show that serum from mice immunized with multiple injections of 2219KDEL7mut has 80% less antitoxin antibodies than mice immunized with nonmutated parental 2219KDEL and this correlated with a reduction in neutralizing antibodies.

Effects of 2219KDEL7mut in SCID mice with systemic cancer

Injection of the Raji-luc cells i.v. into SCID mice results in a systemic tumor that infiltrates all major organs and is reminiscent of human B-cell malignancy. The Raji-luc model offers a major advantage because imaging can be done on individual animals weekly to monitor tumor progression in real time. Malignant progression measured by imaging correlates with histologic progression and mortality in the model.

To determine if 2219KDEL7mut was effective against established systemic cancer, Raji-luc cells were injected i.v. in mice and i.p. treatments were started on day 3. The overall pooled survival of all treated mice (experiments 1 and 2) is shown in Fig. 3D. The Kaplan-Meier plot shows the long-term results after four courses of every other day i.p. treatment with 2219KDEL7mut. The plot shows seven of nine animals surviving >225 days after treatment. There were two late unexplained deaths on days 125 and 150 that occurred without weight loss (toxicity or tumor relapse).

Figure 4 shows detailed imaging and aggressiveness of the model. In Fig. 4A, 25 days after injection with 10^6 cells, organs were removed and imaged for tumor presence. High Raji-luc signal levels were detected in

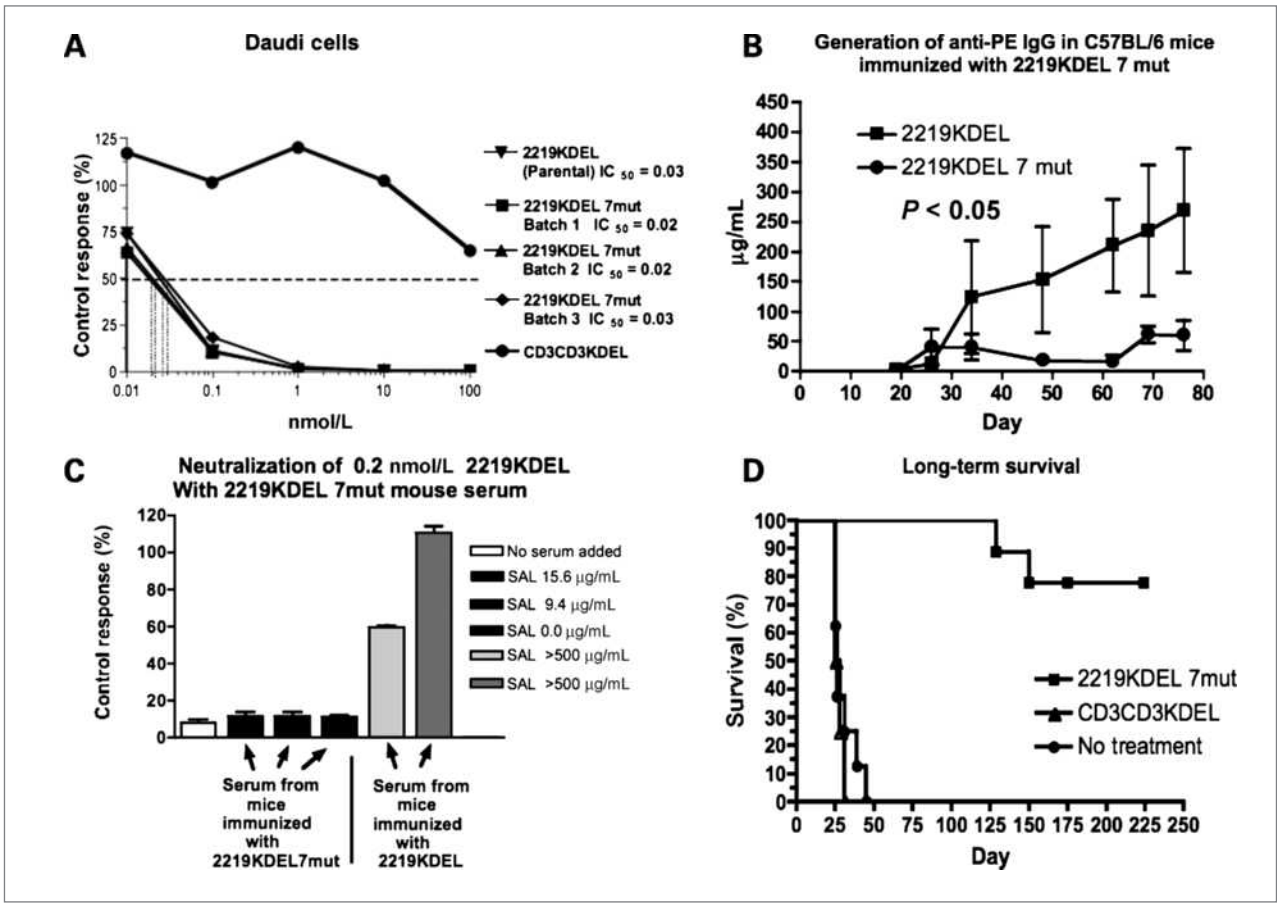

Figure 3. 2219KDEL7mut has reduced immunogenicity but not reduced activity. A, to determine the effect of 2219KDEL7mut on Daudi cells, increasing concentrations of 2219KDEL7mut were compared with nonmutated 2219KDEL in a proliferation assay. Counts for untreated Daudi cells were 68,613 ± 4,535 cpm/20,000 cells. Data are presented as % control response. B, to detect antitoxin antibodies, immunocompetent C57BL/6 mice were immunized with either nonmutated parental EGF4KDEL or mutated EGF4KDEL. Sera from individual mice were analyzed in a modified ELISA measuring μg/mL antitoxin IgG. Data were represented as the average μg IgG/mL. The two groups significantly differed ($P < 0.05$). C, to determine whether neutralizing antibodies were present, serum from the immunized mice was tested for their ability to neutralize a constant inhibitory concentration of 2219KDEL. Data are depicted as percent control response. SAL, serum antitoxin levels. D, to determine whether 2219KDEL7mut was efficacious, SCID mice given a lethal injection of Raji-luc cells were treated with three courses of 2219KDEL7mut or control CD3CD3KDEL beginning on day 3. Pooled data (experiments 1 and 2) are shown as a Kaplan-Meier plot.

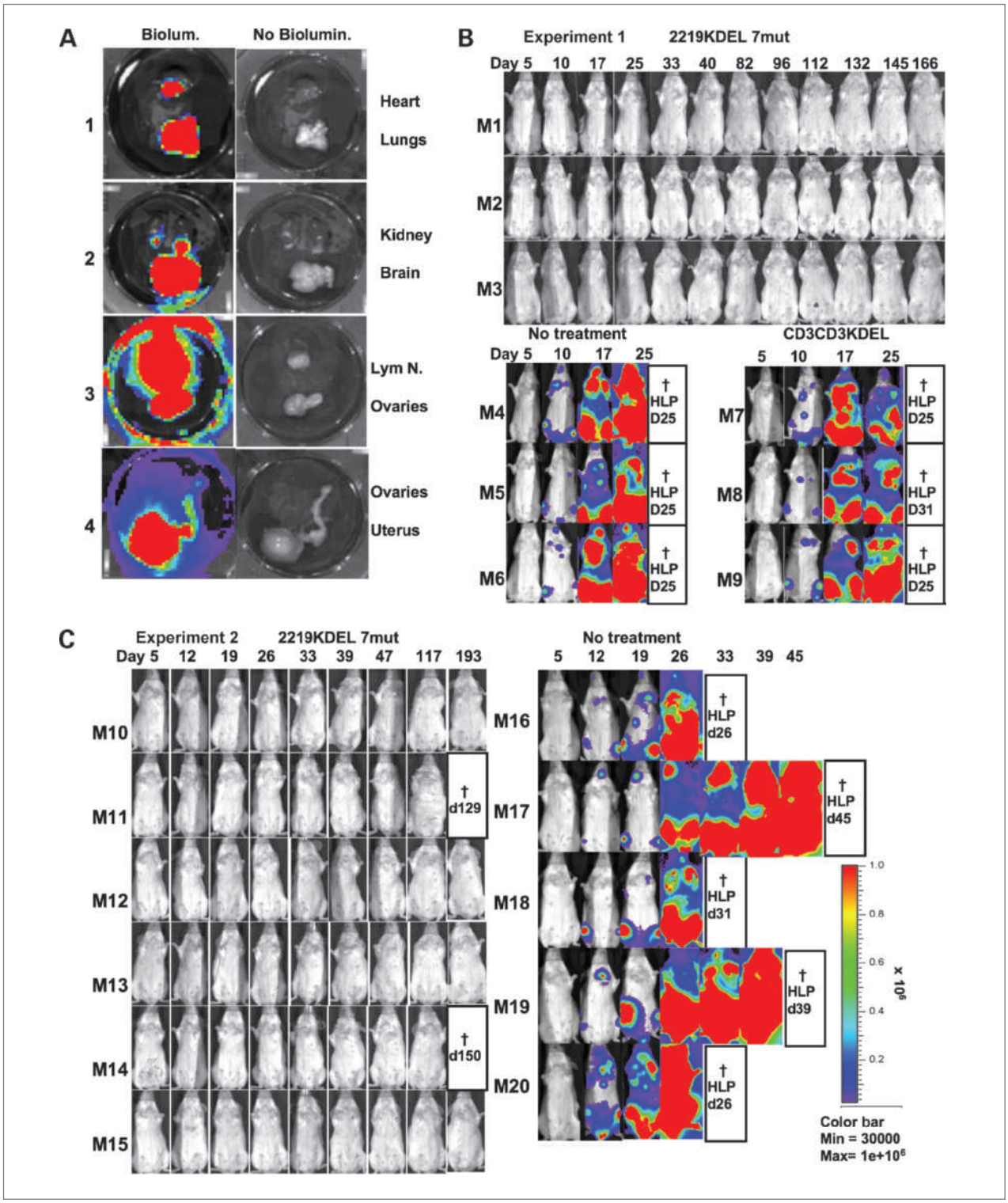

Figure 4. Efficacy of 2219KDEL7mut *in vivo*. A, to determine the metastatic ability of the Raji-luc lymphoma, SCID mice were injected i.v. with Raji-luc. A representative mouse was organ imaged on day 25 following Raji-luc injection on day 0. Two images are shown for each organ. The image on the left is with bioluminescent imaging, and on the right is the same organ without bioluminescent imaging. Bioluminescence intensity is shown as a function of photons/s/sr/cm. B, bioluminescent imaging is shown for experiment 1 in which SCID mice were given Raji-luc cells i.v. Mice M1 to M3 were treated i.p. with three courses of 2219KDEL7mut, whereas mice M4 to M6 were untreated. Mice M7 to M9 received treatment with the identical dose and schedule but with anti–T-cell Bic3 instead. C, experiment 2 was done exactly as experiment 1 only using more animals. Mice M10 to M15 were treated with 2219KDEL7mut, and mice M16 to M20 were not treated. HLP, hind limb paralysis.

the lymph node, ovaries, lung, brain, pancreas, and intestines of mouse 1 (photos 1–3). Low levels were measured in the heart and kidneys (data not shown). A tumor with a photon count of 2.6×10^8 photons/s/cm^2/sr is shown in the uterus of a second mouse (photo 4). Thus, we estimate that 2.6×10^8 photons/s/cm^2/sr roughly correlate with a tumor size of 0.75 cm^3.

In experiment 1 (Fig. 4B), tumor progressed quickly because it was detected in all untreated mice by day 10 and all untreated mice developed hind limb paralysis on days 25 to 31. Animals injected with Raji-luc develop this central nervous system complication with a 100% incidence. All three 2219KDEL7mut-treated mice were completely tumor-free on day 166. Animals treated with Bic3 as an irrelevant control drug developed cancer at the same rapid rate as the untreated controls, indicating that the activity of 2219KDEL7mut was specific. Experiment 1 was repeated with more mice in experiment 2 (Fig. 4C). Mice (n = 6 per group) were given Raji-luc and then treated with 2219KDEL7mut. All of these mice were long-term disease-free survivors, some surviving >225 days. Untreated controls rapidly developed tumor as before.

Figure 5 shows that treatment induced minimal weight loss because 2219KDEL7mut-treated mice (M10–M15) lost <10% of their body weight or did not lose any weight at all after the first treatment course. The same was true after the second treatment course.

Together, these data show that the reduced immunogenicity variant 2219KDEL7mut shows a high level of *in vivo* activity and retained its powerful anticancer activity, resulting in long-term disease-free survivors at dosages that are not toxic, although animals were treated multiple times.

Discussion

If targeted toxins synthesized with bacterial toxins are to be effective for therapy, then a means must be found to reduce their immunogenicity so that multiple treatments can be given without neutralization by patient antitoxin antibodies. Diphtheria toxin may be the most problematic based on lifetime immunization. For example, in phase III clinical trial of ONTAK (DT_{389}-IL-2), by the end of two courses, 59 of 60 patients had developed antitoxin antibodies and 32% of the patients had detectable levels of anti-DT antibodies before treatment even had begun (22). The use of PE has not greatly improved the situation. In a phase 1 trial of anti-mesothelin-PE38 monospecific immunotoxin, 88% developed neutralizing antibodies by day 29 of cycle 1 therapy and were not eligible for retreatment (8). Other approaches for reducing immunogenicity have been investigated including RNase (29), the use of scFvs (30), alternative toxins such as saporin (16), and PEGylation (31). After years of testing, none of these have reached the mainstream as viable possibilities for a reduced immunogenicity drug.

One logical alternative approach would be the genetic alteration of the toxin gene to eliminate peptide regions that interact with T and/or B cells to generate antitoxin responses. A major concern is that deletion of oligopeptide sequences would alter enzyme conformation enough to inactivate the catalytic active site. Recently, Onda et al. (9, 10) reported a potential solution and introduced point mutations in key eptitopic regions. This approach would only work if immunogenic amino acids could be identified in limited areas and if mutation did not involve excision of peptides that would dramatically alter tertiary structure. A large library of anti-PE mAbs was used to map the immunogenic epitopes of PE toxin, and seven major epitopes were located on the toxin recognized by B cells (9). Eight amino acids were removed in the seven regions without compromising toxin activity. The resulting toxin had reduced immune activity and could be used to synthesize immunotoxins with reduced activity (10).

Studies reveal that simultaneously targeting CD22 and CD19 with BLT results in higher levels of killing perhaps due to binding more drug on the cell surface and/or better drug internalization (especially when two highly internalizing markers such as CD22 and CD19 are targeted; refs. 3, 21). The fact that the BLT was more effective than a mixture of its monospecific components indicates the importance of having both ligands on the same single-chain molecule. DT2219 is the first BLT that has reached phase 1 clinical trials and has been well tolerated, but adequate dose levels have not yet been achieved. The synthesis of 2219KDEL7mut, reported herein, combined the BLT discovery with "deimmunized" toxin.

Studies in mice with deimmunized 2219KDEL7mut showed that immunogenicity was reduced at least 80% while eliminating the progression of a highly aggressive metastatic human B-cell lymphoma in a luciferase reporter gene model that permitted the assessment of systemic tumor development in real time with minimal toxicity. A key question is: Is the mouse an acceptable model

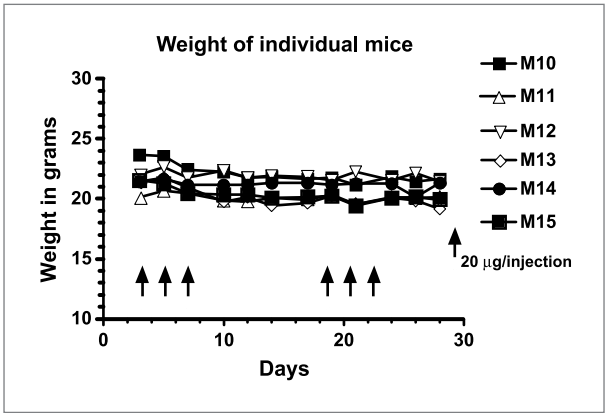

Figure 5. Toxicity of 2219KDEL7mut. The individual weights of 2219KDEL7mut-treated mice (M10–M15) from experiment 2 were plotted over time. The black arrows indicate the days of treatment with 20 μg 2219KDEL7mut.

for the immunogenicity of PE? Onda et al. determined whether treatment with PE immunotoxins induces human antibody responses to the same immunogenic epitopes recognized by mice (9, 32). Sera was taken from eight patients in phase 1 trials with pancreatic, colon cancer, or mesothelioma treated with two different PE immunotoxins: LMB-9 (anti-CD25 PE; ref. 33) or SS1P (anti-mesothelin PE; ref. 8). These patients had developed neutralizing antibodies that prevented further drug administration. Serum samples were analyzed in a published competition assay (9). Most patients with solid tumors produced neutralizing antibodies after one cycle of immunotoxin treatment. Competition analysis of paired serum samples showed that before treatment, the sera contained almost no specific antibody to any of the PE38 epitopes. In contrast, the sera obtained after immunotoxin treatment contained anti-PE38 antibodies to every topographical epitope recognized by the mouse antisera as shown by their ability to inhibit the binding of the corresponding mAbs to each epitope. These results show that human immunotoxin treatment induces human antibodies against the same seven immunogenic epitopes identified by the mouse mAb panel, and affirm that the mouse is an acceptable immunogenicity model for humans.

The anti-CD19 and anti-CD22 scFvs of 2219KDEL7mut are of mouse origin and so they are still immunogenic, although investigators do believe that the majority of the immune response is directed against the bacterial toxin rather than the scFv (32). This likely relates to the higher homology (~90%) between mouse and human scFv and the aggressive response of the human immune system against foreign bacterial proteins. Still, to eliminate any human anti-mouse antibodies, we are currently veneering the scFv, thereby converting any mouse amino acid to human amino acid that does not compromise the activity of the molecule.

The new reduced immunogenicity form of 2219KDEL7-mut showed the same *in vitro* killing ability as nonmutated parental 2219KDEL and about a log greater activity than DT2219ARL. This finding is likely attributed to the inclusion of amino acids KDEL at the COOH-terminus of PE in place of REDLK. The presence of KDEL promotes the specific transport of proteins from the Golgi to the ER. KDEL is recognized within the lumen of the Golgi by its receptor, Erd2p, and retrograde transport occurs via Erd2p in Golgi-derived coat protein 1 (COPI) vesicles (34–36). Recently, investigators showed (with MHC class I as a model) that an additional function of KDEL is to mediate recycling of chaperones escaping from the ER to the *cis*-Golgi intermediate compartment or the *cis*-Golgi (37). Thus, terminal KDEL can be viewed as locking the protein into a transport loop, favoring cytosolic location and thereby EF-2 target access. Other genetic modifications were used in the development of 2219KDEL7mut, including V_H/V_L reversal, inclusion of ARL linkers, and hotspot mutagenesis, to enhance affinity (3, 38).

Monospecific HL22, an anti-CD22 scFv spliced to truncated PE, has proven strikingly effective against hairy cell leukemia (20) but not against other B-cell malignancies. This likely relates to the high level of CD22 expression on patient cells. In our studies, Table 1 examines 23 patients with B-cell malignancies and shows at least three patient categories: (*a*) those containing high levels of both CD22 and CD19 blasts, (*b*) those containing lower levels of both CD22 and CD19 blasts, and (*c*) and those containing one marker at a higher level and the other at a lower level. Although this study did not directly measure the expression of CD22 and CD19 on the same cell, the large subgroup of patients with high expression of both markers indicates that a large cohort simultaneously expresses both markers. Targeting with dual ligand BLT could benefit all of these because superior levels of kill are achieved when targeting both markers simultaneously.

Our observation that BLTs are superior is not unique to hematopoietic cancer. The BLT framework also benefits solid tumor targeting. For example, a BLT assembled with scFvs simultaneously targeting EpCAM and Her2/neu had superior activity compared with its monomeric counterparts in a colorectal cancer model (4). In addition, BLT constructed with dual cytokines epidermal growth factor and interleukin-4, and deimmunized PE showed ability to inhibit the growth of established aggressive systemic breast cancer in mice even after waiting 26 days to begin therapy (7) and was effective against mesothelioma (6).

Eighty percent of the serum IgG antitoxin antibody level was reduced by immunizing immunocompetent mice with deimmunized drug. Studies also showed in Fig. 3C that neutralizing antibodies were reduced. Although we cannot distinguish neutralizing antibodies from nonneutralizing antibodies, it is just as important to reduce nonneutralizing antibodies because they still form drug antigen-antibody complexes and enhance drug clearance.

In summary, low-immunogenic BLTs were synthesized that have potent anti–B-cell cancer effects. These drugs are important alternative cancer therapies. One major advantage of targeted toxins is that they have a unique mechanism of action (protein synthesis inhibition) that differs from the mechanism of conventional chemotherapeutic drugs. A common problem with continually relapsing patients is that they will reach the limits of chemotherapy either because the drugs have become ineffective or because patients reach their toxic thresholds. Dangerously low blood counts are a common reason for discontinuing chemotherapy regimens. We are describing a new alternative drug with reduced immunogenicity and increased potency that can be used in place of chemotherapy. The relevancy of targeted toxins has been rightfully criticized because of their diminished potency, ability to reach tumor, and immunogenicity. However,

genetic engineering has been used to address these issues and 2219KDEL7mut stands out in its ability to inhibit the metastatic progression of highly aggressive B-cell lymphoma with reduced immunogenicity. Our group has recently reported other BLTs that likewise show similar benefits for the treatment of other types of cancer (5–7, 39, 40).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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