A Bispecific Recombinant Immunotoxin, DT2219, Targeting Human CD19 and CD22 Receptors in a Mouse Xenograft Model of B-Cell Leukemia/Lymphoma

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Abstract
A novel bispecific single-chain fusion protein, DT2219, was assembled consisting of the catalytic and translocation domains of diphtheria toxin (DT₃₉₀) fused to two repeating sFv subunits recognizing CD19 and CD22 and expressed in Escherichia coli. Problems with yield, purity, and aggregation in the refolding step were solved by incorporating a segment of human muscle aldolase and by using a sodium N-lauroyl-sarcosine detergent-based refolding procedure. Problems with reduced efficacy were addressed by combining the anti-CD19 and anti-CD22 on the same single-chain molecule. DT2219 had greater anticancer activity than monomeric or bivalent immunotoxins made with anti-CD19 and anti-CD22 sFv alone and it showed a higher level of binding to patient leukemia cells and to CD19⁺CD22⁺ Daudi or Raji cells than did anti-CD19 and anti-CD22 parental monoclonal antibodies. The resulting DT2219, mutated to enhance its avidity, was cytotoxic to Daudi cells in vitro (IC₅₀ = 0.3 nmol/L). In vivo, DT2219 was effective in a flank tumor therapy model in which it significantly inhibited tumor growth (P < 0.05) and in a systemic model in which it significantly prolonged survival of severe combined immunodeficient mice with established Daudi (P < 0.008) compared with controls. DT2219 has broader reactivity in recognizing B-cell malignancies, has more killing power, and requires less toxin than using individual immunotoxin, which warrants further investigation as a new drug for treating B leukemia/lymphoma.

Immunotoxins are synthesized by coupling an antibody to a potent, mostly catalytic toxin, capable of inhibiting protein synthesis such as diphtheria toxin (DT; ref. 1). For CD19 targeting, investigators using conventional biochemically linked anti-CD19 immunotoxins have reported anticancer effects (2–6). However, these agents have not reached the mainstream as antileukemia/lymphoma drugs mostly because of varied degrees of effectiveness. This prompted others to clone recombinant anti-CD19 immunotoxins that are genetically modifiable so that important problems with these immunotoxins such as potency, clearance, toxicity, and generation of human antimurine antibody or human antimouse antibody, might be addressed (7). Unfortunately, recombinant anti-CD19 immunotoxins containing a single sFv were less effective than anti-CD19 immunotoxins made with conventional antibodies (7, 8). Studies have related this observation to the lack of the Fc region in these constructs and showed the importance of components of the Fc domain in conventional anti-CD19 immunotoxins by mutating it (9).

The anti-CD22 immunotoxin, on the other hand, has done more favorably and has been used successfully to treat rare hairy cell leukemia (10). However, these represent a narrow sampling of patients with leukemia and expanding the use of the drug to the wider population of patients is critical. One possibility would be to combine anti-CD19 and anti-CD22 therapy. In fact, investigators have shown that a mixture of an anti-CD19 immunotoxin and an anti-CD22 immunotoxin delivers a broader antileukemia effect (11, 12) than the individual immunotoxin. However, there are disadvantages. For example, giving patients more immunotoxins will enhance toxin-related side effects. Thus, we set out to develop a new recombinant bispecific antibody-targeted toxin with wider reactivity against B-cell leukemias and lymphomas that could be delivered as a single molecule. The molecule would contain the toxin as well as sFv directed against both CD19 and CD22.

The 95-kDa CD19 membrane glycoprotein is considered by many to be the most ubiquitous marker expressed on B cells. CD19 is expressed not only on mature B cells but also on late pre-B cells. It is broadly expressed on B cell leukemia/lymphoma (13, 14) including B-lineage lymphoblastic leukemia, which is the most common form of childhood leukemia. Experiments with CD19-deficient mice and mice overexpressing the CD19 antigen have shown its major role for CD19 in the development of B cells as well as their activation, proliferation, and differentiation (15–17). Among the identified members of the CD19 signal transduction complex, CD19 is uniquely responsible for the induction of calcium mobilization (18).
CD22 is a 135-kDa B lymphocyte–specific glycoprotein and a member of the sialoadhesin family of molecules (19–21). It first appears at the late pro–B cell stage of differentiation as a key regulatory cytoplasmic protein and is expressed simultaneously with immunoglobulin D as a surface membrane receptor on most mature B cells (20). It is expressed in 60% to 70% of B-cell lymphomas and leukemias. The principal function of CD22 is to regulate B-cell responses, which is likely accomplished by recruiting key signaling molecules to the antigen receptor complex (22, 23). Experiments in knockout mice have confirmed the importance of CD22 in modulating B-cell responses and show augmented antibody responses, expanded peritoneal B-1 cell populations, and increased levels of circulating autoantibodies (24–26).

The high level of expression of CD19 and CD22 on B-cell malignancies have made them both attractive targets for immunotoxins. DT is a good choice for immunotoxin production because it has first order Michaelis-Menten kinetics and a single molecule in the cytosol is sufficient for cell killing (27). Intact DT contains two fragments, A and B. The A fragment catalyzes the ADP ribosylation of elongation factor 2 (EF-2) leading to protein synthesis inhibition and cell death (28, 29). Fragment B contains the native binding domain for all human cells and must be replaced by an appropriate ligand (anti-CD19/anti-CD22) to render it a cell-specific immunotoxin. DT was chosen because a series of internal frame deletions that established 389 as the optimum site for genetic fusion of DT and target ligands were used in these studies (30).

In this study, we have found for the first time that a bispecific immunotoxin made with both anti-CD22 sFv and anti-CD19 sFv (called DT2219) has more killing power and broader reactivity than individual immunotoxin. DT2219 had greater anticancer activity than monomeric or bivalent immunotoxins made with anti-CD19 and anti-CD22 sFv alone. If the absence of Fc has diminished the activity of the anti-CD19 moiety of the DT2219 immunotoxin, then it seems that the addition of the anti-CD22 sFv has redeemed it because DT2219 delivers significant anticancer effects against CD19+CD22+ Daudi cancer cells in a nude mouse flank tumor model and in a systemic severe combined immunodeficient (SCID) cancer model. In addition, the fact that this agent was genetically modifiable permitted us to perform additional modifications which improved yield, purity, and activity indicating that DT2219 may serve as a new and useful agent for treating B-cell malignancies.

Materials and Methods

Construction of DT2219. DNA shuffling and DNA ligation were used to synthesize and assemble a hybrid gene encoding a single-chain bispecific immunotoxin. From the 5′ end to the 3′ end, the assembled gene (shown in Fig. 1) consisted of an Ncol restriction site, an ATG initiation codon, the first 389 amino acids of the DT (DT390), the VH and VL regions of anti-CD22 (sFv) and anti-CD19 (31) linked by a 20-amino-acid segment of human muscle aldolase (hma), and a Xho1 compatible restriction site. The resulting 2,760-bp Ncol/Xho1 fragment hybrid gene was spliced into the pHET21d expression vector. The hma fragment was used as a nonimmunogenic linker to connect the two sFvs and was used to enhance the level of protein production and ultimately the level of purity of the molecule. HMA is 363 amino acids in length and we used the final 20 amino acids (PSQAGAAAA-SESFLFSNHAY). DNA sequencing analysis (Advanced Genetic Analysis Center, University of Minnesota) was used to verify that the gene had been cloned in frame and was correct in sequence. Other genes were assembled using the same DT390 cassette spliced to a single anti-CD19 sFv (DTCD19), DT390 bivalent anti-CD19 (DTCD19CD19), DT390 spliced to a single anti-CD22 sFv (DTCD22), and DT390 bivalent anti-CD22 (DTCD22CD22).

As specificity controls for these studies, we constructed a bivalent fusion protein consisting of DT390 fused to two repeating sFvs recognizing human CD3 epsilon called Bic3 and the same DT390 linked to one sFv (Mo3; ref. 32). Anti-CD3 ε recognizes a domain of the T-cell receptor (33). Also used were monovalent DT390 anti-ErbB2sFv (DTe23) and a bivalent immunotoxin consisting of DT390, spliced to two repeating molecules of anti-ErbB2sFv (DTe23e23). Anti-ErbB2sFv has been used by others to synthesize recombinant immunotoxins (34, 35). ErbB2 is a tumor-associated antigen belonging to the epidermal growth factor receptor family and implicated in poor prognosis and more aggressive course of many human cancers including breast, lung, ovary, and stomach (36).

Inclusion body isolation. Plasmid was transformed into the Escherichia coli strain BL21(DE3) (Novagen, Madison, WI). Bacteria were grown in 600 mL Luria broth supplemented with 100 μg/mL carbenicillin in a 2-liter flask at 37°C with shaking. Expression of the hybrid gene was induced by the addition of isopropyl-β-D-thiogalactopyranoside (FisherBiotech, Fair Lawn, NJ). 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% glicerin, 50 mmol/L Tris, 50 mmol/L NaCl, 5 mmol/l EDTA (pH 8.0) and washed.

Refolding and purification. The proteins were refolded using a sodium N-lauroyl-sarcosine (SLS) air oxidation method modified from a previously reported procedure for isolating sFv (37). Isolation bodies were dissolved at 20.1 mg wet weight/mL in 100 mmol/L Tris (pH 10) and 2.5% SLS (Sigma, St Louis, MO). Pellets were removed by centrifugation. CuSO4 (50 mmol/L) was added to the solution and then incubated at room temperature with rapid stirring for 20 hours for air oxidation of ~SH groups. Removal of SLS was done by adding 6 mol/L urea and 10% AG 1-X8 (200–400 mesh, chloride form; Bio-Rad Laboratories, Hercules, CA) to the detergent-solubilized protein solution. (AG 1-X8 was removed using Whatman 1 filter paper). Then 7 mol/L guanidine-HCl were added to the sample. After incubation of the solution at 37°C for 1 hour, the solution was diluted 20-fold with refolding buffer and incubated at 4°C for 2 days. Refolding buffer consisted of 50 mmol/L Tris-HCl, 50 mmol/L NaCl, 0.8 mol/L l-arginine, 20% glycerin, 5 mmol/l EDTA (pH 8.0). Refolded DT2219 was purified by fast protein liquid chromatography ion exchange chromatography (Q Sepharose Fast Flow, Sigma) using a continuous
gradient from 0.2 to 0.5 mol/L NaCl in 20 mmol/L Tris-HCl (pH 9.0) over four column volumes.

Monoclonal antibodies and biochemical immunotoxins. The anti-CD19 monoclonal antibody hybridoma HD37 that secretes mouse IgG1 has been previously described by Dorken et al. (38) and has been studied as a targeted toxin conjugated to ricin toxin A chain (3). RFB4 (anti-CD22) was provided by Dr. Ellen Vitetta (University of Texas Southwestern Medical Center, Dallas, TX). Anti-ly5.2, 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.

ADP ribosylation assay. Duplicate samples of intact DT, DT390, or DT2219 were examined for their ADP ribosyl transferase activity as previously described (39). Briefly, ADP ribosylation was carried out in 50-μL reaction mixtures containing 10 μL of 0.01 mol/L Tris-HCl buffer with 1.0 mmol/L DTT, 0.005 mol/L EDTA (pH 8.0), 10 μL of rabbit reticulocyte lysate (containing EF-2), 1 μL (106 cpm/reaction) [3H]NAD+ (Perkin-Elmer Life Sciences, Boston, MA), and 19 μL H2O. The reaction was initiated by the addition of 10 μL of immunotoxin diluted in 10 mmol/L Tris-HCl and 0.1% bovine serum albumin. Reaction mixtures were incubated at room temperature for 1 hour and the reaction was stopped by the addition of 1 ml of 5% trichloroacetic acid. The precipitate was collected by centrifugation and washed with 1 mL of 10% trichloroacetic acid. The radioactivity was counted by standard scintillation techniques.

Bioassay to measure proliferation. To determine the effect of DT2219 on normal B and malignant B-cell function, the Daudi CD19−CD22+ Burkitt's lymphoma cell line was used. Flow cytometry shows that Daudi is >98% positive for both CD19 expression and CD22 expression (data not shown). Cells (104) were plated in a 96-well flat-bottomed plate in RPMI 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, 10% CO2 for 72 hours. [Methyl-3H]-thymidine (1 μCi per well) for 8 hours was added during the last 8 hours of culture. Cells were harvested onto glass fiber filters, washed, dried, and counted using standard scintillation methods.

Flow cytometry studies. To assess binding, the Daudi cell line or the CD19−CD22+ Raji B cell line were incubated with recombinant FITC-labeled proteins at saturating concentrations for 30 minutes at 4°C. For studies involving patient cells, bone marrow samples were taken from two patients diagnosed with B lineage lymphoblastic leukemia under informed consent. Cells were reacted with DT2219-FITC and then stained with PE-labeled proteins at saturating concentrations for 30 minutes at 4°C. Flow cytometry studies. Monoclonal antibodies and biochemical immunotoxins.

Results

Construction and purification of DT2219. After digestion, the final DT2219 gene was cloned into the pET21d plasmid under the control of the isopropyl-β-d-thio-β-d-galactopyranoside–inducible T7 promoter to create the pDThma2219EA-pET21d plasmid (Fig. 1). This final construction included the hma fragment and the "hotspot" mutation in the anti-CD22sFc. DNA sequencing verified that the DT2219 gene had been cloned in frame. DNA sequencing analysis was done by the University of Minnesota Microchemical Facilities (University of Minnesota, Minneapolis, MN).

Cytotoxicity of various immunotoxins on the Daudi cancer cell line. Daudi was selected as a target cell line in these studies because our flow cytometry studies showed it was >95% positive for both CD19 and CD22 (data not shown). To determine the ability of individual anti-CD19 and anti-CD22 immunotoxins to kill Daudi, DTCD19, DTCD19CD19, DTCD22, and DTCD22CD22 were tested in a proliferation assay and a representative experiment is shown (Fig. 2A). The monovalent anti-CD19 immunotoxin, DTCD19, did not kill Daudi. Only the bivalent form DTCD19CD19 killed Daudi. However, the bivalent immunotoxin was not very potent with an IC50 of 32 nmol/L. When DTCD22 was tested, it killed in a similar manner as DTCD19CD19 with a similar IC50. The DTCD22CD22 immunotoxin was not cytotoxic. Based on their marginal level of activity, none of these immunotoxins would be considered suitable for clinical development. Thus, a new immunotoxin was synthesized consisting of DT390 spliced to the same anti-CD22 sFv, but in this instance, a bispecific immunotoxin was cloned with the downstream addition of the anti-CD19 sFv. This first construct did not include the hma fragment. Figure 2B shows a separate experiment comparing DT2219 to DTCD22. The IC50 of DT2219 was improved by a log to 2 nmol/L. An immunotoxin Mo3 recognizing CD3 was included as a nonspecific control and did not inhibit. Together, these data showed that this new DT2219 configuration improved the potency of the immunotoxin against CD19*CD22+ Daudi and that cytotoxicity was selective.

Binding of the various immunotoxins to Raji. To determine whether the cytotoxicity data related to the ability of the various immunotoxins to bind their target, the recombinant immunotoxins were labeled with FITC and tested for Daudi binding using flow cytometry. Although DTCD19 and DTCD22CD22 tested at the same molar saturating concentration both bound Daudi, DTCD19 bound better (Fig. 2C). The best binding was obtained with DT2219. The high level of binding shown for DT2219 correlated with its high level of cytotoxicity. DTCD22 and DTCD19CD19 also bound Daudi but not as well as DT2219 (data not shown).
The addition of a human muscle aldolase fragment enhances yield and purity. Despite its improved activity, refolding and purification of DT2219 by FPLC were major problems. The SDS-PAGE gel analysis shown in Fig. 3 indicated the marked difference between ion exchange column purified, unmodified DT2219, and DT2219hma. Unmodified DT2219 showed a very low yield from the column (0.05 mg/L) and low purity (10%) determined by densitometry. The problem was addressed by incorporating a fragment of hma into the construct. DT2219hma had a much better yield (1.1 mg/L), an improvement of 20-fold. There was also a higher degree of purity (80%). Unmodified DT2219 and DT2219hma had nearly identical activity curves when assayed against Daudi cells (IC\textsubscript{50} = 2.9 and 3.3 nmol/L, respectively) indicating that the introduction of hma had no effect on protein activity. However, the introduction of this modification permitted us to produce DT2219 in scale-up quantities sufficient for animal studies.

Fig. 2. A, effect of anti-CD19 and anti-CD22 IT on the CD19\textsuperscript{+}CD22\textsuperscript{+} Daudi cell line. Cells were cultured with fusion proteins and proliferation was measured by uptake of tritiated thymidine. Percentage of control response where control response is untreated cells. Points, mean; bars, ± SD. Mean values of untreated Daudi cells were 61,179 ± 3,174. The DTCD22 and DTCD19hma curves differed significantly from the other curves at 100 nmol/L by Student’s t test (P < 0.001). B, effect of DT2219 on Daudi was determined. Mean cpm of untreated Daudi in this experiment was 63,962 ± 2,382. The DTCD2219 differed significantly from the negative control Mo3 group at 10 and 100 nmol/L by Student’s t test (P < 0.001). C, FITC-labeled recombinant IT were reacted with CD19\textsuperscript{+}CD22\textsuperscript{+} Raji cells and binding was measured by flow cytometry. Histograms were plotted to represent increasing cell number versus increasing intensity of binding. 3A1e control (solid light gray, left), DTCD19 (heavy black line) DTCD22CD22 (light dotted line), and DT2219 (solid gray).

Fig. 3. A comparison of antiproliferative activity of unmodified low yield DT2219 and the modified high yield DT2219hma on Daudi. Comparative proliferation data of DT2219 and DT2219hma (A) and comparative purities (B). A, cells were cultured with either agent at various concentrations and then tritiated thymidine was used to measure cell proliferation. Points, mean of triplicate determinations; bars, ± SD. The two groups did not differ significantly as determined by Student’s t test. The comparative purity and yield (mg/L) shown on the table was derived from densitometry measurements of (B). B, SDS-PAGE analysis of unmodified DT2219 and DT2219hma was also performed. Gel was stained with Coomassie blue. Lane 1, unmodified DT2219, a 95-kDa protein that was 10% pure eluted from the FFO-FPLC ion exchange column following refolding. Lane 2, DT2219hma treated identically. DT2219hma, also 95 kDa, was 80% pure. Lane 3, molecular weight standards. Lanes 4 and 5, same as lanes 1 and 2 only run under reducing conditions. Each lane contains ~5 μg protein.
Mutating DT2219 to enhance avidity. To enhance binding avidity and improve toxicity, three amino acids in the anti-CD22 VH heavy region were mutated as previously reported by Salvadore et al. (40). To determine whether mutation had made the agent more potent, we did four experiments and then reported the average IC50. Figure 4 (inset) shows that the average IC50 of the CDR3-mutated DT2219 (DT2219) was $0.3 \pm 0.1$ nmol/L. In comparison, the IC50 of nonmutated DT2219 averaged $3.1 \pm 1.1$ nmol/L. This IC50 represented a significant gain of at least a log in activity ($P < 0.001$) and proved that the anti-CD22 sFv is involved in DT2219 binding. Also shown in the figure is the mean dose response curve averaged from the four experiments for DT2219. Points, mean percent control; bars, $\pm$SD.

Fig. 4. The effect of mutagenizing the CDR3 region of CD22 sFv in the DT2219 construct. Daudi cells were cultured with either CDR3-mutated (DT2219) or nonmutated DT2219 and proliferation was measured by uptake of tritiated thymidine. The IC50 was plotted from each dose response curve. IC50 values plotted from a mean of four experiments $\pm$ SD of the mean in the inset. The two sets of means were compared by Student’s t test ($P < 0.0013$). Mean cpm values of untreated control Daudi cells was $92,007 \pm 10,618$. The graph itself shows the mean dose response curve averaged from the four experiments for DT2219.

To confirm that the anti-CD19 sFv and anti-CD22 sFv ligands were both still active in DT2219, blocking experiments were done. In Fig. 5A, DT2219 was labeled with FITC and blocked with the anti-CD19 antibody HD37. The histogram is shifted towards the left by addition of 150 μg/mL of antibody. However, the addition of an irrelevant control antibody anti-Ly5.2 has no effect. In Fig. 5B, 0.5 or 5 nmol/L anti-CD19 (HD37) or anti-CD22 (RFB4) antibodies were used to block the killing of CD19”CD22” Daudi cells by 10 nmol/L mutated DT2219 in a separate experiment. Both antibodies were capable of blocking some of the antiproliferative effect, but neither of the antibodies completely blocked. As little as 5 nmol/L of a combination of both antibodies almost completely blocked the killing of DT2219. The addition of anti- mouse Ly5.2 had no blocking effect (data not shown). In Fig. 5C, the binding activity of mutated DT2219 was best blocked by a CDR3-mutated 2219 fragment (identical to mutated DT2219 but did not include the DT390 moiety). A nonmutated 2219 fragment, also devoid of DT, blocked, but not as well. The fact that blocking was about a log less with the nonmutated fragment correlated well with the proliferation data in Fig. 4 which also indicated there was a log difference. The addition of control monovalent or bivalent e23 (anti-HER-2/neu) did not have any blocking activity. These data confirmed the higher avidity binding of mutated DT2219. Together, these findings indicated that both sFvs were active on the DT2219 molecule and that mutation caused DT2219 to bind better.

Fig. 5. The activity of mutated DT2219 is mediated by both anti-CD19 sFv and the anti-CD22 sFv ligands. A, mutated DT2219-FITC was reacted with Daudi and binding was measured by flow cytometry (solid gray). Another sample was treated identically and blocked with HD37 monoclonal antibody (150 μg/mL; solid black) or irrelevant anti-Ly5.2 (negative blocking control; heavy black line). 3A1 e-FITC was the irrelevant binding control (dotted line). B, proliferation studies were also performed in which Daudi cells were treated with mutated 10 nmol/L DT2219 and blocked with HD37 (gray), RFB4 (hatched), or a mixture of both antibodies (black). Thymidine uptake was then measured. Columns, mean of triplicate determinations; bars, $\pm$SD. Percent blocking was calculated in comparison with the unblocked control and graphed versus either 0.5 or 5 nmol/L blocking agent. C, a separate proliferation assay was performed, only this time cultures treated with mutated DT2219 were blocked with increasing concentrations of the mutated 2219 fragment (■), nonmutated 2219 fragment (▲), or e23 negative control fragment (● and ●). Counts for untreated Daudi cells were 97,700 $\pm$ 13,900.
Cancer Therapy: Preclinical

Enzymatic activity and in vitro cytotoxicity. Protein synthesis inhibition by DT is due to fragment A catalyzed ADP ribosylation of cytoplasmic EF-2. To determine whether CDR3 mutagenesis affected the enzymatic activity of the DT moiety of the mutated DT2219 molecule, a cell-free assay system was employed in which rabbit reticulocyte lysate, a source of EF-2, was exposed to DT2219, DT390, or intact DT in the presence of [32P]-NAD. Studies revealed a similar dose dependent increase in [32P] incorporation into the trichloroacetic acid–precipitable fraction of rabbit reticulocyte lysate (EF-2; Fig. 6) and a high level of activity. DT2219, DT390, or intact DT all had identical activities confirming that modification did not affect the enzymatic activity of the DT portion of the molecule.

Treatment of flank tumors with mutated DT2219. To determine whether DT2219 had any antitumor effect against established flank tumors, Daudi tumors were established via s.c. injection. Mice were then given i.p. injections of 20 μg per injection DT2219 four times (as stated in Materials and Methods) beginning day 12 after tumor injection. The mean tumor volume of DT2219-treated tumors was significantly (P < 0.05) less than the mean tumor volume of control Bic3-treated tumors or untreated control tumors (Fig. 7A). A second experiment was done to reproduce this one with similar results (data not shown). These studies indicate that DT2219 has activity against an established primary tumor mass.

Effects of DT2219 in severe combined immunodeficient mice with systemic cancer. One advantage of this model is that injection of the same cells i.v. into SCID mice results in a systemic tumor that infiltrates all major organs and is more reminiscent of human leukemia. To determine if DT2219 was effective against established systemic leukemia, SCID mice were injected i.v. with Daudi cells and treatments were started on day 3. Figure 7B shows that mice given nine i.p. injections of 20 μg DT2219 (as stated in Materials and Methods) survived significantly longer (P < 0.008) than control mice receiving the same injection schedule of an irrelevant control anti–T cell immunotoxin Bic3. Untreated controls showed a similar effect as Bic3 and there was no statistical difference between the Bic3 and untreated control groups. A second experiment indicated that the findings were reproducible (data not shown). These data indicate that DT2219 was able to significantly delay the onset of fatal systemic cancer in a highly aggressive SCID model.

Reactivity of DT2219 with patient leukemia samples. To determine if DT2219 was reactive with patient leukemia cells, the recombinant protein was labeled with FITC and then directly assayed on two patient samples by flow cytometry (Fig. 8). Patient one was an 11-year-old male and patient 2 was a 13-year-old female. Both patients had profiles consistent with a diagnosis of precursor B lymphocytic leukemia (precursor B lineage lymphoblastic leukemia) because >90% of all nucleated cells were blasts positive for HLA-DR, CD10, CD19, CD3, and TdT. Cytogenetics were also consistent with the diagnosis. The mutated, high affinity form of DT2219 was not used for these studies. The highest measurements of binding to patient cells were obtained when cells were reacted with DT2219-FITC compared with either anti–CD19-FITC or anti–CD22-FITC alone. In patient 1, the mean fluorescence channel, an indicator of binding intensity, was 425 for DT2219, 186 for anti-CD19, and 13 for anti-CD22. In patient 2, the mean fluorescence
channel was 557 for DT2219, 126 for anti-CD19, and 10 for anti-CD22. These data indicate that DT2219 is more reactive with patient leukemia cells.

**Discussion**

The major contribution of this article is the design and synthesis of a new recombinant bispecific immunotoxin with improved yield and with separate sFvs that recognize CD19 and CD22 on the surface of malignant and nonmalignant B cells. The major advantages of this new construct were (a) its broader reactivity with B-cell malignancies, (b) more binding and killing power using less toxin because both ligands were combined with a single toxin molecule on the same single-chain molecule, and (c) the fact that the addition of CD19 to the DTCD22 molecule enhanced the activity of the molecule greater than each individual component.

DT2219 had greater anticancer activity in proliferation assays than monomeric or bivalent immunotoxins made with anti-CD19 and anti-CD22 sFv alone. This correlated with a higher level of binding to patient leukemia cells. In proliferation studies with CD19'CD22' Daudi cells, a combination of the parental anti-CD19 and anti-CD22 were used to block the binding of DT2219. The combination was more effective at blocking the proliferation of Daudi than the individual anti-CD19 and anti-CD22 antibodies. In fact, Fig. 5B showed by adding 0.5 nmol/L of the combination, 35% blocking of DT2219 activity was achieved, whereas 14% and 8% were achieved with the individual antibodies. By adding 5 nmol/L of the combination, 90% blocking of DT2219 activity was achieved, whereas 41% and 20% were achieved with the individual antibodies. This indicated that the combination inhibited in more than an additive fashion. Because as little as 5 nmol/L of the combination could almost entirely block the activity of 10 nmol/L DT2219, this indicates that both sFvs are critical to the function of this molecule and strengthens the argument that dual antigen targeting is superior.

In its initial design, DT2219 activity was good, but yields and purity were very poor. The two modifications which solved this problem are (a) the use of a detergent based refolding system and (b) introduction of a fragment of hma. Others have cloned sFv-recognizing CD19 with limited success because attempts resulted in low specific avidity and in some cases, complete failure to bind (41). Usually, structural aberrations were blamed. In our hands, it was the refolding step that was mostly responsible for our failed sFv purification attempts.
sFvs are most commonly purified from inclusion bodies refolded by solubilizing them in guanidine or urea and then dialysing the denaturant in the presence of redox buffers (42). The process is inefficient resulting in a wide range of yields, some of them very low, and sFvs with impaired function. These hardships prompted us to investigate refolding as a potential limiting step in our production process. To address this problem with bispecific immunotoxins, we modified a procedure reported to optimize the refolding of sFv from inclusion bodies in E. coli. Previously, we reported these modifications (32) and the procedure is based on inclusion body solubilization in a weak detergent, SLS in the presence of metal ion catalysts which dramatically reduces the incidence of aggregate formation. Kurucz et al. (37) explain that the procedure results in high yields with affinities that are similar to native sFvs because the SLS allows correct disulfide pairing and prevents intermolecular aggregation, behaving as prototypic chaperone proteins that do assist in proper refolding of polypeptide chains off the ribosome in vivo. When used with the hma modification, this procedure resulted in profound improvements in yield and purity with at least two proteins we are seeking to develop for phase 1 studies (32).

Besides the use of detergent-based refolding, the addition of a portion of hma helped increase yields well beyond those obtained with constructs made without this sequence. The reason is not known, but shifts in the isoelectric point or a protein conformation that favors greater stabilization of disulfide bonding frequently improves the refolding and purification process in our hands. The use of the hma fragment and an SLS-based refolding procedure permitted high levels of expression of DT2219 in inclusion bodies, providing adequate quantities for scale-up at a higher level of purity. Although the exact contribution of hma insertion versus the SLS-based refolding procedure to the enhanced yield is undetermined, refolding of the new construct using the old glutathione-based redox procedure did not result in a good yield. Thus, both seem necessary to maximize the effect with DT2219.

In our study, monovalent immunotoxin made with a single anti-CD19 sFv or a bivalent immunotoxin made with two anti-CD19 subunits were either not very effective or not effective at all. This does not seem to relate to refolding because these immunotoxins bound to Daudi. Perhaps, this relates to internalization differences. Only the bispecific DT2219 was reproducibly cytotoxic with an IC_{50} of <1 nmol/L. As in other studies in which combinations of anti-CD19 and anti-CD22 immunotoxins have been effective (43–47), no mechanism for this observation is known. However, Daudi cells express similar densities of CD19 and CD22 (48) and we can speculate that more of the bispecific DT2219 might be concentrated on the cell surface resulting in higher levels of killing. Higher densities of immunotoxins may lead to greater internalization rates and a greater cytotoxic effect.

However, previous studies have shown that activity of anti-CD22 immunotoxin can be enhanced by anti-CD19 antibody alone (49). Investigators in these studies ruled out obvious explanations such as Fc-mediated ADCC, complement, and apoptotic mechanisms. Their studies prompted them to propose a novel type of death similar to that described for an anti-immunoglobulin M antibody (49) or perhaps a down-regulatory effect specific to DNA and not on RNA (50). Whatever the mechanism, our studies indicate that simultaneous triggering of CD19 and CD22 on the cell surface results in enhanced delivery of toxin into critical intracellular pathways that culminate in rapid toxin entry into the cytosol. It is clear from our studies that this can be accomplished with the two different sFvs and a toxin on the same single-chain molecule, a rather efficient means of drug delivery. A major advantage of this approach is that toxicity of immunotoxins is attributed to the toxin moiety of the hybrid molecule and a specific immunotoxin constructed in this manner permits us to target two receptors on the cell surface with a single molecule of toxin. We have no data to support this as yet, but perhaps less toxin may be needed in comparison to studies where CD19 and CD22 are targeted with two separate immunotoxins. Toxicity studies are in progress.

Several reports have addressed the use of anti–CD19-toxin conjugates (51). All of these were constructed with conventional monoclonal antibodies. Using anti-CD19 class switch variants which differed only in their Fc regions, investigators showed that it is the Fc region that plays a role in the internalization of anti-CD19 immunotoxin and it is the CD32 (Fc) receptor which is a likely candidate to be involved in the enhanced uptake of antibody (9). This is supported by studies with immunotoxins made from anti-CD19 fragments, Fab' and F(ab')₂ (8) which have reduced activity. In our hands, removal of the Fc also resulted in reduced immunotoxin activity. Somehow, the addition of the CD22 sFv to DTCD19 restores the ability of these immunotoxins to internalize and enter the intracellular compartment.

The animal studies of DT2219 were quite striking. First, Daudi tumor (expressing >95% CD19 and >95% CD22 as determined by flow cytometry) formed flank tumors on nude mice. High Daudi doses were used to expedite tumor onset and render the model more aggressive. The established tumor xenografts were inhibited by the i.p. administration of DT2219. To test the effect against systemic cancer, high Daudi doses were given i.v. to SCID/hu mice as in other studies where advanced leukemia caused hind limb paralysis indicating the malignant nature of the cancer. Animals showed a significant antileukemia effect when given DT2219. No animals were cured in our model, but results may be improved by reducing the Daudi dose or by altering the dose and dose schedule. Together, these studies indicated that DT2219 reduced established primary tumors and inhibited established systemic malignancy.

Altering a mutational hotspot sequence in the heavy-chain complementarity determining region 3 (V_{H1} CDR3) further improved the activity of DT2219. A hotspot alteration was employed that was previously reported by Salvatore et al. (40). Hotspots are DNA sequences that are frequently mutated during the in vivo affinity maturation of the antibody (51, 52). By focusing on these hotspots, it is necessary to make only small libraries of mutants to find mutations that markedly enhance affinity. This mutation added an extra log kill to our protein and shows that a greater understanding of protein conformation will result in improved performance of these recombinant proteins. The log gain observed in the proliferation studies correlated with the log gain measured in the blocking/binding studies indicating that this measurement was indeed accurate.

Immunotoxins made with anti-CD22 sFv have been very effective in other studies (10), but mostly have been limited to...
the therapy of extremely rare leukemias such as hairy cell leukemia. In this application, they seem more effective against drug refractory hairy cell leukemia in clinical studies than any other agent tested to date. Because of these findings and the findings shown in this study, it is probable that the addition of the anti-CD19 sFv to a DTCD22 single-chain molecule will broaden the reactivity of this agent against most lymphomas and B-cell leukemia, the most common leukemia occurring in children.

In summary, a novel bispecific single-chain fusion protein, incorporating two repeating sFv subunits recognizing CD19 and CD22 was synthesized which heightened yield and improved efficacy of kill. Interestingly, the presence of CD22 sFv somehow promotes internalization and activity of the chimeric protein. The new agent proved extremely effective in animal models and even if it did not prove curative, its superior activity may render it valuable for maintenance therapy in clinical situations where chemotherapy is ineffectual or the all too common situation where chemotherapy must be discontinued because its overuse has damaged the hematopoietic system. Further studies of this unique bispecific immunotoxin are warranted.

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References


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A Bispecific Recombinant Immunotoxin, DT2219, Targeting Human CD19 and CD22 Receptors in a Mouse Xenograft Model of B-Cell Leukemia/Lymphoma

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