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Improved Cytotoxic Activity toward Cell Lines and Fresh Leukemia Cells of a Mutant Anti-CD22 Immunotoxin Obtained by Antibody Phage Display

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Abstract

Recombinant immunotoxins are fusion proteins composed of the Fv domains of antibodies fused to bacterial or plant toxins that are being developed for the targeted therapy of cancer. RFB4 (Fv)-*Pseudomonas* exotoxin 38 (PE38) is an immunotoxin that targets CD22 expressed on B cells and B-cell malignancies. A disulfide-stabilized form of RFB4 (Fv)-PE38 is being evaluated in a Phase I clinical trial. The aim of the present study was to improve the activity of RFB4 (Fv)-PE38 to more effectively treat patients with leukemias and lymphomas. To increase the affinity of RFB4 (Fv), we used the techniques of phage display and hot spot mutagenesis. We identified mutational hot spot sequences in heavy chain complementary determining region 3 (V_H CDR3) and randomized these in a phage display library. Mutant phages were panned on CD22-positive Daudi cells. A variety of mutant Fvs were obtained, and the corresponding immunotoxins were prepared. Several mutant immunotoxins with increased binding affinity and cytotoxic activity were obtained. The most active immunotoxin contained amino acid residues Thr-His-Trp (THW) in place of Ser-Ser-Tyr (SSY) at positions 100, 100A, and 100B of the Fv and had an affinity improved from 85 nM to 6 nM. The THW mutant had a 5- to 10-fold increase in activity on various CD22-positive cell lines and was up to 50 times more cytotoxic to cells from patients with chronic lymphocytic leukemia and hairy-cell leukemia.

Introduction

Hematological malignancies are a major public health problem. It has been estimated that in the year 2000, 54,900 new cases of non-Hodgkin's lymphoma and 30,800 new cases of leukemia will have occurred in the United States (1). The expected deaths from these diseases are 26,100 and 21,700, respectively. Many more patients live with chronic disease-related morbidity. Thus, conventional therapies are not able to induce long-term complete remissions in a high percentage of patients.

In the past several years, immunotoxins have been developed as an alternative therapeutic approach to treat these malignancies. Immunotoxins were originally composed of an antibody chemically conjugated to a plant or a bacterial toxin. The antibody binds to the antigen expressed on the target cell, and the toxin is internalized and causes cell death by arresting protein synthesis and inducing apoptosis (2). Hematological malignancies are an attractive target for immunotoxin therapies because tumor cells are easily accessible, and the target antigens are highly expressed (3). One of these antigens is CD25. A clinical trial with immunotoxin LMB-2 [anti-Tac(Fv)-PE38],[2] which targets CD25, showed that the agent was well tolerated at 40 μg/kg given every other day for three doses, and that it had substantial antitumor activity (4, 5). A complete response was observed in one patient with HCL, and partial responses were observed in patients with HCL, CLL, cutaneous T-cell lymphoma, Hodgkin's disease, and adult T-cell leukemia (4, 5). Another antigen that has been used as an immunotoxin target is CD22, a lineage-restricted B-cell antigen expressed in 60–70% of B-cell lymphomas and leukemias. CD22 is not present on the cell surface in the early stages of B-cell development and is not expressed on stem cells (6). Clinical trials have been conducted with an immunotoxin containing an anti-CD22 antibody, RFB4, or its Fab fragment, coupled with deglycosylated ricin A. In these trials, substantial clinical responses have been observed; however, severe, and in certain cases fatal, vascular leak syndrome was dose limiting (7–9).

As an alternative approach, the RFB4 antibody has been used to make a RIT in which the Fv fragment in a single-chain form is fused to a truncated form of *Pseudomonas* exotoxin A (MR = 38,000; PE38; Ref. 2). PE38 contains the translocating and ADP ribosylating domains of *Pseudomonas* exotoxin but not the cell-binding portion (10). RFB4 (Fv)-PE38 is cytotoxic toward CD22-positive cells and has a K_d of 50 nM, measured by a displacement assay (11). To stabilize the scFv immunotoxin and to make it more suitable for clinical development, cysteine residues were engineered into framework regions of the V_H and V_L (12) generating the molecule RFB4 (dsFv)-PE38. RFB4

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[2] The abbreviations used are: PE38, *Pseudomonas* exotoxin 38; HCL, hairy-cell leukemia; dsFv, disulfide stabilized Fv; DPBS, Dulbecco's phosphate buffered saline; scFv, single-chain Fv; CLL, chronic lymphocytic leukemia; RIT, recombinant immunotoxin; CDR3, complementary determining region 3; WT, wild type.

(dsFv)-PE38 is able to kill leukemic cells from patients and induced complete remissions in mice bearing lymphoma xenografts (13, 14). RFB4 (dsFv)-PE38 (BL22) is currently being evaluated in a Phase I clinical trial at the National Cancer Institute in patients with hematological malignancies. Sixteen patients with purine analogue-resistant HCL were treated with BL22 and 11 (86%) have achieved complete remissions (15). BL22 is the first agent that is able to induce high complete remissions rate in patients with purine analogue-resistant HCL and establishes the concept that immunotoxins can produce clinical benefit to patients with advanced malignancies. Because of the clinical benefits obtained with BL22, we decided to improve this molecule by increasing its affinity and consequently its activity. This should lead to an increase in its activity in patients with malignancies such as CLL, in which the cells have relatively small amounts of CD22, and also to decrease the amount that needs to be given. To increase the affinity of RFB4, we have used the technique of phage display, which has been successfully used to increase the affinity of other Fvs. We have mutagenized residues in V_H CDR3 because this region is important for antigen binding. In addition, we targeted only those residues in CDR3 that contained hot spots. Hot spots are DNA sequences that are frequently mutated during the *in vivo* affinity maturation of an antibody (16, 17). By targeting hot spots, it is necessary to make only a relatively small library of mutants to find mutations that result in Fvs with increased affinity. In the current study, several mutant immunotoxins were obtained that had an increased affinity for CD22 and an increased cytotoxic activity toward leukemic cell lines and leukemic cells from patients.

Materials and Methods

Construction of the Library. The V_H CDR3 of RFB4 consists of 14 amino acids. DNA oligomers were designed to generate a library randomizing 12 nucleotides (4 consecutive amino acids). Degenerate oligomers with the sequence NNS were used (N randomizing with all four nucleotides, S introducing only C or G).

An RFB4 phagemid was constructed by using the PCR to amplify RFB4 Fv from the plasmid pEM10 [RFB4 (scFv)-PE38KDEL]. The following oligomers, which introduced *Sfi*I and *Not*I restriction sites, were used: RFB4S 5′-TTCTATGCGGCCCAGCCGCCATGGCCGAAGTGCAGCTGGTGGAGTCT-3′ and RFB4N 5′-CGGCACCGGCGCACCTGCGGCCGCCCGTTGATTTCCAGCTTGGTGCC-3′. The PCR product was digested with *Sfi*I and *Not*I and inserted into the vector pCANTAB5E (Pharmacia). The phagemid pCANTAB5E-RFB4 was modified by inserting a stop codon (TAA) at position 99 (GGT) using site-directed mutagenesis (Quick Change site-directed mutagenesis kit; Stratagene). The resulting phagemid pCANTAB5E-RFB4–1 was used as a template to introduce four amino acid randomizations in the CDR3 heavy chain in a two-step PCR reaction. The following oligonucleotides were used: S1–2 5′-CAACGTGAAAAAATTAATTATTCGC-3′; RMUT 5′-AGCAAACAAACCCCSNNSNNSNNSNNGTAGCCACTATGTCT-3′; AMBN 5′-GCTAAACAACTTTCAACAGTCTATGCGGGCAC-3′. In the first PCR, 50 pg of the phagemid pCANTAB5E-RFB4–1 was used as the template in a reaction using 20 pmol of DNA oligomers S1–2 along with 20 pmol of the DNA oligomers RMUT. The template and oligonucleotides were mixed with two Ready-To-Go PCR beads (Pharmacia) in a 50-μl volume and then cycled using the following profile: 1 cycle at 95°C for 5 min, followed by 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. This reaction generated 402-bp product that contained the mutations. The product was purified using a Qiagen Quick Spin column and then quantitated by visualization on a 1% agarose gel. The purified product generated in the first PCR was used as primers in a second PCR. In this reaction, ~2 pmol of the product from the first reaction was used with 20 pmol of the DNA oligomer AMBN with 50 pg of phagemid pCANTAB5E-RFB4–1 as template. The primers and template were mixed with two PCR beads in a 50-μl volume and cycled using the profile described above. The reaction generated an 884-bp insert library. The PCR product was digested with *Sfi*I and *Not*I and purified using a Qia quick column (Qiagen). Purified PCR product (150 ng) was ligated with 250 ng of the phage display vector pCANTAB5E and desalted. Forty ng of the ligation was used to transform *Escherichia coli* TG1. Ten transformations were performed to give a library containing 8×10^5 clones. The phage library was rescued from the transformed bacteria, as described previously (18), titered, and stored at 4°C.

Panning on Cells. Panning was performed on Daudi cells. Cells (2×10^7) were pelleted, resuspended in 10 ml of cold blocking buffer (DPBS + 0.5% BSA + 5 mM EDTA) and rotated slowly for 90 min at 4°C. The cells were then pelleted and resuspended in 1 ml of cold blocking buffer. Phage (1×10^{12}) from the library were added to the cell suspension and the mixture was rotated slowly at 4°C for 90 min. The cells were washed five times with 10 ml of cold blocking buffer. Bound phage were eluted by resuspending the washed cells in 1.5 ml of ice-cold 50 mM HCL and incubating on ice for 10 min. Daudi cells were pelleted and the eluted phages were transferred to a tube containing 200 μl of 1 M Tris (pH 8). The eluted phages were titered to determine the number of phage captured. The eluted phage (1.5 ml) were then amplified by reinfecting *E. coli* TG1 for use in the next round of panning.

Analysis of Selected Clones. Binding of phage obtained after the second round of panning was determined by flow cytometric analysis. Phage were prepared from single colonies of *E. coli* TG1 containing phagemids selected in the second round of panning as described previously (18). Equal amounts of phage (8×10^8) were used to study binding to Daudi cells using flow cytometry.

Flow cytometry was carried out as follows. Five $\times 10^5$ Daudi cells were incubated with 8×10^8 phage at room temperature for 60 min, cells were washed two times with blocking buffer, and 5 μg of mouse anti-M13 antibody (Amersham) were added to each sample. The mixture was incubated at room temperature for 20 min, then washed two times with blocking buffer. A goat-antimouse-FITC-labeled antibody (Jackson ImmunoResearch) was added, and cells were incubated for 20 min at room temperature. Cells were washed two times, and analysis was performed in a FACSort flow cytometer (Becton Dickinson). Data were acquired using Cell Quest software. For the competition experiment, 5×10^6 cells were incubated with 8×10^{10} WT RFB4 scFv phage and with 63 μg of RFB4 immuno-

Table 1 DNA and amino acids sequences of CDR3 heavy chain RFB4

The nucleotide and amino acid sequence of the entire CDR3 heavy chain is shown. Hot spots with the sequence Pu-G-Py-A/T are underlined. The amino acids mutagenized are in bold. The residues are numbered according to the Kabat database (21).

95	96	97	98	99	100	100A	100B	100C	100D	100E	100F	101	102
H	S	G	Y	**G**	**S**	**S**	**Y**	G	V	L	F	A	Y
CAT	AGT	GGC	TAC	GGT	AGT	AGC	TAC	GGG	GTT	TTG	TTT	GCT	TAC

toxin (100-fold excess), and then the sample was processed as cells incubated only with the phage.

DNA Sequencing. DNA sequencing was performed using PE Applied Biosystems Big Dye Terminator Cycle Sequencing kit. The samples were run and analyzed on a PE Applied Biosystem Model 310 automated sequencer.

Construction and Expression of Immunotoxin. ScFvs from selected phagemids were PCR-amplified using primers that introduced *Nde*I and *Hin*dIII restrictions sites. The products of the reaction were purified, digested with *Nde*I and *Hin*dIII, and cloned into a T7 expression vector in which the scFv was fused to a truncated version of *Pseudomonas* exotoxin A (PE38; 2). The expression and purification of RITs was performed as described previously (18).

Cytotoxicity Assays. Cytotoxicity on cell lines was measured by protein synthesis inhibition assays. Cells were plated in 96-well plates at a concentration of 5×10^4 cells/well. Immunotoxins were serially diluted in PBS/0.2% human serum albumin and 20 μl of the resulting mixture were added to each well. Plates were incubated for 20 h at 37°C and then pulsed with 1 μCi/well [^{3}H]leucine in 20 μl ofPBS/0.2% human serum albumin for 2.5 h at 37°C. Radiolabeled material was captured on filter-mats and counted in a Betaplate scintillation counter (Pharmacia, Gaithersburg, MD). Triplicate sample values were averaged and the inhibition of protein synthesis was determined by calculating percentage of incorporation compared with control wells without added toxin. The activity of the molecule is defined by IC_{50}, *i.e.*, the toxin concentration that reduced incorporation of radioactivity by 50% compared with the cells that were not treated with the toxin.

For patient experiments, blood was collected from patients as part of approved clinical protocols at the NIH. Patients 1, 2, 3, and 5 had CLL, and patient 4 had HCL. Samples were then processed as described previously (13).

Preparation and Purification of CD22 Extracellular Domain. The extracellular domain of CD22 protein was expressed as a fusion to human IgG Fc in transfected 293T cells. The human Fc fragment was amplified using PCR from plasmid Ret-Fc (provided by M. Billaud, Laboratoire de Genetique, Lyon, France) with primers FCCOD: 5′-GAGTGAGTGCGGCCGCGG TGGTCGTCGTGCATCCGT-3′ and FCNON:5′ TCACTCACTCTAGACGGCCGTCGCACTCATTTAC-3′ introducing 5′ *Not*I and 3′ *Xba*I restriction sites. After digestion with *Not*I and *Xba*I, the PCR product was purified and cloned into the multiple cloning site of vector pCDNA1.1 between *Not*I and *Xba*I sites creating plasmid pCDNA1.1-Fc. The extracellular portion of CD22 was cloned into pCDNA1.1-Fc creating an inframe fusion with the Fc. CD22 was amplified from plasmid pRKm22 using the following oligomers: 22COD 5′GTGAGTGAGAATTCATGCATCTCCTCGGCCCCTG-3′ and 33NON 5′TCACTCACTCGCGGCCGCTTCGCCTGCCGATGGTCTC-3′. pRKm22 is a plasmid encoding full-length human CD22β obtained by cloning from a Daudi cDNA Quick clone library (Clontech). The oligomers introduced *Eco*RI and *Not*I restriction sites. After digestion with *Not*I and *Eco*RI, the PCR product was purified and cloned into vector pCDNA1.1-Fc between *Not*I and *Eco*RI sites creating plasmid pCDNA1.1–22-Fc.

The 293T cells were transfected with plasmid pCDNA1.1–22-Fc by standard $CaPO_4$ precipitation.

Surface Plasmon Resonance. Binding kinetics of the RITs were measured using BIAcore 2000 Biosensor. CD22-Fc protein was diluted to 50 μg/ml in amine coupling buffer (BIAcore, Uppsala, Sweden) and immobilized to a BIAcore sensor chip CM5. RITs were diluted to 25 μg/ml in HEPES-buffered saline. On-and-off rates were measured by injecting 50 μg of immunotoxins over the chip surface at 10 μl/min, and then allowing the bound material to dissociate for 5 min or more. The remaining bound material was removed from CD22 protein by injecting 10 μl of 20 mM phosphoric acid. Each immunotoxin was injected and analyzed at least three times. Binding kinetics were determined using BIA evaluation 2.1 software.

Cell Culture. CA46, JD38, Daudi, Raji, Namalwa, Ramos, and 293T cells were obtained from American Type Culture Collection (Manassas, VA). HUT-102 cells were a gift from T. Waldmann (NIH, Bethesda, MD). Cells were grown as described previously (12).

Results

Construction and Panning of the Library. To increase the affinity of RFB4 (Fv)-PE38, we mutated CDR3 of the heavy chain because V_H CDR3 usually makes significant contacts with antigen (19). The amino acid sequence of CDR3 is shown in Table 1; it contains 14 residues. All of the residues were not randomized together because that would have required a library with 1.6×10^{18} clones. Instead, we targeted only mutational hot spots (16, 18). The hot spot residues in V_H CDR3 are S96, G97, Y98, G99, S100, S100A, Y100B, G100C, and V100D (Table 1). We decided to mutate first G99 (GGT), S100 (AGT), S100A (AGC), and Y100B (TAC). To mutate these four residues randomly, it was only necessary to produce a library of 1.6×10^5 clones. The strategy to create the mutant library and its insertion into the pCANTAB5E vector is described in "Materials and

Methods." To ensure that the library was properly randomized, the DNA sequence of 16 clones was determined in the region that was mutated. Each clone had a different DNA sequence, which demonstrated that the library was well randomized (data not shown).

Phage were rescued from the library and panned on Daudi cells, which have 1×10^5 CD22 binding sites (20). We decided to carry out just two rounds of panning because Fvs with a high affinity for antigen can be lost during several rounds of panning (18). The enrichment in round 1 was not determined, but between round 1 and round 2, there was a 60-fold enrichment (data not shown).

Analysis of Selected Clones. After the second round of panning, phage stocks were prepared from 24 individual clones and tested for their ability to bind to Daudi cells by flow cytometry. Fig. 1*A* shows the background fluorescence intensity of cells incubated with secondary antibody (goat antimouse FITC) without phage and of cells incubated with WT phage (*GSSY*) and secondary antibody. It is evident that the presence of phage caused a large increase in signal. When the cells were incubated with GSSY phage in the presence of the parental GSSY containing immunotoxin [RFB4 (Fv)-PE38], the fluorescence intensity shifted to the left close to the level of cells incubated with no phage. This finding indicates that phage binding is specific and that the assay can discriminate between good binders and poor binders. Fig. 1*B* shows the fluorescence intensity of Daudi cells incubated with phage, which display the WT RFB4 scFv (*GSSY*) and of cells incubated with three other phage, which display three different mutant scFvs (*lines A*, *B*, and *C*) selected by panning. The fluorescence intensity of phage A and B is somewhat greater than cells incubated with WT phage GSSY, which indicates that these mutants are good binders. Cells incubated with phage C had a fluorescence intensity similar to that of control cells incubated without phage. We classified this mutant as a poor binder. Twenty-two of the phage analyzed behave like phage A and B. Only 2 of 24 phage did not bind to the cells. The Fvs of the 22 phage that bound to Daudi cells were sequenced. The deduced amino acid residues of the region mutated in V_H CDR3 are shown in Table 2. A variety of different mutants were obtained. The mutant Fvs are named according to the amino acid sequence obtained. The only mutant phage found three times was GKNR. GSTR was found twice. The remaining sequences were present only once. Nevertheless, a common pattern of substitution can be identified. In position 99 (according to Kabat database; Ref. 21) the glycine was conserved in all of the binding phage recovered. In position 100B, three major groups of mutants can be identified: a group with arginine substitution (31% of clones) a group with tryptophan (13% of clones), and a group that conserved the tyrosine (36% of clones). Positions 100 and 100A were quite variable.

Construction, Expression of the Immunotoxins and Cytotoxic Activity on Cell Lines. Our goal was to obtain an Fv with increased affinity for CD22, which when converted to an immunotoxin would improve cytotoxic activity to cells displaying CD22. Therefore, we chose several mutant Fvs to convert into RITs. Because we obtained many different sequences, we decided to prepare mutant immunotoxins based on the substitution the Fvs had at position 100B. In this position, there was a restricted pattern of mutation. We made immunotoxins from

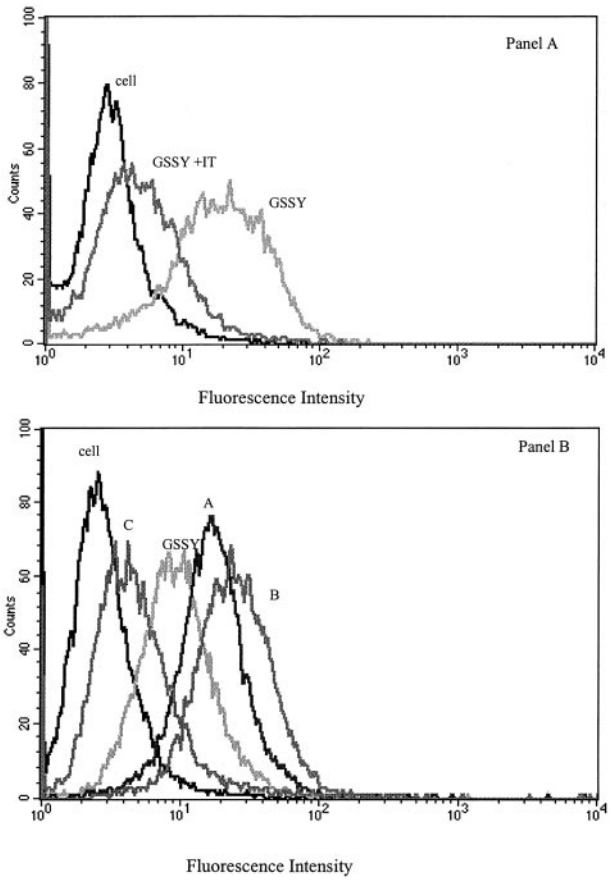

Fig. 1 Binding of phage to Daudi cells. Phage were prepared from single colonies eluted after the second round of panning and titered as described in "Materials and Methods." Five $\times 10^5$ Daudi cells were incubated with 8×10^8 phage for 60 min at room temperature; washed with DPBS + 0.5 mM EDTA + 0.5% BSA, and incubated with 5 μg of anti-M13 antibody (Amersham) for another 20 min. Successively, a FITC-conjugated goat antimouse IgG (H+L) antibody was added to the cells, and analysis was performed using a flow cytometer (Becton and Dickinson). *A, cell*, cells were not incubated with phage or with anti-M13 antibody but only with FITC-conjugated goat antimouse IgG (H+L) antibody; *GSSY*, WT phage; *GSSY+IT*, 63 μg of RFB4 immunotoxin were added, together with GSSY phage to Daudi cells, and processed because the sample incubated only with the phage. *B, cell*, cells were not incubated with phage or with anti-M13 antibody but only with FITC-conjugated goat antimouse IgG (H+L) antibody; *GSSY*, WT phage; *line A*, mutant phage A, binder; *line B*, mutant phage B, binder; *line C*, mutant phage C, binder.

each of the three major 100B substitutions (Y, R, W). The immunotoxins were prepared with the following Fvs: GTTW, GYNW, GTHW, GSTY, and GKNR. Each immunotoxin was constructed by PCR-amplifying of the Fvs from the phage display vector and subcloning them into the immunotoxin expression vector. Each immunotoxin was purified to over 95% homogeneity and eluted as a monomer in TSK gel filtration chromatography (result not shown). The purified immunotoxins were used in cytotoxicity assays on a panel of six antigen-positive lymphoma cell lines. In Table 3 are shown the IC_{50} values representative of several experiments, each done in triplicate. *P*s indicating that IC_{50}s were significantly different are

Table 2 Sequences of mutant phage obtained after panning

Amino acid sequences of mutant phage isolated after two rounds of panning are listed. The entire Fv of each phage was sequenced. Only amino acids in the target region are shown. Each residue is numbered according to the Kabat database (21).

99	100	100A	100B
G	S	S	Y[a]
G	T	H	W[b]
G	Y	N	W[b]
G	T	T	W[b, c]
G	S	T	Y[b]
G	K	N	R[b, c]
G	S	T	R[d]
G	H	T	F
G	N	R	Y
G	T	A	Y
G	T	N	Y
G	L	H	Y
G	F	L	Y
G	S	R	Y
G	R	N	Y
G	V	H	R
G	A	L	R
G	V	R	A
G	T	A	K
G	R	T	S

[a] WT.
[b] Immunotoxin made.
[c] Clone found three times.
[d] Clone found two times.

also indicated in Table 3. All of the mutant immunotoxins except GKNR were more cytotoxic to the antigen-positive cell lines than the WT. Because of its lower activity, we did not conduct any additional experiments with this mutant. The GSSY-containing immunotoxin had an IC_{50} ranging from 2 to 252 ng/ml on the different cell lines compared with the most active mutant GTHW, which had an IC_{50} ranging from 0.2 to 32 ng/ml Mutant immunotoxins were not cytotoxic to the CD22-negative cell line HUT-102, which demonstrated that the cytotoxic effect of the immunotoxins is selective to antigen-positive cells. Although cytotoxicity in this study was assessed by a protein synthesis inhibition assay, it has been shown in many cell types, including Raji cells and fresh leukemic cells obtained from patients, that protein-synthesis inhibition produced by RFB4 (Fv)-PE38 is associated with cell death. (13, 22).

Cytotoxic Activity of Mutant Immunotoxins on Patient Cells. We next investigated the cytotoxic activity of the mutant immunotoxins on malignant cells isolated from patients. Mononuclear cells from four patients with CLL and from one patient with HCL were incubated with immunotoxins for 3 days and pulsed with [^{3}H]leucine for 6–8 h. All of the mutant immunotoxins analyzed were more cytotoxic than WT and *P*s were calculated for many of the samples (Table 4). Fresh leukemic cells from patient 2 had an IC_{50} of 490 ng/ml with GSSY (WT) toxin and an IC_{50} of 22 ng/ml with mutant GTHW. In the same patient, mutant GYNW had an IC_{50} of 40 ng/ml and mutant GTTW of 95 ng/ml. Fig. 2 shows an example of a cytotoxicity assay on cells isolated from patient 1. The immunotoxin with GTHW had an IC_{50} of 29 ng/ml, whereas the IC_{50} of the WT was >1000 ng/ml In most of the patients, the parental immunotoxin GSSY was not able to inhibit protein synthesis by 50% at the concentrations tested. We, therefore, calculated an IC_{40}, the toxin concentration that reduced incorporation of [^{3}H]leucine by 40%, compared with untreated cells. In patient 5, WT immunotoxin (GSSY) had an IC_{40} of 854 ng/ml, whereas mutant GTHW had an IC_{40} of 18 ng/ml. This corresponds to a 47-fold increase in activity; in the same patient, immunotoxin GYNW had an IC_{40} of 28 ng/ml.

Binding Properties of Mutant Immunotoxins. To determine whether the increased activity of the mutant immunotoxins was attributable to increased binding affinities, we measured affinity by plasmon surface resonance (BIAcore). We prepared CD22 recombinant protein as described in "Materials and Methods" and immobilized it on a CM5 chip. Mutant and WT immunotoxins were analyzed for their binding activities using the CM5 chip coated with CD22. Fig. 3 shows binding profiles of GSSY (WT), GTTW, GYNW, and GTHW immunotoxins. The binding constants, K_{on}, K_{off}, and K_d are tabulated in Table 5.

The sensograms in Fig. 3 show that the mutant immunotoxins had much slower dissociation rates compared with WT GSSY-containing immunotoxin. The association rates also were increased. All of the mutants had increased affinity. The mutant with the highest affinity was GTHW with a K_d of 6 nM compared with WT GSSY with a K_d of 85 nM. Mutant GYNW had a K_d of 10 nM and mutant GTTW had a K_d of 24 nM. Mutant GSTY had a K_d of 49 nM.

Discussion

In the current study, we improved the activity and the affinity of an anti-CD22 immunotoxin by mutating residues in V_H CDR3. Several of the mutant immunotoxins obtained showed increased cytotoxicity to CD22-positive cell lines and to cells directly isolated from patients with CLL and HCL, and these differences were statistically significant (Tables 3 and 4). The most active mutant immunotoxin (GTHW) had up to a 50-fold improvement in activity toward cells from patients with CLL. CLL cells often have very few CD22 binding sites (23). In the current study, patient 1 had only 130 sites/cell, patient 2 had 700 sites/cell, and patient 5 had 4,000 sites/cell (data not shown). Nevertheless, the GTHW-containing immunotoxin was very cytotoxic to these cells and, therefore, could be very useful in the treatment of CLL and other malignancies that express low levels of CD22. Moreover the increased cytotoxic activity of the mutant immunotoxin should produce a clinical benefit with a lower dose and in turn lead to a decrease in nonspecific toxicities in patients. For all of these reasons, the GTHW mutant immunotoxin merits further preclinical development.

The approach used to increase the affinity of RFB4 Fv was to mutagenize hot spot residues in V_H CDR3. Hot spots are DNA sequences naturally prone to mutation during the *in vivo* affinity maturation of antibodies. Other strategies that are used to increase the affinity of antibodies, such as codon-based mutagenises (24), CDR walking (25, 26), error-prone replication (27), and synthetic CDR construction (28), require the construction of large libraries because all CDRs residues are mutagenized. Such libraries are difficult to make and to handle (16). By targeting hot spots, we had to make only a small library

Table 3 Cytotoxic activity (IC_{50}) in ng/ml of selected RFB4 (Fv)-PE38 mutants on six different CD22-positive cell lines

Cells were seeded at 5×10^4/well in 96-well plate 24 h before the assay. Immunotoxins were added to the plate, and cells were incubated at 37°C for 20 h and then were pulsed with [^{3}H]leucine for 2 h. [^{3}H]leucine incorporations were determined. Each assay was done in triplicate, and, except as noted below, at least three different assays were performed with each cell line. IC_{50} (expressed in ng/ml) is the toxin concentration that reduced incorporation of radioactivity by 50% compared with the cells that were not treated with the toxin. Mean values of three experiments ± SD are shown. SDs are not listed when the immunotoxins were tested only once. Below each IC_{50} is the significance in its difference from GSSY (WT) and GTHW when differences by Student's *t* test were significant ($P < 0.05$).

	JD38	Ca46	Raji	Daudi	Namalwa	Ramos
GSSY (WT) *vs.* GTHW	2.3 ± 0.5	3.1 ± 0.2	5.1 ± 0.15	8.1 ± 2.3	10.6 ± 1.2	252 ± 3
	$P < 0.01$	$P < 0.05$	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$
GTHW *vs.* WT	0.2 ± 0.09	1.4 ± 0.5	1 ± 0.14	1.7 ± 0.1	2.8 ± 0.4	32 ± 3
	$P < 0.01$	$P < 0.05$	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$
GYNW *vs.* WT *vs.* GTHW	0.6 ± 0.1	0.8	0.6 ± 0.07	2.1 ± 0.5	5.6 ± 3.3	N.D.[a]
	$P < 0.01$		$P < 0.01$	$P < 0.01$		
	$P < 0.01$		$P < 0.05$			
GTTW *vs.* WT *vs.* GTHW	0.7 ± 0.03	1.7 ± 0.4	2.75 ± 0.3	2.0 ± 1.2	5.3 ± 0.2	N.D.
	$P < 0.01$	$P < 0.05$	$P < 0.01$	$P < 0.01$	$P < 0.01$	
	$P < 0.01$		$P < 0.01$		$P < 0.01$	
GSTY *vs.* WT *vs.* GTHW	1.1	2	4.1	8.5 ± 0.7	9.5	N.D.
				$P < 0.01$		
GKNR *vs.* WT *vs.* GTHW	5	6	10	55 ± 7	25	N.D.
				$P < 0.01$		
				$P < 0.01$		

[a] N.D., not determined.

Table 4 Cytotoxic activity (IC_{50}) in ng/ml of mutant immunotoxins on patient cells

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
GSSY (WT) *vs.* GTHW	>1000	490 ± 70	34 ± 5	6.7 ± 2.3	>1000
	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$
GTHW *vs.* GSSY (WT)	29 ± 10	22 ± 2	1.5 ± 0.4	<1	28 ± 6
	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$
GYNW *vs.* GSSY (WT) *vs.* GTHW	105 ± 48	40 ± 5	3.4 ± 0.7	N.D.[a]	41 ± 2
		$P < 0.01$	$P < 0.01$		$P < 0.01$
		$P < 0.01$	$P < 0.05$		$P < 0.05$
GTTW *vs.* GSSY (WT) *vs.* GTHW	>1000	95 ± 9	8.5 ± 3	1.5 ± 0.6	76 ± 9
	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.05$	$P < 0.01$
	$P < 0.01$	$P < 0.01$	$P < 0.01$		$P < 0.01$
GSTY *vs.* GSSY (WT) *vs.* GTHW	N.D.	N.D.	15 ± 2	2.1 ± 0.7	129 ± 50
		$P < 0.01$	$P < 0.01$	$P < 0.05$	$P < 0.01$
			$P < 0.01$		$P < 0.05$

Ficoll-purified mononuclear cells from patients were obtained by an approved protocol at the NIH. Cells were incubated with immunotoxins for 3 days at 37°C and pulsed with [^{3}H]leucine for 6–8 h; protein synthesis was measured. IC_{50}s are expressed in ng/ml; SDs are shown. Each assay was done in triplicate. Diagnosis for patients 1, 2, 3, and 5 was CLL; diagnosis for patient 4 was HCL variant.

[a] N.D., not determined.

Significance in difference between IC_{50}s as in Table 3.

containing 1.6×10^5 clones to cover all of the possible mutations. We performed panning on CD22-positive cells, which express the antigen in its natural configuration. Therefore, we should not have missed mutant antibodies recognizing conformational or carbohydrate epitopes.

An analysis of mutant phage obtained after panning showed a variety of different sequences. This is different from results obtained in previous studies in which a restricted number of mutant sequences were found (16, 18). It is possible that, if we used more rounds of panning or more stringent washing, we would have selected a limited set of sequences. Although many different mutant sequences were obtained, a pattern of substitution can be identified. All of the binders conserved the glycine at position 99, possibly indicating that it is the best residue needed for interaction with the antigen. The selection for a glycine residue at this position 99 may also indicate that the V_H CDR3 loop requires conformational flexibility to achieve increased affinity or that no other residues can be physically located in this position. In position Y100B, most of the binders selected had an Arg(R) or a Trp (W). Immunotoxins with a W substitution had the best activity. Mutants GTTW, GYNW, and GTHW had an increase in affinity of 3.5-, 8.5-, and 14-fold, respectively. Tryptophan is a bulky hydrophobic residue; changing a Tyr to a Trp increases hydrophobicity. Residues in V_H CDR3 interact with CDR residues in the light chain (29); V_H Y100B often interacts with V_L Y49. In some antibodies, a W substitutes for residue Y100B (30). However, it was not only the Y100B/W substitution that lead to increased affinity: mutant GTTW and GTHW differ in only one amino acid but have a large difference in affinity (K_d, 24 nM and 8 nM). This observa-

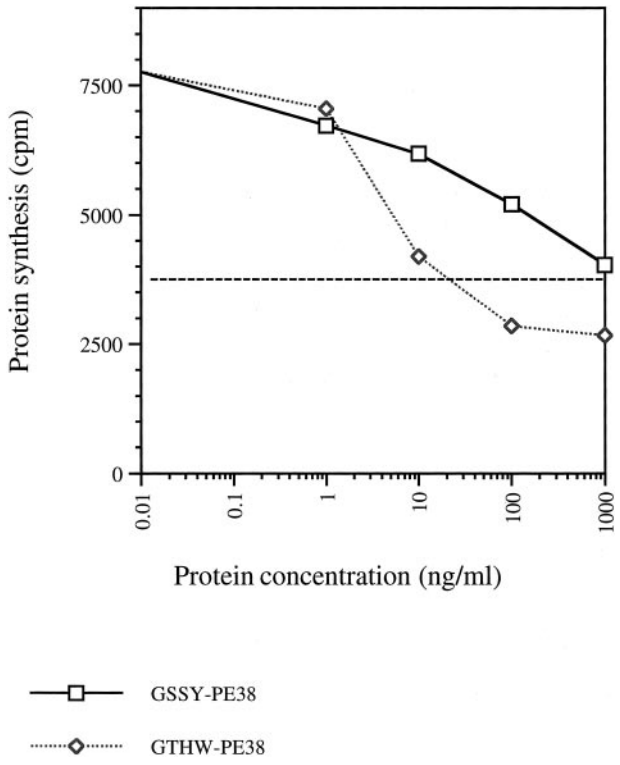

Fig. 2 Cytotoxicity assay on cells isolated from patient 1. Ficoll-purified mononuclear cells from the patient were incubated with immunotoxins for 3 days at 37°C and pulsed with [^{3}H]leucine for 6–8 h. Radiolabeled material was captured on filter-mats and counted in a Betaplate scintillation counter (Wallac). The graph shows, protein synthesis as measured by cpm of [^{3}H]leucine incorporated into protein. The *horizonial dashed line* indicates, 50% inhibition of protein synthesis, which is halfway between the level of incorporation in the absence of toxin and that in the presence of 10 μg/ml of cycloheximide.

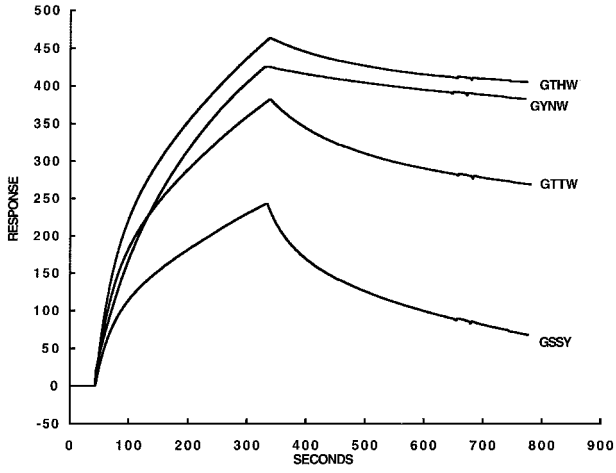

Fig. 3 Biacore sensograms of RFB4(Fv)-PE38 mutants. Binding profile of WT immunotoxin GSSY is compared with mutants GTTW, GTHW, and GYNW. RITs were diluted to 25 μg/ml in HEPES buffered saline buffer and injected over a chip surface containing CD22. Binding kinetics were analyzed using BIA evaluation 2.1 software. *Y axis*, the resonance units bound. *X axis*, the time in seconds.

Table 5 Summary of BIAcore analysis mutants of RFB4-PE38

Binding kinetics were analyzed on a BIAcore 2000 Biosensor (BIAcore). On and off rates were measured by injecting immunotoxins at a concentration of 380 nM over a CM5 sensor chip (BIAcore) containing immobilized CD22. Bound material was allowed to dissociate for 5 min or more.

Immunotoxin	K_{on} ($M^{-1}S^{-1}$)	K_{off} (S^{-1})	K_d[a] (nM)
GSSY (WT)	2.08×10^4	1.77×10^{-3}	85
GTHW	3.27×10^4	2.07×10^{-4}	6
GYNW	1.84×10^4	1.91×10^{-4}	10
GTTW	2.62×10^4	6.5×10^{-4}	24
GSTY	3.15×10^4	1.55×10^{-3}	49

[a] K_d was calculated by dividing K_{off} by K_{on}.

tion suggests that mutation S100A/His also contributes to the increase in affinity. Mutant GSTY has only one amino acid difference compared with WT GSSY in position 100A and also has an increased affinity (K_d, 49 nM).

Almost all of the immunotoxins studied had an increase in cytotoxic activity, except for mutant GKNR. It was the most common Fv sequence obtained (3 of 22 clones screened) but when converted into an immunotoxin, it resulted in a less active molecule. Phage display selects for both increased affinity and increased Fv expression. Therefore, it can confer an avidity effect leading to an apparent increase in affinity. To test whether this might be the case, we performed a dot blot analysis in which serial dilutions of WT GSSY and mutant GKNR phage were spotted on a membrane. The amount of Fv present was detected with an anti-E TAG antibody. When equal amounts of phage were spotted, the mutant GKNR had a stronger signal than GSSY (WT) phage. This indicates that increased numbers of scFvs are displayed on the GKNR phage (data not shown). Thus, the selection of mutant GKNR is likely caused by the increased display of the Fv fusion protein.

The IC_{50}s of the mutant immunotoxins varied greatly among the six cell lines tested. This is probably attributable to different numbers of CD22 receptor sites on the cells but could also be caused by differences in proteolytic processing. Daudi cells contain 1×10^5 sites/cell, but this cell line does not efficiently process *Pseudomonas* exotoxin (11). It is also possible that different variants of CD22 are present on different cell types. Two different isoforms of CD22 have been described. CD22 β is a full-length molecule with seven extracellular domains and CD22 α lacks extracellular domains 3 and 4 because of alternative splicing (6).

In the present work, we confirmed that targeting hot spots is an effective method to increase the affinity of the Fv of immunotoxin RFB4 (Fv)-PE38. Four mutant immunotoxins were isolated that had increased activity and affinity. This is the third antibody in which targeting hot spots has been effective, which indicates that targeting hot spots is a general method that can be used to increase the affinity of antibodies. The results obtained in this study also could have important consequences in a clinical setting in that immunotoxins with increased affinity might be administered in lower amounts with fewer side effects, and leukemic cells could be killed even if they have very low numbers of CD22 binding sites, as is the case in CLL.

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