Immunotoxins with Increased Activity against Epidermal Growth Factor Receptor vIII-expressing Cells Produced by Antibody Phage Display

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ABSTRACT

Recombinant immunotoxins are fusion proteins composed of Fv regions of antibodies and bacterial or plant toxins that are being developed for the targeted therapy of cancer. MR1(Fv)-PE38 is a single-chain recombinant immunotoxin that targets a mutant form of the epidermal growth factor receptor (EGFR), EGFRvIII, that is frequently overexpressed in malignant glioblastomas. We have used random complementarity determining region (CDR) mutagenesis to obtain mutants of MR1(Fv) with an increased affinity for EGFRvIII and an increased activity when converted to a recombinant immunotoxin. Initially, nine residues of heavy chain CDR3 were randomly mutagenized, and several mutants with increased binding affinity were isolated. All mutations were located at amino acids 98 and 99, which correspond to a DNA hot spot, a DNA sequence that mutates at high frequency during natural antibody maturation. A specific region of variable region of antibody light chain CDR3 was mutagenized that corresponded to a hot spot and a mutant (MR1-1) with an additional increase in affinity, and cytotoxic activity was isolated. These studies show that targeting hot spots in the CDRs of Fvs is an effective approach to obtaining Fvs with increased affinity. The increased affinity of MR1-1(Fv) makes it an attractive candidate for the targeted therapy of glioblastomas.

INTRODUCTION

MR1 is a scFv[2] that binds specifically to a mutant form of the EGFR, EGFRvIII. The MR1 scFV was isolated from an antibody phage display library (1). The scFv library containing MR1 was prepared from the splenic mRNA of a mouse that was immunized with an EGFRvIII-specific peptide plus the extracellular domain of EGFRvIII purified by affinity chromatography from EGFRvIII-expressing tumor xenografts (2). The EGFRvIII mutation is often highly expressed in glioblastoma and is also present in carcinoma of the breast, ovary, and lung (3). Approximately 60% of glioblastoma multiforme cases and 25% of breast carcinoma and non-small cell lung carcinoma cases express EGFRvIII (3). The mutation consists of an in-frame deletion of exons 2–7 near the NH_2 terminus of the extracellular domain that results in the expression of an EGFR mRNA with an 801-base deletion. The mutant protein contains a new glycine codon at the splice junction (4). The mutant receptor is expressed on the cell surface and creates a new tumor-specific cell surface sequence at the deletion junction. The receptor has constitutive tyrosine kinase activity that enhances the tumorigenicity of glioblastomas *in vivo* (5). Because of the tumor-specific extracellular sequence, the mutant receptor is an attractive target for cancer therapy. MR1 was developed with the prospect of developing a therapeutic agent that would be specifically targeted to EGFRvIII-positive cancer cells.

Our laboratory is focused on the development of immunotoxins for cancer therapy. Immunotoxins are made by fusing a targeting moiety, such as an antibody or a portion of an antibody, to a protein toxin such as *Pseudomonas* exotoxin.

PE is a potent bacterial toxin composed of three major domains (6): (*a*) domain Ia (amino acids 1–252) is the cell binding domain; (*b*) domain II (amino acids 253–364) is responsible for translocation into the cytosol; and (*c*) domain III (amino acids 400–613) ADP-ribosylates elongation factor 2, arresting protein synthesis and causing cell death, and also contains the COOH-terminal sequence REDLK, which directs the endocytosed toxin to the ER. Domain Ib (amino acids 365–399) is a minor domain, and its function is unknown. PE38 is a modified form of PE in which all of domain Ia and amino acids 365–380 of domain Ib have been deleted.

In a previous study, we described the properties of immunotoxin MR1(Fv)-PE38, which was constructed by fusing the scFv of MR1 to PE38, a truncated form of PE (1). Our laboratory has developed many different recombinant immunotoxins, several of which are now in clinical trials (7–9). Generally, the scFv genes are joined with the PE38 gene by a short linker and cloned into a T7-based expression vector. The recombinant immunotoxins are expressed in *Escherichia coli*, where they accumulate in inclusion bodies. After the inclusion bodies are washed extensively, they are dissolved in guanidine hydrochloride, and the protein is renatured and purified by ion-exchange chromatography and gel filtration. The resulting molecules are active and are directed to a cell-specific antigen by the scFv. Cell death is caused by the activity of the toxin.

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[2] The abbreviations used are: scFv, single-chain antibody variable domain; EGFR, epidermal growth factor receptor; CDR, complementarity determining region; PE, *Pseudomonas* exotoxin A; PEG, polyethylene glycol; V_L, variable region of antibody light chain; V_H, variable region of antibody heavy chain; 2xYT, 16 g of bacto-tryptone, 10 g of bacto-yeast extract, and 5 g NaCl per liter in H_2O.

To be useful as therapeutic agents, immunotoxins should have a high affinity for the antigen, resulting in a high cytotoxicity toward cells expressing the antigen. It is also necessary that the immunotoxin be produced with a high yield. MR1(scFv)-PE38 falls short in both of these criteria, with a K_d of 8 nM and a yield of 2%.

Antibodies bind to antigens via residues in their CDRs. Consequently, CDR mutagenesis is a widely used technique for improving the affinity of Fab and Fv fragments of antibodies. There are a number of different approaches to CDR mutagenesis. Most of these approaches, such as codon-based mutagenesis (10), CDR walking (11, 12), error prone replication (13), and synthetic CDR construction (14), require the construction of large libraries that are technically difficult to make and hard to handle. Lately, the trend in antibody affinity maturation has been toward the isolation of high-affinity binders from relatively smaller-sized libraries (15–17). All of these approaches involve the construction of expression libraries of antibodies with mutations in the CDRs and selection for better binders. Of the several different approaches to achieve this, filamentous phage display technology is the best approach in terms of efficiency and speed.

Phage display technology has become a useful tool for screening large peptide or protein libraries (18–20). scFvs can be expressed on phagemid vectors as fusions with M13 gene 3 protein using the pCANTAB5E or similar vectors (1). The fusion proteins are expressed in *E. coli* and, in the presence of helper phage, are displayed on the tips of the M13 phage, which can be collected from culture media. Phages that display scFv fusion proteins and bind to specific antigens are selected by panning the phage libraries on cells expressing the antigen or on a surface to which the antigen is coupled, such as magnetic beads. Phages that do not bind are washed away. Bound phages are eluted and amplified by reinfecting *E. coli*. Several rounds of panning result in an enrichment of specific binders. By making the panning conditions more stringent, better binders can be separated more effectively from poor binders.

In the current study, a phage display library was made with MR1 scFv. Initially, we introduced random mutations in the heavy chain CDR (V_HCDR3), an area that has a major role in antigen binding (21). Panning on cells expressing EGFRvIII produced several mutants which, when used to construct immunotoxins, had improved affinity, cytotoxicity, and yield. Analysis of these variants revealed that they all had mutations localized to a region of the V_HCDR3 that qualifies as a hot spot for hypermutation (22–24). Hot spots are defined by the consensus sequence G/A-G-T/C-A/T or A-G-C/T. The latter contains serine codons found in the variable domain genes of antibodies (25). Hot spots are regions that mutate at a high frequency during the maturation of antibodies in mice. Panning the V_HCDR3 library did not yield any clones containing mutations outside the hot spots. This result confirms our previous data demonstrating that randomizing hot spots is more likely to produce mutants with improved affinity (17). Therefore, we used the best binder obtained from panning the V_HCDR3 library to make a phage display library randomizing a hot spot in the light chain CDR3. Panning this library produced several more mutant clones. One of these Fvs, when expressed as an immunotoxin, showed a further increase in binding to EGFRvIII peptide and increased cytotoxicity to cells expressing the mutant receptor.

MATERIALS AND METHODS

Construction of the V_H Mutant Library. The V_HCDR3 of MR1 consists of 11 amino acids. DNA oligomers were designed to generate three libraries, each randomizing nine nucleotides (three consecutive amino acids). Using degenerate oligomers with the sequence NNS for randomizing each amino acid codon creates 32 possible codons for each position [N randomizing with all four nucleotides, and S introducing only C or G ($4 \times 4 \times 2 = 32$)]. Three consecutive codons randomized in this manner result in 32^3 (32,768) possible combinations. MR1 phagemid was used as a template to introduce three amino acid randomizations in the CDR3 heavy chain in three separate two-step PCRs (Fig. 1). The following oligonucleotides were used: (*a*) CDR3Hb, 5′-CTTGGCCCCA-SNNSNNSNNAGAGGTACTAGAATAGCCTC TTGTGCA-3′; (*b*) CDR3Hd, 5′-CTTGGCCCCACATAGCATASNNSNNSN-NAGAATAGCCTCTTGTGCA-3′; (*c*) CDR3Hf, 5′-CTTGGC-CCCACATAGCATAAGAGGTACTSNNSNNSNNTC TTGTG-CA-3′; (*d*) S1, 5′-CAACGTGAAAAAATTATTATTCGC-3′; and (*e*) AMBN, 5′-GCTAAACAACTTTCAACAGTCTATGCGGG-CAC-3′.

In the first PCR, 50 pg of the phagemid pCANTAB5E-MR1 DNA, in which the MR1(Fv) is fused to gene 3, were used as the template in three separate reactions using 20 pmol of DNA oligomer S1 along with 20 pmol of the DNA oligomer CDR3Hb, CDR3Hd, or CDR3Hf. 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 of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. These reactions generated 407-bp products that contain the mutations. The products generated in the first step were purified using Qiagen Quik Spin columns and then quantitated by visualization on a 1% agarose gel. The purified products generated in the first PCR were used as primers in a second PCR. In these reactions, approximately 2 pmol of the product from the first reaction were used with 20 pmol of the DNA oligomer AMBN with 50 pg of phagemid pCANTAB5E-MR1 as the template. The primers and template were mixed with two PCR Beads in a 50-μl volume and cycled using the profile described above. Each reaction generated an 876-bp library. The PCR products were digested with restriction enzymes *Sfi*I and *Not*I and purified. Purified PCR products (150 ng) were ligated with 250 ng of the phage display vector DNA pCANTAB5E (predigested as supplied by Pharmacia). The ligation mixtures were desalted, and 40 ng of each ligation were used to transform *E. coli* TG1. Each transformation resulted in approximately 1.5×10^6 clones. The phage libraries were then rescued from the transformed bacteria. Cells from each transformation were grown in 10 ml of 2xYT containing 2% glucose at 37°C with shaking at 250 rpm. After 1 h, ampicillin (100 μg/ml, final concentration) and 1×10^{10} plaque-forming units of M13KO7 helper phage were added. The cultures were grown for 1 h, pelleted, resuspended in 10 ml of 2xYT plus ampicillin (100 μg/ml) and kanamycin (50 μg/ml), and grown for 16 h at 37°C with shaking at 250 rpm. The bacteria were pelleted by centrifugation in a Sorval SS34 rotor

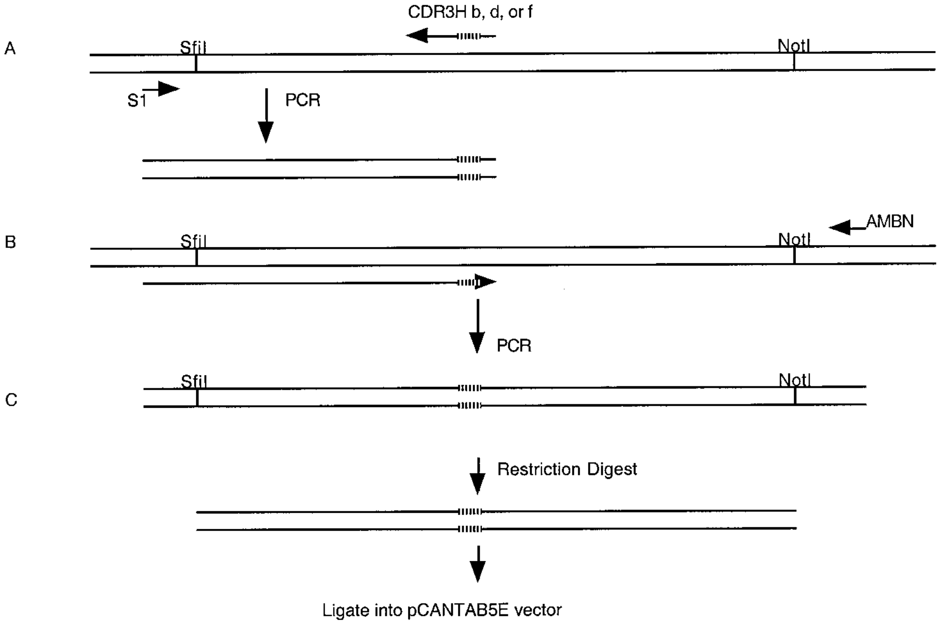

Fig. 1 Strategy for PCR construction of mutant libraries. *A*, pCANTAB5E-MR1 was used as a template for amplification using upstream primer S1 and downstream primer CDR3H b, d, or f containing degeneracies (NNS) in the targeted areas. *B*, pCANTAB5-EMR1 was used as a template for amplification using the products of the reaction step in *A* as upstream primers and downstream primer AMBN. *C*, the products from *B* are digested and cloned into pCANTAB5E.

at 8,000 rpm for 20 min. The phage-containing supernatants were filtered using a 0.45-μm syringe filter unit. The phages were precipitated by adding 2 ml of PEG/NaCl [20% PEG8000 in 2.5 M NaCl (w/v)] and incubated on ice for 30 min. The precipitated phages were pelleted by centrifugation in a Sorvall SS34 rotor at 10,000 rpm for 20 min and resuspended in 1 ml of NTE [100 mM NaCl, 10 mM Tris (pH 7.5), and 1 mM EDTA]. The rescued phage libraries were titered and stored at 4°C.

Panning the V_HCDR3 Library. NR6M cells grown in DMEM containing 10% fetal bovine serum plus 750 μg/ml G418 were harvested using 0.02% EDTA (Sigma E-8008). Cells (2×10^7) were pelleted, resuspended in 10 ml of cold blocking buffer (2% BSA and 0.02% NaN_3 in Dulbecco's PBS), and rotated slowly for 1 h at 4°C. The cells were pelleted and resuspended in 5 ml of cold blocking buffer. Phages (1×10^9) from each of the heavy chain CDR3 libraries were added to the cell suspension, and the mixture was rotated slowly at 4°C for 2 h. The cells were washed twice with 10 ml of cold blocking buffer and resuspended in 5 ml of cold blocking buffer. MR1dsFVPE38 was added (2 μM, final concentration) as a competitive inhibitor, and the suspension was rotated slowly at 4°C for 2 h. The cells were washed three times with 10 ml of cold blocking buffer. Bound phages were eluted by resuspending the washed cells in 1.5 ml of ice-cold 50 mM HCl and incubated on ice for 10 min. The NR6M cells were pelleted, and the eluted phages were transferred to a new tube containing 200 μl of 1 M Tris (pH 8.0). The eluted phages were titered to determine the number of phages captured. The eluted phages (0.5 ml) were then amplified by reinfecting *E. coli* TG1 for use in the next round of panning.

Construction of the V_L Mutant Library. Heavy chain CDR3 mutant (S98P-T99Y) was used as a template in a two-step PCR that introduced randomizations in the hot spot located in the light chain CDR3 (Fig. 1). In the first reaction, 50 pg of the phagemid containing heavy chain mutant (S98P-T99Y) were mixed with 20 pmol of the DNA oligomers VLMUT (5′-GATTACTACTGTTTGCAANNSNNSAACGTGCC TCTTACA-3′) and AMBN in a 50-μl volume. The mixture was cycled using the same profile used to generate the heavy chain CDR3 library. The reactions generated 150-bp products containing randomization of the hot spot in the light chain CDR3. After purification and quantitation, 2 pmol of the reaction products generated in the first PCR were used with 20 pmol of DNA oligomer S1 in a second PCR with 50 pg of phagemid DNA containing the heavy chain mutant (S98P-T99Y) as the template. The template and primers were mixed with two PCR Beads in a 50-μl volume and cycled using the above-mentioned profile. The reactions generated a 876-bp library which contains the V_HCDR3 mutation (S98P-T99Y) and randomization of the hot spot in CDR3L. The PCR products were digested with restriction enzymes *Sfi*I and *Not*I, purified, and ligated with the pCANTAB5E vector as described for the heavy chain CDR3 libraries. The ligation was desalted, and one-tenth (40 ng) of the reaction was used to transform *E. coli* TG1. The phage library containing 3×10^5 clones was rescued as described in the heavy chain CDR3 construction. One-fourth of it was amplified and used for the first round of panning.

Panning the V_LCDR3 Library. NR6M cells were harvested as described in the V_HCDR3 panning procedure. All steps and washes were done in cold blocking buffer, except as noted. Cells (5×10^6) were resuspended in 1 ml of blocking buffer and rotated for 1 h. The cells were pelleted and resuspended, rescued phage was added, and the suspension was rotated slowly at 4°C for 2 h. The cells were washed three times and then resuspended in blocking buffer containing 2 μM MR1 heavy chain mutant (S98P-T99Y) scFv-PE38 and rotated slowly at 4°C for 2 h. The cells were washed three times, and bound phages were eluted and neutralized as described for the heavy chain CDR3 library. The eluted phages were titered and rescued for the next round of panning.

Phage Rescue. The phages captured after each round of panning were amplified for use in the next round of panning. For rescue, *E. coli* TG1 was grown in 10 ml of 2xYT containing 2% glucose incubated at 37°C with shaking at 250 rpm. When the $A_{600\ \mathrm{nm}}$ reached 0.3, 0.5 ml of the captured phage was added to the culture, and the incubation was continued. After 1 h, ampicillin (100 μg/ml, final concentration) and 1×10^{10} plaque-forming units of M13KO7 helper phage were added, and incubation continued for 1 h. The culture was then centrifuged, and the pelleted bacteria were resuspended in 10 ml of fresh 2xYT media containing ampicillin (100 μg/ml) and kanamycin (50 μg/ml) and incubated at 37°C with shaking at 250 rpm for 16 h. The bacteria were pelleted by centrifugation in a Sorvall SS34 rotor at 8,000 rpm for 20 min. The phage-containing supernatants were filtered using a 0.45-μm syringe filter unit. The phages were then precipitated by adding 2 ml of PEG/NaCl and incubated on ice for 30 min. The precipitated phages were pelleted by centrifugation in a Sorvall SS34 rotor at 10,000 rpm for 20 min and then resuspended in 1 ml of NTE. The rescued phage library was titered and stored at 4°C.

Detection of Positive Clones. After the third round of panning, 48 clones from the heavy chain CDR3 library were analyzed for binding to EGFRvIII peptide (LEEKKGNYVVT-DHSGGK-biotin) using ELISA. Twenty-two clones that gave the strongest signal were subjected to DNA sequencing and further analyzed. After the fourth round of panning, 48 clones were analyzed by ELISA, and the DNA sequence of the 10 clones with the strongest ELISA signal was determined. For the light chain library, after the second round of panning, 48 clones were analyzed by ELISA, and the 17 clones with the strongest signal were sequenced. After the third round, 20 clones were analyzed, and 10 clones were subjected to DNA sequencing. To rescue the phage from the individual clones, single colonies were picked from the final panning titer plate and inoculated into 150 μl of 2xYT with 2% glucose and 100 μg/ml ampicillin in a 96-well culture dish. The dish was incubated at 37°C with shaking at 150 rpm. After 3 h, 20 μl of the culture were transferred to the wells of a second dish containing 100 μl of 2xYT with 2% glucose, 100 μg/ml ampicillin, and 1×10^8 M13KO7 helper phage and incubated for 2 h. The cultures were pelleted, resuspended in fresh 2xYT plus ampicillin and kanamycin, and then grown for 16 h. The cells were pelleted, and 50–100 μl of the phage-containing supernatants were assayed in ELISA. Phage ELISA was performed as described previously (1), except that 100 μl of 3,3′,5,5′-tetramethyl benzidine (BM blue; Boehringer Mannheim) were used as a substrate for detection. After blue/green color developed, 100 μl of 2 M H_2SO_4 were added to stop color development. Absorption was measured at 450 nM.

DNA Sequencing. DNA sequencing was preformed using the PE Applied Biosystems Rhodamine Terminator Cycle Sequencing Kit. The samples were run and analyzed on a PE Applied Biosystems Model 310 automated sequencer.

ScFv Immunotoxin Plasmid Construction, Expression, and Purification. ScFvs from selected phagemid clones were PCR-amplified using primers that introduced *Nde*I and *Hin*dIII restriction sites. The products were then digested and cloned into a T7-based expression vector in which the scFv is fused to a truncated version of PE. The plasmids were transformed into the expression host BL21 (λDE3). The MR1 mutant immunotoxins were expressed and prepared as described previously (26).

Surface Plasmon Resonance. Binding kinetics were measured using a BIAcore 2000 Biosensor. Steptavidin was bound to a CM5 research-grade sensor chip using amine coupling reagents provided by BIAcore. Biotinylated EGFRvIII peptide was bound to streptavidin by injecting 10 μl of a 10 nM solution of the peptide over the chip. Immunotoxins were diluted to 25 μg/ml in HEPES-buffered saline. On and off rates were measured by injecting 50 μl of the diluted immunotoxin over the chip surface at 10 μl/minute, and then allowing the bound material to disassociate for 5 min or more. The remaining bound material was removed from the EGFRvIII peptide by injecting 5 μl of 100 mM phosphoric acid. Each immunotoxin was injected and analyzed at least three times. Binding kinetics were analyzed using BIAevaluation 2.1 Software.

Cell Culture and Cytotoxicity Assays. NR6M cells were cultured in DMEM plus 10% fetal bovine serum supplemented with 750 μg/ml G418. Cytotoxicity assays measured the inhibition of $[^3H]$leucine incorporation as described previously (27). The IC_{50} of each of the immunotoxins was determined using three wells for each point. Each immunotoxin was assayed at least twice, and critical ones were assayed more frequently.

Bacteria and Cell Lines. *E. coli* TG1 is a K12 strain with the genotype supE *thi1* Δ(*lac-proAB*) Δ(*mcrB-hsdSM*)5/F′[*traD36 proAB lacI*^{q}ZΔM15]. *E. coli* BL21 (DE3) is a B strain with the genotype F^- *ompT gal [dcm] [lon] hsdS*$_B$ ($r_B^- m_B^-$) with DE3, a prophage carrying the T7 RNA polymerase gene. NR6 is a Swiss 3T3 mouse fibroblast variant cell line with no detectable EGFR. NR6M is the NR6 cell line transfected with a cDNA for the mutant EGFRvIII receptor under the control of the β-actin promoter. The source of NR6 and NR6M has been described previously (2).

RESULTS

Construction of the V_HCDR3 Library. To increase the affinity of the MR1(Fv) and the activity of the corresponding immunotoxin MR1(FV)-PE38, we chose to mutate V_HCDR3 and V_LCDR3 because these portions of the antibody make significant contact with the antigen (21). The amino acid sequence of V_HCDR3, which contains nine residues, is shown in Table 1. A complete library in which nine amino acid residues were randomized would require more than 3.5×10^{13} individual clones; instead, we prepared three different libraries covering residues 95–97, 98–100, and 101A-101C as described in "Materials and Methods." Library H-CDR3 95–97 contained 1.6×10^6 clones, library H-CDR3 98–100 contained 4×10^5 clones, and library H-CDR3 100A-100C contained 1×10^6 clones. To ensure the libraries were properly made, five clones from each library were sequenced. As expected, each clone had different amino acid combinations in the region targeted for mutations. Because a completely diverse library randomizing three amino acids would require only 33×10^3 independent clones, the size of the libraries ensures that all possible DNA sequences are well represented.

Table 1 DNA and amino acid sequence of heavy chain CDR3 and light chain CDR3 of MR1 Fv[a]

MR1 CDR3H	GGC	TAT	TCT	AGT	ACC	TCT	TAT	GCT	ATG
	G	Y	S	S	T	S	Y	A	M
Position	95	96	97	98	99	100	A	B	C
MR1 CDR3L	TTG	CAA	AGT	TTT	AAC	GTG	CCT	CTT	ACA
	L	Q	S	F	N	V	P	L	T
Position	89	90	91	92	93	94	95	96	97

[a] Hot spots with the sequence Pu G Py A/T are underlined.

Table 2 Phage enrichment during panning

Panning	Phage input	Phage eluted
V_HCDR3		
1	3×10^9	1.5×10^5
2	3×10^{11}	1.0×10^6
3	5×10^{11}	1.5×10^8
4	5×10^{11}	2.0×10^7
V_LCDR3		
1	1×10^{10}	2.4×10^5
2	1×10^{10}	5.5×10^6
3	1×10^{10}	2.0×10^7

Panning of the V_HCDR3 Library and Analysis of Selected Clones. Because the final intended use for an improved MR1 mutant was to target a toxin to EGFRvIII-positive cancer cells, we used EGFRvIII-positive NR6M cells for panning. Panning was done in the presence of immunotoxin MR1(Fv)-PE38, which should act as a competitor against the selection of wild-type MR1(Fv) in the library. The phages from each library were rescued, and equal amounts of phage (1×10^9) from each library were pooled to begin the first round of panning. The eluted phages were titered and rescued for the next round of panning. The subsequent pannings were done without determining the titer of the rescued phages until after the panning was carried out. This was done to save time and to decrease the possibility that unstable binders might be lost during the 24-h period required to titer the rescued phages. Typically, the rescues yielded 1×10^{12} colony-forming unit/ml. Table 2 summarizes the number of phages captured during each of the four rounds of panning. The enrichment peaked at the third round and appeared to decrease at the fourth round.

We initially examined the phages selected after four rounds of panning. Forty-eight clones were analyzed for binding in a phage ELISA, and the DNA sequence of 10 clones with the strongest signal was determined. As shown in Table 3 the only mutations recovered were in positions 98 and 99. DNA sequence analysis showed that the clones belong to five different groups: parental S98-T99 and mutants P98-N99, P98-Y99, P98-F99, and P98-W99. We also examined phages selected after the third round to see whether good binders were lost between rounds 3 and 4. We analyzed 48 individual clones for binding to the EGFRvIII peptide by ELISA, and 22 of these that gave the highest signal were subjected to DNA sequencing. Again, the only mutations recovered were in positions 98 and 99 (Table 3). A variety of amino acids were recovered in these positions. The most frequent was S98-T99 (wild type), followed by P98-S99, P98-Y99, P98-N99, and A98-D99. The fourth round of panning resulted in an enrichment of clones P98-N99 and P98-Y99 over the wild type (ST) and a loss of several clones and also produced a new clone containing P98-F99.

Cytotoxicity and Affinity of V_HCDR3 Mutants. Each of the 12 mutants obtained by panning the MR1 V_HCDR3 library was used to make immunotoxin. Each immunotoxin was constructed by PCR-amplifying the Fvs from the phage display vector and subcloning them into the immunotoxin expression vector. All of the immunotoxins (except one containing the A98-D99 mutation in V_HCDR3) could be highly purified to more than 90–95% homogeneity (results not shown). The purified immunotoxins were used to determine their cytotoxicity on NR6M cells. The binding affinities were measured by the BIAcore method using either peptide immobilized on a chip or a mutant form of the extracellular domain of the EGFR attached to a chip. The results of one set of experiments is shown in Table 3; we find that compared with the parental clone (S98-T99), which had an IC_{50} of 8.0 ng/ml, there were six clones that were more cytotoxic. These were P98-Y99, which had an IC_{50} of 3.5 ng/ml, followed by P98-N99, P98-W99, and P98-I99, which had an IC_{50} of 4.5 ng/ml, P98-F99, which had an IC_{50} of 6 ng/ml, and P98-V99, which had an IC_{50} of 6.5 ng/ml. Four clones, P98-S99, W98-V99, S98-W99, and P98-T99, had IC_{50}s identical to the parental clone.

Construction of the V_LCDR3 Library. Interestingly, all of the mutants recovered after panning the V_HCDR3 libraries were localized to positions 98 and 99. The DNA sequence of these two residues constitutes a hot spot, which is a region that undergoes mutations during the *in vivo* affinity maturation of an antibody. We therefore decided to use the mutant with the highest cytotoxic activity from the V_H library and subject it to mutagenesis, targeting only the hot spot in V_LCDR3. The sequence of V_LCDR3 is shown in Table 1. Our library introduced randomizations in the hot spot located in residues 91 and 92. The V_LCDR3 phage library was constructed as described in "Materials and Methods" and contained 3×10^5 clones. Because a library of only 1024 clones is required to achieve all possible codons in the two positions chosen for mutation, we assume that all possible DNA sequences were abundant in this library.

Panning of the V_LCDR3 Library and Analysis of Selected Clones. Three rounds of panning were carried out, and the phage captured at each step is shown in Table 2. For this library, we obtained more enrichment in the second round of panning than in the third round. After the second round of panning, 48 individual clones were rescued, and binding to the peptide was measured by ELISA. The DNA sequences of 17 clones that gave the strongest signal were determined. As shown in Table 4, five different mutants were obtained. All retained the wild-type serine residue at position 91 and had mutations at

Table 3 Sequences and properties of mutant phage isolated from heavy chain CDR3 library

Position	95	96	97	98	99	100	100A	100B	100C	No.	IC$_{50}$ (ng/ml)	K_d (nM)	Yield (%)
Original amino acid	G	Y	S	S	T[a]	S	Y	A	M		8.0	8.0	2.0
3rd round residues				S	T[a]					5[b]	8.0	8.0	2.0
				P	S[a]					4[b]	8.0	N.D.[c]	17.5
				P	Y[a]					3[b]	3.5	6.0	7.0
				P	N[a]					2[b]	4.5	11.0	10.0
				P	W[a]					2[b]	4.5	4.0	10.0
				A	D[d]					2[b]	N.D.	N.D.	N.D.
				P	I[a]					1[b]	4.5	5.0	10.0
				W	F[a]					1[b]	8.0	N.D.	10.0
				P	V[a]					1[b]	6.5	N.D.	7.0
					W[a]					1[b]	8.0	N.D.	5.0
4th round residues				S	T[a]					1[e]	8.0	8.0	2.0
				P	N[a]					3[e]	4.5	11.0	10.0
				P	Y[a]					3[e]	3.5	6.0	7.0
				P	F[a]					2[e]	6.0	20.0	3.0
				P	W[a]					1[e]	4.5	4.0	10.0

[a] Wild type.
[b] Number of clones of 22 clones.
[c] N.D., not determined.
[d] Could not make immunotoxin.
[e] Number of clones of 10 clones.

Table 4 Sequences and properties of mutant phage isolated from light chain CDR3 library

Position	89	90	91	92	93	94	95	96	97	No.	IC$_{50}$ (ng/ml)	K_d (nM)	Yield (%)
Original amino acid	L	Q	S	F	N	V	P	L	T		3.5	6	7
2nd round residues			S	F						4[a]	3.5	6	7
			S	W						6[a]	1.3	3	7
			S	R						3[a]	6.0	9	8
			S	S						2[a]	15.0	22	16
			S	L						1[a]	5.5	4	8
			S	M						1[a]	9.0	6	2
3rd round residues			S	F						1[b]	3.5	6	7
			S	S						7[b]	15.0	22	16
			S	W						1[b]	1.3	3	7
			S	R						1[b]	6.0	9	8

[a] Number of clones of 17 clones.
[b] Number of clones of 10 clones.

residue 92. The most frequent mutation at residue 92 was F92W (6 of 17 clones) followed by F92R (3 of 17 clones), F92S (2 of 17 clones), F92L (1 of 17 clones), and F92 M (1 of 17 clones). Of the 17 clones analyzed, 4 were found to be of the parental type.

We also analyzed the properties of clones isolated after the third round of panning (Table 4). Twenty clones were picked at random, and the phages were rescued and checked for binding to the peptide by ELISA. Ten clones that gave the strongest signal were chosen for DNA sequence analysis. The DNA sequence analysis revealed that there were three different clones present, all of which were represented in round 2. Besides one parental clone, these clones were F92S (7 of 10 clones), F92W (1 of 10 clones), and F92R (1 of 10 clones). There were no new mutants found, and we did not find mutants F92L and F92M.

Cytotoxicity and Binding Properties of the V_LCDR3 Mutants. The different Fvs obtained were used to make immunotoxins, and their cytotoxic activities and binding affinities were measured using the purified recombinant proteins. Only one mutant, F92W, gave an immunotoxin that was more active than the parent. Its IC$_{50}$ was 1.3 ng/ml, as compared with 3.5 ng/ml for its parent (Table 4). The other mutants had lower activities. The data in Table 4 also show that F92W has a higher affinity (K_d = 3 nM) than the parental clone F92 (K_d = 6 nM). One other mutant, F92L, had a slightly increased affinity (K_d = 4 nM) but did not have an increase in cytotoxicity. Fig. 2 shows a BIAcore sensorgram comparing the binding properties of the parental clone with the most active immunotoxin made from the Fv obtained from the V_HCDR3 library (V_H S98P-T99Y) and the most active clone obtained from the V_LCDR3 library (V_H S98P-T99Y V_L F92W), now called MR1-1. The figure shows that the two mutants have slower dissociation rates than the parental Fv. The analysis of the immunotoxin [MR1-1(Fv)-PE38] with both V_H and V_L mutations showed that it had a decrease in k_{off} and a slight increase in k_{on} resulting in a K_d of 3 nM.

Table 5 shows two experiments in which the cytotoxic

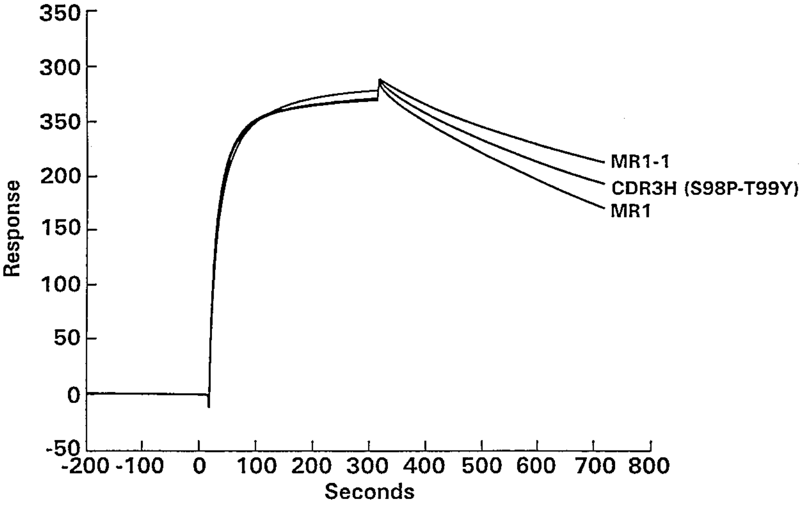

Fig. 2 BIACore sensorgram of MR1 and mutant derivatives.

Table 5 Comparison of the cytotoxic activity of MR1(Fv)-PE38 with two mutants with increased affinity for EGFRvIII

IC_{50}s are calculated from assays using different amounts of immunotoxins. The values are the means ± SD. *P*s are <0.01 when comparing MR1(Fv)-PE38 with MR1-1(Fv)-PE38 and MR1(Fv)(V_H S98P-T99Y)-PE38 with MR1-1(Fv)-PE38 in experiment 1 and when comparing MR1(Fv)-PE38 with MR1(Fv)(V_HS98P-T99Y)-PE38 and MR1(Fv)(V_H S98P-T99Y)-PE38 with MR1-1(Fv)-PE38 in experiment 2.

Immunotoxin	Experiment 1 IC_{50}	Experiment 2 IC_{50}
MR1(Fv)-PE38	9.2 ± 2.1	6.6 ± .2
MR1(Fv)(V_HS98P-T99Y)-PE38	6.0 ± .39	4.6 ± .7
MR1-1(Fv)-PE38	2.6 ± 6.2	2.0 ± 1

activities of MR1(Fv)-PE38 were compared with the two improved variants: MR1(Fv)(V_H S98P-T99Y)-PE38 and MR1-1(Fv)-PE38. In both experiments, MR1(Fv)-PE38 was the least active, and MR1-1(Fv)-PE38 was the most active. The values shown in the table are the means ± SD. *P*s are $<$ 0.01 when comparing MR1(Fv)-PE38 with MR1-1(Fv)-PE38 and MR1(Fv)(V_H S98P-T99Y)-PE38 with MR1-1(Fv)-PE38 in experiment 1 and when comparing MR1(Fv)-PE38 with MR1(Fv)(V_H S98P-T99Y)-PE38, and MR1(Fv)(V_H S98P-T99Y)-PE38 with MR1-1(Fv)-PE38 in experiment 2.

Immunotoxin Yield. A notable increase in the yield of different mutant immunotoxins was observed during the purification process. After refolding, the proteins are dialyzed and purified by chromatography (26). The protein is first batch-purified on a Q-Sepharose anion exchange resin that removes gross protein aggregates, nucleic acids, and other contaminants. Next, the eluted protein is loaded onto a MonoQ column, and the proteins are eluted with a 0–0.3 M NaC1 continuous gradient. This step removes the smaller aggregates. The properly folded monomeric immunotoxin elutes from MonoQ at a characteristic NaCl concentration of 280 mM and is then applied to a TSK G3000SW size exclusion column from which it elutes as a single peak. We calculate the yield of each mutant immunotoxin MR1(Fv)-PE38 based on the amount of protein present in the dissolved inclusion bodies and the amount recovered as a single peak on the size exclusion chromatogram (TSK 3000). Purity was assessed by SDS-PAGE. All immunotoxins studied were at least 98% pure by SDS-PAGE. Tables 3 and 4 show the yields when 100 mg of inclusion body protein are subjected to purification. It is evident that the yield of immunotoxin is greatly affected by the mutations in the CDRs and increased from 2% with the parental MR1(Fv)-PE38 to as much as 10–17.5% with many of the mutants. MR1(Fv)-PE38 has been prepared on at least eight different occasions, and the yield was never more than 2%. Each of the mutant molecules was only prepared one time because the yields were very high, and there was sufficient material for all of the experiments that were performed.

DISCUSSION

Construction of the Libraries. The limiting factor in the construction of antibody libraries with randomizations in the CDRs is the large number of residues that constitute the CDRs. Because X-ray structures are not available for most antibodies, there is usually no attempt to identify the few CDR residues where mutations are likely to yield higher-affinity variants. Consequently, it is necessary to construct extremely large randomized libraries to ensure the isolation of higher-affinity variants. In our efforts to make useful small libraries, we have focused on CDR3 because residues in CDR3 are known to make important contributions to antigen binding (21). In the present study, we have used two different approaches to make small libraries and demonstrate that better affinity variants can be isolated easily from small libraries. Initially, three libraries were constructed that randomized the nine residues making up the V_HCDR3 of MR1. Three residues were randomized at a time, creating three independent libraries. In the first library, residues 95–97 were randomized, in the second library, residues 98–100 were randomized, and in the third library, residues 100A-100C were randomized. It was relatively easy to make these libraries because each had a size requirement of only about 33,000 clones to achieve all possible codons. Therefore, the libraries could be made by using three degenerate oligonucleotides in three cloning experiments.

Small libraries can be constructed by separately randomizing every amino acid residue of each CDR with the corresponding number of oligonucleotides and an equivalent number of transformations (10). Making a library with 20 variants of a single residue and making one with a size requirement of 33,000 clones requires almost the same effort. Our approach of randomizing three residues at a time decreased the number of oligonucleotides needed, as well as the number of libraries that needed to be handled. By constructing and panning a V_HCDR3 library made in this way, we isolated a variant that improved the cytotoxicity by more than 50% (IC_{50} was reduced from 8 to 3.5 ng/ml). Analysis of the clones isolated from this library and our previous study (17) showed that it should be sufficient to target mutations only to particular amino acids of the CDRs rather than trying to mutate each one. These residues are coded by DNA sequences called hot spots, which are prone to mutation during affinity maturation of antibodies (22). We used this knowledge to focus the random mutations to the hot spot in V_LCDR3 that involved only two residues. Thus, this library had a size requirement of only 1,024 clones. This library was made using a single degenerate oligonucleotide and one transformation. It yielded a clone that, when used for making an immunotoxin, had another 65% increase in cytotoxicity (from 3.5 to 1.3 ng/ml). Thus, we demonstrate that it is possible and relatively easy to make small libraries to fish out higher-affinity variants. This approach is not restricted to MR1 but has been found to work in two other Fvs we are studying (17).[3]

Panning and Enrichment of the Libraries. As stated previously, our aim was to isolate a variant of MR1 that would bind to EGFRvIII with an improved affinity. Because we were trying to substitute all of the positions with all 20 amino acids, it is likely that the library would contain wild-type MR1. To try and decrease the reisolation of these clones, we did our panning in the presence of MR1(Fv)-PE38, a protein that should compete for wild-type MR1 phage binding. As shown in Table 3, we could not completely eliminate MR1 interference. A probable reason for this is that various Fvs are displayed to a different extent on the surface of phages, and none of them had a much greater affinity than the parental MR1.

The pattern of enrichment (Table 2) obtained with each of the two libraries is very different. In the case of the V_H library, there is slow enrichment at the beginning that peaks at round 3. With the V_L library, enrichment peaks at the beginning. We believe this is due to of the nature of the two libraries. In the case of the V_H library there were no binders recovered from the libraries that randomized residues 95–97 and 100A-100C. This indicates that more than two-thirds of the initial mixture made by combining the three libraries contained nonbinders. Because the few binders restricted to residues 98–100 were being slowly selected from a large population of nonbinders, the initial panning led to poor enrichment. In the third round, enrichment was greater because the population of input phage was dominated by the binders. The appearance of a new mutant (P98-F99) despite a lack of enrichment after the fourth round of panning is interesting. We see from our cytotoxicity and BIACore analysis with purified monomeric protein that this mutant has low affinity and cytotoxicity.

The V_L library introduced randomizations into a hot spot region that is apparently tolerant to mutagenesis. This library had fewer nonbinders; hence, it was possible to enrich the binders after only two rounds of panning. Once these binders were selected, there was no further enrichment as observed in the larger V_H library. These results suggest that the best time to analyze clones is early in the process. Panning after the enrichment peaks can be deleterious because of the risk of losing clones. It is possible that Fvs with low affinity but high expression may be preferentially enriched, whereas good binders may be lost. Evidence supporting this was observed while panning the light chain CDR3 libraries; mutant F92S with a low affinity (K_d = 22 nM) was found in 7 of 10 clones examined after the third round, whereas the best binder (F92W) was present only once. In contrast, in the second round, F92S was found in only 2 of 17 clones, whereas F92W was present in 6 of 17 clones.

Analysis of the mutant clones obtained from the three V_HCDR3 libraries revealed that we never obtained any mutants from regions outside the hot spot that had any binding ability. This suggests that hot spots are regions that tolerate mutations and, at the same time, influence affinity. An analysis of many mutants that arise during *in vivo* antibody maturation reveals that many of these localize to the hot spots (22, 28).

Cytotoxicity and Affinity of Immunotoxins Made with Mutated MR1 Fvs. Because our goal was to isolate a Fv with a higher cytotoxic activity, we first measured cytotoxic activity and then measured affinity using the BIAcore method. We found that increased cytotoxic activity did not necessarily correlate with increased affinity (Tables 3 and 4). There is no obvious explanation for this lack of correlation. Besides binding affinity, which is measured at 22°C, there are many steps in the intoxication process that could be affected by these mutations including stability at 37°C, rate of internalization, proteolytic processing, and transfer to the compartment required for translocation. It is possible that one or more of these steps is affected.

One very striking difference observed among the CDR mutants is the final yield of active monomeric protein. Recombinant toxins accumulate in inclusion bodies as insoluble aggregated protein (immunotoxin). Active monomers are produced by dissolving the inclusion bodies in 6 M guanidine-HCl, followed by controlled renaturation in a redox system and separation of monomers from multimers and aggregates. Our data show that mutations in one or two amino acids in the CDRs can greatly increase yields (Table 3). The yield of MR1(Fv)-PE38 is only 2%, but it was dramatically increased to 17% with heavy chain CDR3 S98P-T99S mutations. Presumably, these mutations have a profound effect on the folding pathway. In general, all of the heavy chain mutants isolated in the initial mutagenesis of the heavy chain had a better yield than the parental MR1, suggesting that the heavy chain of CDR3 is very important for proper folding and that the phage expression system may select in some way for proteins that fold more efficiently. Consequently, phages containing better-folding Fvs would be present in larger numbers and would be preferentially enriched during panning on antigen. Thus, phage display may be useful for selecting Fvs with an increased production yield.

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[3] G. Salvatore and I. Pastan, unpublished results.

In summary, we have produced a recombinant immunotoxin with increased cytotoxic activity toward a cell line expressing a mutant form of the EGFR by mutagenizing CDR3 of the heavy and light chains. The mutations obtained were all located in typical hot spots that have the DNA sequence Pu G Py A/T. This sequence is present in other CDRs of the MR1(Fv), and mutations in these regions may lead to further increases in cytotoxic activity.

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