Recombinant immunotoxin BL22, containing the Fv portion of an anti-CD22 antibody, produced complete remissions in most patients with drug-resistant hairy cell leukemia but had less activity in leukemias with low CD22 expression. Complementarity-determining region (CDR) mutagenesis is used to increase antibody affinity but can be difficult to perform successfully. We previously showed that antibodies with increased affinity and immunotoxins with increased activity could be obtained by directing mutations at specific DNA residues called hot spots. Because hot spots can arise either by somatic mutation or be present in the germline, we examined which type of hot spot is preferred for increasing antibody affinity. Initially, a second generation antibody phage-display library targeting a germline hot spot (Ser36→Asn31) within CDR1 of the light chain was mutated. Substitution of serine 30 or asparagine 31 with arginine produced mutant immunotoxins with an affinity (0.8 nM) increased 7-fold over BL22 (5.8 nM) and 3-fold over the first generation mutant HA22 (2.3 nM). More importantly, a 10-fold increase in activity over BL22 and a 2-3-fold increase over HA22 were observed in various B lymphoma cell lines including WSU-CLL that contains only 5500 CD22 sites per cell. For comparison, two phage-display libraries targeting non-germline hot spots in heavy chain CDR1 and CDR3 were generated but did not produce Fv with increased affinity. Our results demonstrate that germline hot spots but not non-germline hot spots are effective for in vitro antibody affinity maturation.

Hematological malignancies remain a major public health problem. In the past several years, immunotoxins have been developed as a new therapeutic approach to treat these malignancies. Immunotoxins are composed of antibodies or their Fv biochemically or genetically linked to toxins, toxin subunits, or ribosome-inactivating proteins from bacteria, plants, or fungi (reviewed in Refs. 1–3). The antibody portion 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.

Hematological malignancies are attractive targets for immunotoxin therapy because the tumor cells are easily accessible to the therapeutic agent (4). CD22 is a lineage-restricted B cell antigen expressed in about 70% of B cell lymphomas and leukemias. Because CD22 is not expressed on stem cells and in the early stages of B cell development (5), it is an attractive target for cancer therapy. Clinical trials have been conducted with RFB4(dsFv)-PE38 (BL22), a recombinant immunotoxin containing an anti-CD22 murine monoclonal antibody RFB4 (6) Fv fused to a truncated form of Pseudomonas exotoxin A (PE38). BL22 was engineered by inserting a disulfide bond in the framework region so that it holds the Fv together but does not interfere with binding to antigen (7, 8). BL22 is able to kill leukemic cells from patients and induced complete remissions in mice bearing lymphoma xenografts (9, 10). BL22 has been evaluated in a phase I clinical trial at the NCI, National Institutes of Health, in patients with hematological malignancies. Sixteen patients with purine analogue-resistant hairy cell leukemia (HCL) were treated with BL22 and 11 (86%) achieved complete remissions (11). BL22 is the first agent that is able to induce a high complete remission rate in patients with purine analogue-resistant HCL and confirms 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 chronic lymphocytic leukemia (CLL), in which the cells have relatively small amounts of CD22 on the cell surface (10, 12).

Efforts have been made to improve the affinity of antibodies by random or semi-random mutation (13–19). Recently, a strategy using hypermutating B cell lines mimicking the natural somatic hypermutation has been developed (20). We have developed an approach mimicking somatic hypermutation to increase antibody affinity by using small antibody phage-display libraries (21). We used a few hot spots in the antibody complementarity determining regions (CDR) that are naturally prone to hypermutations (22). Hot spots RGYW (R = A or G, Y = C or T, and W = T or A) are DNA sequences that are frequently mutated during the in vivo affinity maturation of an antibody (22–25). We introduced random mutations around these sites by PCR and made phage-display libraries with a minimum size of only 10^2 and 10^4 independent clones (26). Panning of these small hot spot libraries has yielded mutant immunotoxins with an increase in activity (21, 26, 27). One of these is immunotoxin HA22 that has a mutation in a hot spot region of heavy chain CDR3 (27). We hypothesized that a directed evolution approach targeting a different hot spot in each generation would allow us to sequentially increase the activity of immunotoxins. This uphill-climbing strategy has the major advantage that in each generation the library size is small and easy to make, whereas...
it is technically very difficult to produce large phage libraries where many nucleotides and amino acids in the Fv are randomly mutated. Because large changes in antibody affinity can result from accumulating point mutations, each with small effects (28), the capability to identify slight improvements in each generation could be of significant value. Here we have used immunotoxin HA22 as a starting point, and we examined the effect of mutating hot spots in germline residues or non-germline residues that have already undergone somatic mutation. We observed that mutation of germline hot spots but not non-germline hot spots resulted in Fv with increased affinity and immunotoxins with increased activity.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**Daudi, Namalwa, Ramos, MCF7, and 293T cells were obtained from American Type Culture Collection (Manassas, VA) and grown as described previously (8, 27). The WSU-CLL line was kindly provided by Dr. A. Al-Katib (Wayne State University, Detroit, MI) and grown as described (29).

**Construction of Phage-display Libraries—**In the present study, we constructed 3-generation phage-display libraries by mutagenizing hot spot positions using site-directed mutagenesis (QuickChange site-directed mutagenesis kit; Stratagene, La Jolla, CA). The resulting phagemids pCANTAB5E-G3 for display of HA22 single chain Fv (scFv) on the surface of bacteriophage M13 was made according to a previous procedure (27). The vector was modified by inserting a BspHI site (TCA TGA) containing a stop codon (underlined) at the hot spot positions using site-directed mutagenesis (QuickChange site-directed mutagenesis kit; Stratagene, La Jolla, CA). The resulting phagemids pCANTAB5E-G3-VL30/31BspHI, pCANTAB5E-G3-VH30/31BspHI, and pCANTAB5E-G3-VH30/31BspHI were used as a template to introduce two or three amino acid randomizations in a two-step PCR (26). The following oligonucleotides were used: S1, 5'-CAAGGAGTAATTGATTTATTGCCGC-3'; RFB4V30/31, 5'-AGTCGAGGATCTGGNNSN-STATTTAAGCCTGG-3'; RBF4V30/31, 5'-GGATTGGTTCTTNNSNNTTGAAGCAGTCTT-3'; RBF4VH9697/98, 5'-TACTGTCAGAACAGAT- NNSNNSNNGTGAAGCCTGGGGG-3'; and AMBN, 5'-GGTAAGA- CATCTGGATACCCATCGGCGC-3'. In the first, PCR was performed with the phagemid pCANTAB5E-G3-VL30/31BspHI, pCANTAB5E-G3-VH30/31BspHI, or pCANTAB5E-G3-VH9697/98BspHI was used as the template in each library using 20 pmol of DNA oligomers AMBN along with 20 pmol of the DNA oligomers RBF4V30/31, RBF4VH30/31, or RBF4VH9697/98. The template and oligonucleotides were mixed with two Ready-To-Go PCR beads (Amersham Biosciences) 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 a 344-bp product that contained the mutations. The product was purified using a Qiagen Quick Spin column (Qiagen, Valencia, CA) and then quantitated by visualization on a 1%

**Preparation and Purification of CD22—**The extracellular domain of CD22 protein was expressed as a fusion to human IgG Fc in transfecting 293T cells containing pCDNA1.1-22-Fc plasmid encoding the extracellular domain of human CD22, as described previously (27). The plasmid was transfected into 293T cells by Lipofectamine reagent (Invitrogen), and the CD22-Fc protein was harvested from the culture supernatant and purified with Hi-Trap protein A column (Amersham Biosciences).

**Enzyme-linked Immunosorbent Assay (ELISA)—**Phages or scFvs were prepared from 10 cell lines from each library using E. coli TG1 containing phagemids selected in panning. In order to normalize the scFv expression, we performed a dot blot analysis in which serial dilutions of phage or scFv were spotted on a membrane. The amount of scFv was detected with an anti-E TAG antibody (Amersham Biosciences). The colorimetric intensity of each dot was measured with the NIH Image program (rsb.info.nih.gov/nih-image/Default.html). ELISA were performed using CD22-Fc or CD22-Fc scFv ELISA kit (Amersham Biosciences, Pittsburgh, PA) using an E TAG antibody (Amersham Biosciences). The colorimetric intensity of each dot was measured with the NIH Image program (rsb.info.nih.gov/nih-image/Default.html). ELISA were performed using CD22-Fc or CD22-Fc scFv ELISA kit (Amersham Biosciences, Pittsburgh, PA) using an E TAG antibody (Amersham Biosciences). The colorimetric intensity of each dot was measured with the NIH Image program (rsb.info.nih.gov/nih-image/Default.html). ELISA were performed using CD22-Fc or CD22-Fc scFv ELISA kit (Amersham Biosciences, Pittsburgh, PA) using an E TAG antibody (Amersham Biosciences). The colorimetric intensity of each dot was measured with the NIH Image program (rsb.info.nih.gov/nih-image/Default.html). ELISA were performed using CD22-Fc or CD22-Fc scFv ELISA kit (Amersham Biosciences, Pittsburgh, PA) using an E TAG antibody (Amersham Biosciences). The colorimetric intensity of each dot was measured with the NIH Image program (rsb.info.nih.gov/nih-image/Default.html). ELISA were performed using CD22-Fc or CD22-Fc scFv ELISA kit (Amersham Biosciences, Pittsburgh, PA) using an E TAG antibody (Amersham Biosciences). The colorimetric intensity of each dot was measured with the NIH Image program (rsb.info.nih.gov/nih-image/Default.html). ELISA were performed using CD22-Fc or CD22-Fc scFv ELISA kit (Amersham Biosciences, Pittsburgh, PA) using an E TAG antibody (Amersham Biosciences). The colorimetric intensity of each dot was measured with the NIH Image program (rsb.info.nih.gov/nih-image/Default.html). ELISA were performed using CD22-Fc or CD22-Fc scFv ELISA kit (Amersham Biosciences, Pittsburgh, PA) using an E TAG antibody (Amersham Biosciences). The colorimetric intensity of each dot was measured with the NIH Image program (rsb.info.nih.gov/nih-image/Default.html). ELISA were performed using CD22-Fc or CD22-Fc scFv ELISA kit (Amersham Biosciences, Pittsburgh, PA) using an E TAG antibody (Amersham Biosciences). The colorimetric intensity of each dot was measured with the NIH Image program (rsb.info.nih.gov/nih-image/Default.html).
(Amersham Biosciences). Triplicate sample values were averaged, and the inhibition of protein synthesis was determined by calculating the percentage of incorporation compared with control wells without added toxin. The activity of the molecule is defined by IC50, i.e. the toxin concentration that reduced incorporation of radioactivity by 50% compared with the cells that were not treated with the toxin. All experiments were performed in triplicate on three separate occasions.

Cell death was assessed by WST-8 conversion using the Cell Counting Kit-8 according to recommendations from the supplier (Dojindo Laboratories). A volume of 10 μl of WST-8 (5 mM WST-8, 0.2 mM 1-methoxy-5-methylphenazinium methylsulfate, and 150 mM NaCl) was added to each well, and the incubation was carried out for 4 h at 37 °C. The absorbance of the sample at 450 nm was measured with a reference wavelength of 650 nm. Cytotoxicity was expressed as 50% inhibition of cell viability, which is halfway between the level of viability in the absence of toxin and that in the presence of the highest concentration that reduced incorporation of radioactivity by 50% compared with the cells that were not treated with the toxin. All experiments were performed in triplicate on three separate occasions. Statistical analyses were performed with the Prism software (version 3.0.2) for Windows (GraphPad software, San Diego, CA). Within each cell line, raw data were analyzed by application of one factor (treatment) repeated measures analysis of variance with Dunnett's and Student-Newman-Keuls post-tests. The confidence interval was set at 95% for all statistical tests.

Both the heavy and light chains variable region sequences were numbered following the Kabat rule (33). The scFv sequences were analyzed by online V-Quest software provided by the International ImMunoGeneTics data base (IMGT, imgt.cines.fr/textes/vquest/) for identification of germline origin of VH/VL regions (34).

**RESULTS**

Sequence Analysis of the RFB4 Antibody and Identification of DNA Hot Spots for in Vitro Affinity Maturation—High affinity antibodies were generated in mice and humans by somatic hypermutation. Somatic hypermutation does not occur randomly within immunoglobulin V genes but is preferentially targeted to certain nucleotide positions (hot spots) and away from others (cold spots) (23, 36). Cold spots often coincide with residues essential for V gene folding. Hot spots, which appear to be strategically located to favor affinity maturation, are most frequently located in the CDRs (particularly CDR1). This process mainly results in the introduction of mutations that are located at or very near (A/G)(C/T)(A/T) (RGYW) and AG(C/T) (AGY) sequences. A total of 50–60% of all somatic hypermutations are found around RGYW motifs (24). To improve the affinity of the antibody portion of recombinant immunotoxins and thereby increase their cytotoxic activity, we have proposed that hot spots are the preferred regions to target new mutations (21). The first step in this process is to identify the DNA hot spot

![Sequence alignment of the VH and VL domains of RFB4.](Image)
sequences in all the CDRs of the antibody under investigation.

A homology search using V-Quest at IMGT (imgt.cines.fr/textes/vquest/) (34) shows that the light chain variable region of antibody RFB4 used to make immunotoxin BL22 is of the Vx10 class and has 96% identity at the amino acid level with the germline light chain IGKV10-96*01 (GenBankTM accession number M15520). The light chain variable region gene probably originated from recombination of the Vκ gene (IGKV10-96*01) and the Jκ1 gene IGKJ1*01 (GenBankTM accession number V00770) without a somatic insertion. The DNA sequence of the germline V and J gene fragments were manually assembled and translated into an amino acid sequence. Fig. 1 showed alignment of the germline sequence with the amino acid sequence of the RFB4 V_L region.

The heavy chain variable region of RFB4 was found to be of highest homology with the germline VH gene IGHV5S21*01 (GenBankTM accession number AF120463). It has 94% amino acid identity with this germline sequence. The C-terminal region probably originated from recombination of the germline D gene IGHD-FL16.1*01 (GenBankTM accession number J00434) and J gene IGHJ3*01 (GenBankTM accession number V00770) with an insertion resulting in one additional amino acid at the 5′-end and three amino acids at the 3′-end of the D gene. The germline V, D, and J gene fragments were manually assembled, converted into the amino acid sequence, and aligned with the RFB4 VH region (Fig. 1).

We identified two types of hot spot residues in the nucleotide sequences of the CDR domains of RFB4. One type contains hot spots in germline residues; the other type contains hot spots in residues that have already undergone mutational events. Germline hot spot residues have the same nucleotide sequence as the germline gene, whereas in non-germline hot spot residues the nucleotide sequence at or very near the RGYW motifs differs from the germline gene. The non-germline hot spots were presumably produced during somatic hypermutation of B cells. Five germline hot spot clusters (Fig. 1, red) were identified in the CDR domains of RFB4. Among them, four contain RGYW in their nucleotide sequences. They are located in light chain CDR1 (L1) and CDR3 (L3), and heavy chain CDR2 (H2) and CDR3 (H3) (Fig. 1). Three non-germline hot spot clusters were also found. All of them contain RGYW in their nucleotide sequences. They are located in heavy chain CDR1, CDR2, and CDR3 (Fig. 1, green).

We generated a three-dimensional molecular model (Fig. 2) for the RFB4 V region using the WAM program. Ribbon diagrams of the superimposed germline and RFB4 V regions (Fig. 2A) demonstrates that heavy chain CDR3 (H3) of RFB4 shows significant displacement from that of the germline, suggesting that heavy chain CDR3 is directly involved in antigen binding. A surface view of the RFB4 V region reveals that most germline (Fig. 2, red) and non-germline (green) CDR hot spot residues are exposed, suggesting that they may be located at the interface between the antibody and antigen (Fig. 2B). Ser{H100}, Ser{100A}, and Tyr{100b} are highly exposed on the tip of heavy chain CDR3 loop (H3).

Selection of a Light Chain Germline Hot Spot in CDR1—As shown in the primary sequence alignment (Fig. 1), almost all the somatic mutations are located within the heavy chain CDR regions of antibody RFB4 except for a change of germline residue Arg{53} to Ile{53} in the light chain of CDR2. In the heavy chain CDR1, germline Thr{28}, Ser{31}, and Ala{33} were mutated to Ala{28}, Ile{31}, and Asp{33}. In the heavy chain CDR2, Asp{55}, Tyr{56}–Ile{57} and Ala{60} were mutated to Gly{55}–Thr{56}–Thr{57} and Pro{60}. In the heavy chain CDR3, in addition to somatic insertion of five amino acids (Ser{96}, Gly{97}, Gly{100c}, Val{100d}, and Leu{100e}) located at the ends of the germline D gene fragment, Arg{95} was mutated to His{95}. In the present study, we first chose a light chain germline hot spot in CDR1 for in vitro mutagenesis because unlike heavy chain CDR regions and light chain CDR2, light chain CDR1 and CDR3 have not been changed by somatic mutations of B cells.

Construction of a Phage-display Library Targeting a Germ-line Hot Spot in Light Chain CDR1—In this work, we used the Fv portion of HA22, a first generation mutant of monoclonal antibody RFB4, in which the affinity of the Fv had already been increased by mutating Ser{100}–Ser{100A}–Tyr{100b} to Thr{100}–His{100A}–Trp{100b} in a hot spot of the heavy chain CDR3 (27). The light chain Fv of HA22 is the same as that of BL22. We then constructed a phage display library (RFB4LibVL30/31) that targeted a germline hot spot (Ser{30}–Asn{31}) located in the light chain CDR1 (L-CDR1) of HA22.

The new library was transduced into E. coli TG1 cells by electroporation and the Fv expressed as a fusion with the minor
FIG. 3. **FACS analysis of pooled phage bound to CD22-positive Daudi cells.** Primary (L): primary phage display library RFB4LibVL30/31 (A), RFB4LibVH30/31 (B), and RFB4LibVH96/97/98 (C). First round (I), second round (II), and third round (III) refer to the round number of each biopanning process. HA22 phage (HA) was used as a standard.

### Table I

**Phage selection in subtractive biopanning**

| Library for panning | Panning cycle | Input      | Output     | Enrichment over previous round | Total enrichment |
|---------------------|---------------|------------|------------|-------------------------------|-----------------|
| RFB4LibVL30/31      | 1             | $1 \times 10^{12}$ | $1.4 \times 10^4$ | 50                            | 10,700          |
|                     | 2             | $1 \times 10^{12}$ | $7 \times 10^5$  | 214                           |                 |
|                     | 3             | $1 \times 10^{12}$ | $1.5 \times 10^6$ | 50                            |                 |
| RFB4LibVH30/31      | 1             | $1 \times 10^{12}$ | $1.2 \times 10^4$ | 50                            | 2650            |
|                     | 2             | $1 \times 10^{12}$ | $6 \times 10^5$  | 53                            |                 |
|                     | 3             | $1 \times 10^{12}$ | $3.2 \times 10^7$ | 53                            |                 |
| RFB4LibVH96/97/98   | 1             | $5 \times 10^{11}$ | $2 \times 10^3$  | 50                            | 100             |
|                     | 2             | $5 \times 10^{11}$ | $2 \times 10^5$  | 50                            |                 |
|                     | 3             | $5 \times 10^{11}$ | $1.5 \times 10^7$ | 50                            | 1000            |
phage coat protein pIII after rescue with a helper phage. The library complexity of RFB4LibVL30/31 consisted of more than $1 \times 10^4$ independent clones. Because it is only necessary to produce a library of 1024 ($32 \times 32$) independent clones to randomly mutate the hot spot sequence (AGCAAT) to NNSNNS, the size of the current library is 10-fold larger than the one required. Sequence analysis of 10 independent clones from the mutant library demonstrated that the targeted hot spot residues were successfully randomized (data not shown).

Subtractive Biopanning and Analysis—To mimic somatic hypermutation in the immune response, which optimizes the differential binding affinity for an antigen relative to other molecules, we decided to screen for high affinity phage that could bind to CD22-positive Daudi but not CD22-negative MCF7 cells. To reduce the number of phage that bound non-specifically, subtractive panning was performed first on CD22-negative MCF7 cells, and then enrichment was performed on CD22-positive Daudi cells as described in detail under “Experimental Procedures.” At the last (third) round of panning, the Daudi cells were washed 16 times with the blocking buffer. Each wash included an incubation for >15 min on ice. The total duration of the off-rate selection was 6 h at the third round of biopanning. Then bound phage were eluted (pH 1.5) (Table I, RFB4LibVL30/31). The overall enrichment for library RFB4LibVL30/31 was very large (10,700-fold). Fluorescence-activated cell sorting (FACS) analysis of the bound phage was used to monitor the enrichment of high binders after each biopanning step (Fig. 3A). The gradual enrichment of phage suggests that a small number of high affinity binders existed in the primary library RFB4LibVL30/31 and were gradually enriched during the process of biopanning.

In addition, we also conducted a phage ELISA on CD22, CD25, and CD30-Fc fusion proteins (Fig. 4A). The ELISA signals on CD22-Fc were increased after each round of panning, but the non-specific background signals on CD25-Fc or CD30-Fc proteins were not. The results indicate that phages with a high affinity for CD22 were successfully enriched during biopanning.

After the third round of biopanning, monoclonal scFvs were prepared from individual phage clones and tested for their ability to bind to recombinant single chain CD22-Fc by ELISA (Fig. 5). Fig. 5, 1st panel, shows the CD22 binding activity of clones selected after panning of RFB4LibVL30/31. Thirty three clones were tested and could be grouped into four types as follows: 1) 23 clones had high ELISA signals; 2) 4 clones had ELISA signals comparable with HA22 scFv phage (Fig. 5, arrow); 3) 4 clones had ELISA signals lower than the starting parental phage; and 4) 2 clones had no signal. Twenty seven ELISA high binders in groups 1 and 2 were further analyzed on Daudi cells by flow cytometry (data not shown). Sixteen mutant scFvs with FACS signals (mean fluorescence intensity) for CD22-positive cells similar to or higher than HA22 and with high phage ELISA signals (Fig. 5) were selected for sequence analysis. The results are shown in Table II. The most frequent residues were Gly$^{30}$–Arg$^{31}$ and Arg$^{30}$–Gly$^{31}$. Each of these appeared six times. His$^{30}$–Gly$^{31}$, Ser$^{30}$–Gly$^{31}$, Arg$^{30}$–Ser$^{31}$, and Ala$^{30}$–Arg$^{31}$ each occurred once. More than one codon was used in the clones containing Gly$^{30}$–Arg$^{31}$ or Arg$^{30}$–Gly$^{31}$ indicating that there was a strong selection for these amino acid residues. Furthermore, basic amino acids such as Arg and His were conserved in all the Fv that bound strongly to CD22 except clone B6.

In addition to the binding affinity, we also examined the specificity of representative mutant phage antibodies by measuring their binding to MCF7 cells. When tested on CD22-negative MCF7 cells, some mutants showed reduced non-specific binding as compared with HA22 (Table III), indicating that subtractive biopanning probably enriches for mutants that have lower non-specific binding than the starting molecule. Based on these results, we decided to choose four Fv clones, E6 (Gly-Arg), B8 (Arg-Gly), B5 (His-Gly), and D8 (Ala-Arg), to make immunotoxins and to examine their binding and biological properties.

Construction, Expression of the Immunotoxins—We subcloned DNA encoding the Fv into a T7 promoter-driven expression vector in which the scFv is fused to a truncated version of Pseudomonas exotoxin A (PE38) to generate a recombinant immunotoxin. These were expressed in E. coli BL21 (DE3) cells, and upon induction with isopropyl-1-thio-D-galactopyranoside, large amounts of recombinant protein accumulated as intracellular inclusion bodies. We then refolded immunotoxins from solubilized inclusion bodies in a redox-shuffling buffer and purified them by ion-exchange chromatography on Q-Sepharose and MonoQ columns followed by TSK gel filtration chromatography. Purified recombinant immunotoxin monomers with the expected size of 63 kDa were obtained as analyzed by SDS-PAGE under nonreducing conditions (Fig. 6).
The purity of each recombinant immunotoxin is above 90%.

Cytotoxic Activity—The recombinant immunotoxins were tested on several CD22-positive cell lines. Cytotoxicity (IC50) was evaluated by both cell death assays (WST-8) in three Burkitt lymphoma cell lines (Daudi, Namalwa, and Ramos) and one B-CLL cell line (WSU-CLL) (Table IV) and protein synthesis inhibition ([3H]leucine incorporation) in Daudi cells (Fig. 7) and Raji, another Burkitt lymphoma cell line (data not shown). Immunotoxins containing the E6, B8, B5, and D8 Fv light chains were 2- or 3-fold more active than HA22 and up to 10-fold more active than BL22 in different B cell lines. Among these mutants, E6 and B8 Fv were found in 6 of 16 clones analyzed after biopanning, whereas D8 and B5 were found only once (Table II). Nevertheless, a common pattern of increase in their immunotoxin activities is present. More importantly, as shown in Table IV, in WSU-CLL, a cell line with low CD22 expression established from a B-CLL patient resistant to chemotherapy (29), the mutant immunotoxins (E6, B8, D8, and B5) were significantly more active than HA22 and BL22. Daudi cells have 1/100,000 CD22 sites/cell (37), but WSU-CLL cells have only 5500 CD22 sites/cell (10). None of the mutant immunotoxins were cytotoxic to the CD22-negative cell line MCF-7, which demonstrates that the cytotoxic effect of these immunotoxins is selective to antigen-positive cells.

Finally, we performed affinity measurement on Daudi cells (Table IV) by using monomeric recombinant immunotoxins. As predicted from the increase in cytotoxic activity, the binding affinity of mutants E6, B8, D8, and B5 for native CD22 on cells is increased from 2.3 to 0.8 nM. The increase in cytotoxicity was generally proportionally related to the increase in affinity.

Phage Display Libraries Targeting Non-germline Hot Spots—The results with phage library RFB4LibVL30/31 and previous data (21, 27) indicate that germline hot spot residues are good candidates for in vitro hot spot-based antibody evolution. To evaluate non-germline hot spot residues as targets, we constructed two other libraries (RFB4LibVH30/31 and RFB4LibVH96/97/98) that are targeted to two non-germline hot spot regions in heavy chain CDR1 (Ser30–Ile31) and CDR3.

### Table II

Sequences of ELISA and FACS-positive mutant phage obtained after panning

Amino acid sequences of mutant phage isolated after three 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 numbering scheme. Only sequences of regions that differ among the clones are shown.

| Phage library | Light chain CDR1 | 30 | 31 |
|---------------|-----------------|----|----|
| Germline      | Ser Asn         |    |    |
| BL22 (RFB4)   | Ser Asn         |    |    |
| HA22 (G3)     | Ser Asn         |    |    |
| E6 (6/16)     | Gly Arg         |    |    |
| B8 (6/16)     | Arg Gly         |    |    |
| D8 (1/16)     | Ala Arg         |    |    |
| B5 (1/16)     | His Gly         |    |    |
| H6 (1/16)     | Arg Ser         |    |    |
| B6 (1/16)     | Ser Gly         |    |    |
| Phage library | Heavy chain CDR1| 30 | 31 |
| Germline      | Ser Ser         |    |    |
| BL22 (RFB4)   | Ser Ile         |    |    |
| HA22 (G3)     | Ser Ile         |    |    |
| H11 (1/7)     | Pro Ile         |    |    |
| E12 (3/7)     | Pro Leu         |    |    |
| E11 (1/7)     | Gln Leu         |    |    |
| C12 (1/7)     | Ser Val         |    |    |
| G10 (1/7)     | Ser Ile         |    |    |
| Phage library | Heavy chain CDR3| 96 | 97 98 |
| Germline      | Tyr             |    |    |
| BL22 (RFB4)   | Ser Gly Tyr     |    |    |
| HA22 (G3)     | Ser Gly Tyr     |    |    |
| A3 (2/3)      | Ser Gly Phe     |    |    |
| C4 (1/3)      | Ser Gly Tyr     |    |    |

* Germline hot spot residues.

b Number of the clones with the same amino acid residues found among the clones sequenced.

* Immunotoxin was made.

d Non-germline hot spot residues (somatic mutations).

The purity of each recombinant immunotoxin is above 90%.

Cytotoxic Activity—The recombinant immunotoxins were tested on several CD22-positive cell lines. Cytotoxicity (IC50) was evaluated by both cell death assays (WST-8) in three Burkitt lymphoma cell lines (Daudi, Namalwa, and Ramos) and one B-CLL cell line (WSU-CLL) (Table IV) and protein synthesis inhibition ([3H]leucine incorporation) in Daudi cells (Fig. 7) and Raji, another Burkitt lymphoma cell line (data not shown). Immunotoxins containing the E6, B8, B5, and D8 Fv light chains were 2- or 3-fold more active than HA22 and up to 10-fold more active than BL22 in different B cell lines. Among these mutants, E6 and B8 Fv were found in 6 of 16 clones analyzed after biopanning, whereas D8 and B5 were found only once (Table II). Nevertheless, a common pattern of increase in their immunotoxin activities is present. More importantly, as shown in Table IV, in WSU-CLL, a cell line with low CD22 expression established from a B-CLL patient resistant to chemotherapy (29), the mutant immunotoxins (E6, B8, D8, and B5) were significantly more active than HA22 and BL22. Daudi cells have 1×10^5 CD22 sites/cell (37), but WSU-CLL cells have only 5500 CD22 sites/cell (10). None of the mutant immunotoxins were cytotoxic to the CD22-negative cell line MCF-7, which demonstrates that the cytotoxic effect of these immunotoxins is selective to antigen-positive cells.

Finally, we performed affinity measurement on Daudi cells (Table IV) by using monomeric recombinant immunotoxins. As predicted from the increase in cytotoxic activity, the binding affinity of mutants E6, B8, D8, and B5 for native CD22 on cells is increased from 2.3 to 0.8 nM. The increase in cytotoxicity was generally proportionally related to the increase in affinity.

Phage Display Libraries Targeting Non-germline Hot Spots—The results with phage library RFB4LibVL30/31 and previous data (21, 27) indicate that germline hot spot residues are good candidates for in vitro hot spot-based antibody evolution. To evaluate non-germline hot spot residues as targets, we constructed two other libraries (RFB4LibVH30/31 and RFB4LibVH96/97/98) that are targeted to two non-germline hot spot regions in heavy chain CDR1 (Ser^{30–}Ile^{31}) and CDR3.
Cytotoxicity was measured by cell death assays (WST-8). Briefly, cells were seeded at 5 x 10^4/well in a 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 the cell viability was measured with WST-8. Each assay was done in triplicate. IC₅₀ (mean values expressed in ng/ml) is the toxin concentration that reduced cell viability by 50% compared with the cells that were not treated with the toxin. The results are represented as means ± S.D. of triplicate determinations, and assays were repeated two or three times. In each CD22-positive cell line, repeated measures, analysis of variance with Dunnett’s and Student-Newman-Keuls post-tests, revealed a significant increase in cytotoxicity (IC₅₀) of E6, B8, D8, and B5 as compared with BL22, HA22, and the H11 and E12 mutants (p < 0.01 in Daudi and WSU-CLL cells, p < 0.05 in Namalwa and Ramos). The H11 and H12 mutants did not show increase of cytotoxicity as compared with HA22 (p > 0.05). No statistically significant differences among the E6, B8, D8, and B5 immunotoxins were observed (p > 0.05).

### Table IV

| Immunotoxin | Kᵦ (nM) | Burkitt lymphoma | B-CLL, WSU-CLL | Breast adenocarcinoma, MCF7 |
|-------------|---------|-----------------|----------------|-----------------------------|
|             |         | Daudi Namalwa Ramos |                |                             |
| BL22        | 5.8     | 2.3 ± 0.15      | 5.8 ± 0.2      | 20 ± 1.5                    |
| HA22        | 2.3     | 1.2 ± 0.3       | 5.3 ± 1.5      | 20 ± 4.5                    |
| E6          | 0.7     | 0.22 ± 0.03     | 0.6 ± 0.1      | 2.6 ± 0.4                    |
| B8          | 0.8     | 0.52 ± 0.02     | 0.8 ± 0.1      | 3.2 ± 0.3                    |
| D8          | 0.8     | 0.22 ± 0.02     | 0.8 ± 0.2      | 3.4 ± 0.3                    |
| B5          | 0.8     | 0.20 ± 0.03     | 0.6 ± 0.1      | 2.6 ± 0.2                    |
| H11         | 2.4     | 1.2 ± 0.2       | 5.2 ± 0.2      | 31 ± 6                      |
| E12         | 2.2     | 0.56 ± 0.05     | 1.4 ± 0.2      | 5.0 ± 0.1                    |

*Affinities were measured on Daudi cells with flow cytometry. Equilibrium constants and Scatchard plots were determined as described under "Experimental Procedures."

(Ser⁹⁶–Gly³⁷–Tyr⁹⁸), respectively. Ile³¹ was mutated from Ser³¹ in somatic hypermutation targeting the germline sequences around the RGYW (AGTAGCT) motifs (Fig. 1). Ser⁹⁶–Gly³⁷ was generated by somatic insertion between VH and D gene fragments. The library complexity of RFB4LibVH30/31 consisted of more than 2 x 10⁴ independent clones. For library RFB4LibVH96/97/98, five independent transformations were performed to produce more than 1 x 10⁵ independent clones. Because theoretically the minimum library size for three hot spot residue randomization is 32,768 (32 x 32 x 32), the actual size of RFB4LibVH96/97/98 is 3-fold larger. Sequence analysis of 10 independent clones from each mutant library demonstrated that the targeted hot spot residues were successfully randomized (data not shown). As shown in Fig. 3, the pattern of enrichment of both libraries was strikingly different from the RFB4LibVL30/31 phage library. More rapid and early (at the second round) enrichment of binders with FACS signals similar to HA22 in library RFB4LibVH30/31 (Fig. 3B) indicated that it contained a large number of binders similar to the starting Fv HA22. But unlike RFB4LibVL30/31, the third round of RFB4LibVH30/31 panning failed to enrich the binders. In the case of RFB4LibVH96/97/98 (Fig. 3C), a large enrichment with FACS signals similar to HA22 occurred only at the third round of biopanning, suggesting that there are a very small number of binders in the phage-display library, and these binders are likely similar to HA22. The pattern of phage enrichment was also demonstrated in an ELISA on soluble CD22-Fc proteins (Fig. 4).

Fig. 5 shows the CD22 binding activity of individual phage clones. Of 33 clones tested from the RFB4LibVH96/97/98 (2nd panel), 14 did not bind to CD22. Of the 19 binders, 9 had ELISA signals greater than the HA22 phage. Fig. 5, 3rd panel, shows the CD22 binding activity of phage clones from library RFB4LibVH96/97/98. Of 33 clones tested, only 7 bind to CD22. Only 2 clones had ELISA signals slightly greater than the HA22 phage clone. ELISA-positive clones from RFB4LibVH30/31 and RFB4LibVH96/97/98 were sequenced (Table II). The sequences of the mutants enriched from the RFB4LibVH30/31 and RFB4LibVH96/97/98 were very similar to that of the parental antibody. Among the mutants of RFB4LibVH30/31, only one clone contains a mutation (Val¹¹) other than Ile/Leu¹¹. It is also interesting to find that mutants selected from library RFB4LibVH96/97/98 targeting heavy chain CDR3 contained the similar sequence (Ser⁹⁶–Gly³⁷–Tyr³⁸) (A3) or the same sequence (C4) as the parental one, Ser⁹⁶–Gly³⁷–Tyr³⁸. Mutants H11 and E12 from LibRFB4VH30/31 were chosen to generate recombinant immunotoxins for further biological characterization. Cell death assays (Table IV) and protein synthesis inhibition assays (Fig. 7) showed that H11 and E12 were not more active than HA22. In fact, FACS analysis using the monomeric H11 and E12 immunotoxins showed that their binding to Daudi cells were similar to HA22 (Table IV). The results may suggest that the phage scFv binding assays are semi-quantitative, and a better indicator of successful enrichment may be that almost all the phage scFv clones bind the antigen strongly and that sequencing analysis shows that enrichment of new sequences.

**DISCUSSION**

The results presented here demonstrate that an evolution strategy using intrinsic mutational hot spots as targets can be used for antibody affinity maturation in vitro and therefore improve immunotoxin activity. More importantly, germline hot spots but not non-germline hot spots are good candidates for in vitro affinity maturation.

**Germline Hot Spots and In Vitro Affinity Maturation**—As shown in Table V, the number of germline RGYW hot spots tends to decrease in antibodies with high affinities, suggesting an overall correlation between affinity and number of hot spot-based somatic mutations that is characteristic of affinity maturation in vivo. The existence of germline hot spots in high affinity antibodies obtained in vivo may provide the possibility that in vitro affinity maturation targeting these hot spots could further increase the binding affinity. Therefore, we hypothesized that the germline hot spots could be good candidates for in vitro antibody affinity maturation. To test this hypothesis we first made a phage-display library targeting a germline hot spot located in light chain CDRI encoding serine-asparagine. This library successfully produced several mutants in which Ser¹⁰–Asn¹¹ is most frequently replaced by either Arg³⁰–Gly³¹ or Gly³⁰–Arg³¹. These Fv have affinities higher than the starting molecule, and immunotoxins made from them showed increased cytotoxic activity.

For comparison, we also made two phage-display libraries (RFB4LibVH30/31 and RFB4LibVH96/97/98) targeting non-germline hot spots in heavy chain CDRI and CDRII. Two immunotoxins made from the H11 and E12 Fv of the RFB4LibVH30/31 library containing mutations in the non-germline hot spots did not show increased affinity and cytotoxic activity on Daudi cells (Table IV; Fig. 7). As shown in Table II, the sequences of the H11 and E12 mutants were very similar to...
that of the parental antibody. Both heavy chain CDR1 mutants have Ile\textsuperscript{31} or Leu\textsuperscript{31}, indicating that in vitro affinity maturation simply regenerated the somatic hypermutation process in vivo which changed Ser\textsuperscript{31} to Ile\textsuperscript{31}. Among the mutants sequenced, only one clone was very different and contains mutation (Val\textsuperscript{31}) in place of Ile/Leu\textsuperscript{31} (Table II). Mutants selected from library RFB4LibVH96/97/98 targeting heavy chain CDR3 contained a similar sequence (Ser\textsuperscript{96–Gly97–Phe98}) (A3) or the same sequence (C4) as the parental one, Ser\textsuperscript{96–Gly97–Tyr98}. In the mutated A3 clone Ser\textsuperscript{96} is encoded by TCG instead of AGT present in the original clone indicating truly successful enrichment and the importance of Ser\textsuperscript{96}.

**Fig. 7. Inhibition of protein synthesis.** Inhibition of protein synthesis was determined as percentage of \(^{3}H\)leucine incorporation in Daudi cells after 20 h of treatment with immunotoxins. B5, B8, E6, D8, H11, and E12 are recombinant immunotoxins (scFv-PE38). The graphs show protein synthesis as measured by counts/min of \(^{3}H\)leucine incorporated into protein. Dashed lines indicate 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 \(\mu\)g/ml of cycloheximide. Error bars, S.D. from the means of triplicate experiments. IC\(_{50}\) values for E6 (A), B8 (B), D8 (C), B5 (D), H11 (E), and E12 (F) were 0.25 ± 0.04, 0.24 ± 0.07, 0.28 ± 0.02, 0.24 ± 0.03, 0.7 ± 0.04, and 0.72 ± 0.08 (ng/ml), respectively, as compared with HA22 (G3) (0.7 ± 0.1 ng/ml). Repeated measures analysis of variance analysis with post-hoc Dunnett’s and Student-Newman-Keuls tests revealed that the E6, B8, D8, and B5 mutants significantly increased their activity by ~2.8-, 3-, 2.5-, and 3-fold as compared with HA22 (p < 0.01). When tested under similar conditions, the H11 and H12 mutants did not show any increase of cytotoxicity (p > 0.05). No statistically significant differences among the E6, B8, D8, and B5 immunotoxins were observed (p > 0.05).
in antigen binding of RFB4. Together, these results may suggest that heavy chain CDR non-germline hot spot residues (Ile$^{51}$, Ser$^{52}$, and Gly$^{53}$), which have been mutated in \textit{vitro} by a somatic process, are residues important for antigen binding and therefore may be unchanged by \textit{in vitro} affinity maturation.

Furthermore, we analyzed mutants obtained from previous \textit{in vitro} hot spot-based affinity maturation studies in our laboratory (21, 27). In the case of first generation mutant HA22, germline hot spot residues Ser$^{50}$–Ser$^{54}$ (IGHV1*01, GenBank accession number J00434) of heavy chain CDR3 were mutated to Thr$^{50}$–His$^{54}$ (IGHD-FL16.1*01, GenBank accession numbers AJ235939, AJ231233, and AJ231211) is mutated. SS is an anti-mesothelin single chain Fv antibody. SS1 is the mutant with an increased affinity selected by phage display (21). In SS1 germline hot spot Gly$^{53}$–Tyr$^{54}$ in the heavy chain CDR3 (V$\alpha$ germline sequences in GenBank accession numbers AJ235939, AJ231233, and AJ231211) is mutated to Lys$^{53}$–His$^{54}$. In contrast, when targeting a non-germline hot spot (Ser$^{53}$–Thr$^{54}$) in the heavy chain CDR2 of the anti-Tac antibody in our laboratory, mutants enriched from the phage-display library have an affinity similar to the original Fv (data not shown). Almost all mutants contain Thr at the 54 position with nucleotide sequences different from the original clone, indicating true enrichment and the importance of Thr$^{54}$ in the antigen binding. It suggests that the phage-display library targeting a non-germline hot spot might simply regenerate the in \textit{vivo} somatic hypermutation process in which the original germline hot spot Ser$^{52}$–Ser$^{54}$ (IGHV1S27*01, GenBank accession number X02459) was mutated into the non-germline hot spot Ser$^{52}$–Thr$^{54}$.

Most interestingly, in a recent study Wittrup and colleagues (19) created a random yeast surface-displayed scFv mutant library of humanized MFE-23 by mutagenic PCR. MFE-23 is an antibody specific for carcinoembryonic antigen. An scFv with a dissociation half-time over 4 days at 37 °C was successfully isolated after four sorts. Two point mutations responsible for this affinity increase were identified and analyzed. The change that led to the greatest improvement in affinity is Ser$^{50}$Leu$^{50}$ to Ser$^{50}$Leu$^{50}$. This mutation in the light chain CDR2 loop. The mutation of the germline hot spot residue (Ser$^{50}$) to Leu$^{50}$ appears to have a great effect on binding affinity and stability of the MFE-23 antibody. Based on the results described in this study and that of others, it is reasonable to expect that germline hot spots may prove valuable in antibody affinity maturation \textit{in vitro}.

### Table V

| Antibody | VL CDR1 | VL CDR2 | VL CDR3 | VH CDR1 | VH CDR2 | VH CDR3 | Germline hot spots | $K_d$ |
|----------|--------|--------|--------|--------|--------|--------|-------------------|------|
| RFB4     | 1      | 0      | 1      | 0      | 1      | 3      | n.a.              |      |
| HA22     | 1      | 0      | 1      | 0      | 1      | 0      | 3                | 2.3  |
| E6       | 0      | 0      | 1      | 0      | 1      | 0      | 2                | 0.7  |
| B8       | 0      | 0      | 1      | 0      | 1      | 0      | 2                | 0.8  |
| B5       | 0      | 0      | 1      | 0      | 1      | 0      | 2                | 0.8  |
| SS       | 2      | 1      | 1      | 1      | 2      | 1      | 8                | 11   |
| SS1      | 2      | 1      | 0      | 1      | 2      | 1      | 7                | 0.72 |
| Anti-Tac | 1      | 0      | 0      | 2      | 0      | 0      | 3                | 0.76 |
| H26      | 2      | 0      | 2      | 1      | 2      | 0      | 7                | 7    |
| H10      | 1      | 0      | 2      | 1      | 2      | 0      | 6                | 0.3  |
| H8       | 1      | 0      | 2      | 1      | 0      | 0      | 4                | 0.2  |

*Number of germline RTGW hot spots and affinity maturation*

Top, immunotoxins containing the Fv portions of RFB4, SS, and Anti-Tac are currently under investigation in our laboratory for affinity maturation. The original Fv (boldface) and its mutants obtained by \textit{in vitro} hot spot mutagenesis are shown. Affinities ($K_d$ values) were measured by flow cytometry (RFB4 and its mutants) or BIAcore (SS, SS1, and Anti-Tac) by using recombinant immunotoxins. Bottom, three independent monoclonal antibodies (H26, H10, and H8) from mice immunized with the same protein antigen (hen egg white lysozyme) that recognize overlapping epitopes on the antigen (42, 43). All three antibodies use the same V$\lambda$ (Igk-v23) and VH (VHM460) germline genes.
dissociation beyond 30 min. In the present study, in the final round of biopanning on Daudi cells expressing the CD22 antigen in the presence of 10-fold excess of phage antibodies, the total duration of the off-rate selection was 6 h, a far longer interval than the 30-min window. In order to inhibit the internalization of CD22, we used NaN₃ and low temperature during the cell panning. However, we were not able to increase the affinity to 0.1 nM or better. It is possible that we would have selected a better mutant if we used more (100-fold) competitors in the panning (18), longer off-rate selection, or more rounds of enrichment. What is more likely is that a mutant with a Kₒₕ of 0.1 nM or less simply does not exist in the current hot spot library and therefore further stepwise evolutionary process targeting hot spots in other CDR regions of RFBy (e.g. L-CDR3) may be needed.

**Linkage of Affinity with Cytotoxicity of Immunotoxins**—In previous works, we found a discrepancy between an increase in affinity and cytotoxicity when panning or measuring the affinities by using recombinant protein made in E. coli or peptides (21, 26). In the present study, we initially performed phage panning on recombinant CD22-Fc proteins with the phage-display library RFByLibVL30/31. Mutants containing Ser³⁰–Lys³¹ and Ser³⁰–Gly³¹ in light chain CDR1 were successfully enriched after the third round panning. Several clones with the same amino acid (Ser³⁰–Lys³¹) but different nucleotide sequences were found. A Ser³⁰–Lys³¹ mutant (A1E) was chosen for further characterization. It bound to the CD22-Fc proteins with an ELISA signal 3-fold higher than HA22. But when tested on CD22-positive cells, a recombinant immunotoxin containing the A1E Fv had cytotoxic activity only slightly (~30%) better than HA22 on Raji cells and almost the same as HA22 on Daudi cells (data not shown). Because of that, we decided to use cells expressing native CD22 molecules on the cell surface for both biopanning and affinity determination. It appeared to improve the linkage of phage-displayed antibody binding affinity with immunotoxin cytotoxicity. By doing that, we should not have missed mutant antibodies recognizing conformational or carbohydrate epitopes. It may be especially important in this study because the precise epitope of the RFBy antibody on CD22 has not been elucidated.

**Structural Implications of Mutations**—The formation of antibody-protein antigen complexes involves a combination of electrostatic and hydrophobic interactions at the interface between the two proteins (40). First generation mutant HA22 replaces the Ser¹⁰⁰–Ser¹⁰⁰a–Tyr¹⁰⁰b (Fig. 2B) with Thr¹⁰⁰–His¹⁰⁰a–Trp¹⁰⁰b on the heavy chain CDR3 loop. Second generation mutants enriched from phage-display library RFByLibVL30/31 replace Ser³⁰–Asn³¹ with Gly³⁰–Arg³¹ (E6) and Arg³⁰–Gly³¹ (B8) in light chain CDR1 (Table II). In both cases, positively charged residues were selected. In fact, we previously found that a single histidine can increase the affinity by 3-fold (27). It is reasonable to expect that the surface-exposed positions of basic residues may strengthen the electrostatic interactions at the interface between the RFBy antibody with the CD22 antigen.

In summary, the uphill-climbing approach using intrinsic germline hot spots successfully evolved several immunotoxins with increased activity on B lymphoma cells including CLL, strongly suggesting that they should be very useful in the treatment of CLL and other cancer that express low levels of CD22. Furthermore, potential applications of this germline hot spot strategy extend beyond antibody affinity maturation and improvement of immunotoxin activity to the modification of receptors, ligands, and other biologically important proteins because somatic hypermutation may be a global phenomenon in the whole genome, and similar DNA hot spot mechanisms are not restricted to immunoglobulin genes (41).
In Vitro Antibody Evolution Targeting Germline Hot Spots to Increase Activity of an Anti-CD22 Immunotoxin

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