HA22 (R490A) Is a Recombinant Immunotoxin with Increased Antitumor Activity without an Increase in Animal Toxicity

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ABSTRACT

Purpose: **RFB4 (dsFv)-PE38 (BL22) is a recombinant immunotoxin containing an anti-CD22 (Fv) fused to truncated *Pseudomonas* exotoxin A, which induces a high complete remission rate in patients with purine analogue–resistant hairy cell leukemia. HA22 is a mutant of BL22 with mutations in heavy-chain CDR3 resulting in increased cytotoxic activity. Our goal was to improve the activity of HA22.**

Experimental Design: **Arg^{490}, which is located in the catalytic domain (III) of the immunotoxin HA22, was mutated to alanine. Purified immunotoxins were produced and tested for cytotoxic activity in cell culture and for antitumor activity and nonspecific toxicity in mice. ADP-ribosylation activity was also measured.**

Results: **HA22 (R490A) is ~2-fold more cytotoxic than HA22 on several CD22-positive cell lines. When injected i.v., HA22 (R490A) has more potent antitumor activity than HA22 against CA46 tumors in mice. HA22 and HA22 (R490A) have similar LD_{50}s (~1.3 mg/kg) and similar plasma half-lives. The R490A mutation also improved the cytotoxicity of the antimesothelin recombinant immunotoxin SS1 (dsFv)-PE38 (SS1P). *In vitro* ADP-ribosylation assays show that HA22 R490A has increased activity. Increased cytotoxic activity is probably related to this increase in ADP-ribosylation activity.**

Conclusion: **Protein engineering can be used to increase the efficacy of recombinant immunotoxins. Because HA22 (R490A) has increased antitumor activity without increased animal toxicity, immunotoxins with this mutation are candidates for clinical development.**

INTRODUCTION

In the past decade, monoclonal antibodies, radioimmunoconjugates, and immunotoxins have emerged as promising agents for the treatment of cancer and particularly for the treatment of hematologic malignancies. Immunotoxins are hybrid proteins that are targeted to cancer cells by monoclonal antibody domains that bind to antigens that are highly expressed on cancer cells and are not expressed on essential normal cells (1). The antibody domains are fused to truncated protein toxins that can kill the cell after the immunotoxin is internalized. Hematologic malignancies are particularly appropriate for immunotoxin therapy because the tumor cells are often present in the blood where they are readily accessible and the target antigens are often highly expressed (2).

CD22 is a lineage-restricted B-cell antigen that is expressed on B-chronic lymphocytic leukemia, hairy cell leukemia, acute lymphocytic leukemia, and Burkitt's lymphoma. The RFB4 antibody that specifically reacts with CD22 has been used to make a recombinant immunotoxin in which the Fv fragment stabilized by a disulfide bond is fused to a 38 kDa truncated form of *Pseudomonas* exotoxin A (PE; refs. 3–5). PE38 (a 38 kDa mutant form of PE A) contains the translocating and ADP-ribosylating domains of PE but not the cell-binding portion. In preclinical studies, RFB4 (disulfide-stabilized single-chain antibody fragment [dsFv])-PE38 (BL22) was shown to kill cell lines expressing CD22 as well as leukemic cells from patients with hairy cell leukemia and chronic lymphocytic leukemia. It also induced complete remissions in mice bearing lymphoma xenografts (6, 7). BL22 has been evaluated in a phase I clinical trial at the National Cancer Institute in patients with hematologic malignancies. In the initial report, 16 patients with purine analogue-resistant hairy cell leukemia were treated with BL22 and 11 (68%) achieved complete remission (1).

HA22 is an improved form of BL22 in which the Fv was mutated and antibody phage display used to isolate mutant phage that bound better to CD22. In HA22, residues SSY in heavy-chain CDR3 have been mutated to THW. HA22 has a 5-fold to 10-fold increase in cytotoxic activity on various CD22-positive cell lines and is up to 50 times more cytotoxic to cells from patients with chronic lymphocytic leukemia and hairy cell leukemia (8).

Based on the crystallographic structure of PE (9) and many functional studies, BL22 and other immunotoxins kill target cells by a complex series of steps that are initiated by binding to a cell surface molecule and followed by endocytosis, proteolytic processing, and translocation into the cytosol. In the cytosol, the ADP-ribosylation activity located within domain III of PE catalytically inactivates elongation factor 2, inhibiting protein synthesis and initiating programmed cell death.

Analysis of the three-dimensional structure of domain III of PE has shown that residues 486 to 493 are arranged in a flexible loop with a poorly ordered structure on the surface of domain III. The functional role of this flexible loop has not been defined (10). Brinkmann et al. (11) found that deletion of a trypsin recognition site (R490) in domain III of native PE made it resistant to proteolytic digestion and prolonged its survival in the circulation of mice.

In the present work, we have investigated the effects of mutating Arg^{490} to alanine on the properties of immunotoxin

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HA22. We investigated its cytotoxic activity, its half-life in the circulation, and its toxicity and antitumor activity in mice. We chose Arg^{490} because this residue constitutes a protease recognition site.

MATERIALS AND METHODS

Site-Directed Mutagenesis

Mutations were introduced using the two-step overlap PCR method and the RFB4 [variable domain heavy chain (V_H)-GTHW]-PE38 plasmid DNA used as the template. Mutagenic primers that contain mutated sites (bold) and restriction endonuclease sites of *Sal*I and *Eco*RI (underlined) are as follows: primer A (5′-GAACCCGACGCA**GCC**GGCCGTATC-CGCAAC-3′, upstream) and B (5′-GTTGCGGA-TACGGCC**GGC**TGCGTCGGGTTC-3′, downstream) and C (5′-GCTGTC GTGGAACCAGGTCGACCAGG-3′) and D (5′-CTTTGTTAGCAGCCGAATTCATAT TCGAT-3′). First, PCR reactions were amplified using primers A and D or primers B and C. A portion (0.01 mL) of each of first reactions was combined and directly used in a second PCR with only primers C and D. This reaction generated a 1,000 bp product that contained the mutation. DNA amplified using this procedure was then cloned into the Invitrogen T/A cloning vector pCR II (Invitrogen, Carlsbad, CA) without further purification, transformed into *E. coli* DH5α, and identified using blue-white screening procedures. Positive clones were sequenced using the primers C and D. The mutated insert was removed from pCR II by digesting the plasmid with *Sal*I and *Eco*RI endonuclase and the fragment was ligated to identically digested V_H-PE38 plasmid.

Expression and Purification of HA22 (R490A)

To produce immunotoxin, the two components, variable domain light chain (V_L) and V_H-PE38 (R490A), were expressed in *E. coli* BL21 (λDE3) where the proteins accumulated in inclusion bodies. Immunotoxins were then purified as previously described (5, 12).

Preparation of SS1P (R490A). R490A was constructed by PCR-based site-directed mutagenesis. After *Sal*I and *Eco*RI endonucleases digestion, the mutated insert was cloned into the identically digested SSV_H-PE38 plasmid DNA. To produce SS1P (R490A) immunotoxin, SS1 V_L and SSV_H-PE38 (R490A) were expressed in *E. coli* BL21 (λDE3) and purified to near homogeneity as previously described (5, 12).

Cell Lines. CD22-positive human Burkitt lymphoma cell lines (CA46, Daudi, and Raji) were obtained from American Type Culture Collection (Manassas, VA). CD-25-positive HUT-102 was obtained from T. Waldmann (NIH, Bethesda, MD) and human umbilical vascular endothelial cells (HUVEC) were purchased from Clonetics (San Diego, CA). A431-K5 cells were derived from the A431 cell line (human epidermoid carcinoma) by cotransfection of A431 cells with a plasmid encoding mesothelin (13). The ovarian cancer cell line A1847 was obtained from Dr. S. Aaronson (National Cancer Institute, Bethesda, MD).

Cytotoxicity Assay. The specific cytotoxicity of HA22 (R490A) was determined by protein synthesis inhibition assays (7, 8). The concentrations of immunotoxin that reduced [^{3}H]leucine incorporation by 50% relative to untreated control culture were defined as the IC_{50}. The cytotoxic effect of SS1P (R490A) was evaluated on two mesothelin-positive cancer cell lines, A431/K5 (an epidermoid carcinoma cell line transfected with full-length mesothelin cDNA) and A1847, using a protein synthesis inhibition assay previously described (11).

Cell Viability Assay. Inhibition of cell growth upon treatment with immunotoxin was determined in standard 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo-phenyl)-2*H*-tetrazolium, monosodium salt (WST) assays based on the reduction of tetrazolium salt to formazan by the enzymes from viable cells (14). Viability was expressed as percentage of untreated controls. CA46, A431/K5, and A1847 cells were plated at 8,000 cells/well in a 96-well plate. HUVECs were seeded in 96-well plates at 3,000 cells/well. Plates were incubated for 40 hours or for 72 hours (HUVEC) at 37°C (15). Five microliters WST-8 solution was added to each well of the plate and incubated for 4 hours at 37°C. To correct for background activity, cells were cultured in the presence of cycloheximide at 10 μg/mL.

ADP-Ribosylation Activity Assay

ADP-ribosylation activity of HA22 and HA22 (R490A) was determined by measuring transfer of ADP-ribose from [^{14}C]NAD to EF-2 (16).

Nonspecific Animal Toxicity Assay. On day 0, female Swiss mice (5-6 weeks, 18-22 g) were given a single injection into the tail vein of various amounts of immunotoxin in 0.2 mL PBS containing 0.2% human serum albumin. Animal mortality was observed over 2 weeks.

Pharmacokinetics

NIH Swiss mice were injected into the tail vein with 10 μg HA22 or HA22 (R490A). Blood samples were drawn at different times (2, 5, 10, 20, 30, 40, 50, 60, and 90 min) and the concentration of immunotoxin determined by ELISA. A standard curve was made with each pure immunotoxin. Briefly, microtiter plates were coated with 50 μL CD22-Fc protein (5 μg/mL; ref. 8) in PBS at 4°C overnight. The plates were blocked with PBS containing 3% bovine serum albumin at room temperature for 2 hours, followed by washing five times in PBS containing 0.05% Tween 20. Standards and samples were diluted 1/100, 1/500, and 1/1,000 in PBS with 1% normal mouse serum. One hundred microliters diluted standards or samples were applied, followed by incubation with 50 μL of 1:250 dilution of horseradish peroxidase–conjugated anti-PE antibody for 3 hours at room temperature. After washing, plates were developed using 3,3′,5,5′-tetramethylbenzidine for 10 minutes and the absorbance read at 450 and 650 nm. The assays were done in triplicate.

Antitumor Activity and Statistics

The antitumor activity of the immunotoxins was determined in severe combined immunodeficiency (SCID) mice bearing CA46 cells (6). Cells (1×10^7) were injected s.c. into SCID mice (5 weeks, body weight 18 g) on day 0. Tumors ~100 mm^3 developed in animals by day 6 after tumor implantation when treatment was initiated. All data are presented as mean ± SD. Differences in data from animal experiments were analyzed for significance with Wilcoxon scores (rank sum) test. All mentioned changes in distribution qualified for the probability level $P < 0.05$.

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RESULTS

Preparation and Characterization of Immunotoxins. All immunotoxins were constructed and purified as described in Materials and Methods. The final yield of the purified HA22 (R490A) protein is 6% of the starting inclusion body protein and that of HA22 is 8%. Figure 1*A* shows the elution profile of HA22 (R490A) from a Superose-12 gel filtration column (Amersham Pharmacia, Piscataway, NJ). There is one peak eluting in fractions 11 to 15, the position expected of a protein with a molecular weight of M_r 63,000. Figure 1*B* shows the SDS-PAGE analysis of the peak fraction (fraction 12). HA22 (R490A) migrates as a single band with the expected molecular weight of M_r 63,000 in a nonreducing gel. The other immunotoxins had a purity comparable with HA22 (R490A; data not shown).

Cytotoxicity of HA22 (R490A). The cytotoxic activities were evaluated on several CD22-positive B-cell lymphoma cell lines (Daudi, CA46, and Raji) and on a CD22-negative Hodgkin's lymphoma (L540) and a T-cell leukemia line (HUT-102) using a protein synthesis inhibition assay. These values were compared with the activity of BL22, the immunotoxin from which HA22 was derived (Table 1). As shown in Fig. 2 and Table 1, HA22 (R490A) is ~2-fold more active on Daudi and on CA46 cells than HA22 and is 3-fold more active on Raji cells. BL22 is much less active than either immunotoxin. The immunotoxins were also tested on the CD22-negative L540 or HUT-102 cell lines and found to be >1,000-fold or 10,000-fold less toxic, demonstrating that these immunotoxins are specific for CD22-expressing cells.

Cell Viability Assay. The activities of the immunotoxins were also assessed on CA46 cells using a cell viability assay (Fig. 2*C*; ref.14). The concentrations of HA22 (R490A), HA22, or BL22 required to cause 50% inhibition (IC_{50}) of cell viability are 0.2 ± 0.01, 0.36 ± 0.05, and 1.3 ± 0.08 ng/mL, respectively. The magnitude of the differences in activities among these three immunotoxins shows the same dose-response relationship as for the inhibition of protein synthesis assays.

To further assess specificity, we examined whether HA22 (R490A) could result in induction of endothelial cell death. We

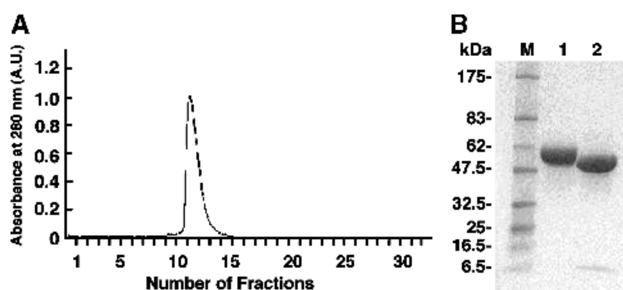

Fig. 1 *A,* purified HA22 (R490A) immunotoxin elution profile from Superose-12 gel filtration column chromatography. *B,* SDS-PAGE of HA22 (R490A) under nonreducing and reducing conditions. HA22 (R490A) was prepared as described in Materials and Methods and elution fraction 12 was analyzed on a 4-20% polyacrylamide gel. *X*-axis, number of fractions marked on the chromatogram. Lanes: molecular weight standards (*lane M*); nonreducing condition (*lane 1*); the purified HA22 (R490A) immunotoxin (*lane 2*) was reduced by boiling for 5 minutes in SDS sample buffer containing DTT. The gel was stained with Coomassie blue. The nonreduced dsFv immunotoxin migrated M_r ~63,000 and dissociated into V_L chain (M_r ~12,000) and V_H-PE38 fusion protein (M_r ~51,000) by reduction.

Table 1 Cytotoxicity activity (IC_{50}) in ng/mL of BL22 and mutant immunotoxins toward various cell lines

	CD22-positive cell line (Burkitt's lymphoma)			CD-22-negative cell line	
	Daudi	CA46	Raji	HUT-102*	L540†
BL22	2.78 ± 0.11	0.85 ± 0.04	2.63 ± 0.24	>2,000	>10,000
HA22	0.46 ± 0.02	0.24 ± 0.01	1.02 ± 0.09	>2,000	>10,000
HA22 (R490A)	0.18 ± 0.01	0.18 ± 0.02	0.27 ± 0.02	~2,000	>10,000

NOTE. Cytotoxicity data are given as IC_{50}'s, which are the concentrations of immunotoxin that cause a 50% inhibition in protein synthesis compared with controls after incubation with cells for 20 hours. Means values of three experiments ± SD are shown.

*CD22-negative, human T-cell lymphotrophic virus-1–positive T-cell lymphomas.

†CD22-negative, CD30-positive Hodgkin's lymphomas.

examined endothelial cells because they do not express CD22 but may have a role in the toxic side effects of some immunotoxins (15). The endothelial cell line HUVEC was treated with either HA22 (R490A), HA22, BL22, HB21 (Fv)-PE40, or LMB-7. HB21 (Fv)-PE40 targets the transferrin receptor that is widely expressed on many cell types and was expected to be cytotoxic to HUVEC. LMB7 targets the Le^Y antigen previously shown to be expressed on HUVEC (16). HA22 (R490A), HA22, or BL22 did not decrease the viability of HUVECs (Fig. 2*D*). However, as expected, both HB21 (Fv)-PE40 and LMB7 were cytotoxic to the cells (16). These results further confirm the specificity of HA22 (R490A).

Mouse Studies. Because HA22 (R490A) was more cytotoxic to the CD22-positive cell lines than HA22, we compared its antitumor activity with HA22. Before doing this, we determined its LD_{50}. Groups of five or more mice received a single i.v. injection of various doses of HA22 (R490A), HA22, or BL22 and were observed for 2 weeks. HA22 and HA22 (R490A) have very similar animal toxicities with LD_{50}s of ~1.3 mg/kg (Table 2). BL22 was slightly more toxic with all mice dying at a dose of 1.25 mg/kg. The calculated (interpolated) LD_{10} and LD_{50} are 1.01 and 1.12 for BL22, 1.06 and 1.33 for HA22, and 1.08 and 1.38 for HA22 (R490A). Almost all of the deaths occurred within 72 hours after treatment. These data show that the R490A mutation has very little effect on mouse toxicity.

Pharmacokinetics. Another important parameter of antitumor activity is the length of time the immunotoxin remains in the circulation and can interact with the tumor cells. To determine the $t_{1/2}$ of HA22 or HA22 (R490A), mice were injected i.v. with a single dose of 10 μg HA22 or HA22 (R490A). Blood samples were drawn at different times over a 90-minute period and the concentration in the plasma was measured. Each data point used to determine half-life is the average of samples from four animals. In Fig. 2*F*, the plasma levels of HA22 and HA22 (R490A) are shown beginning 2 minutes after a single bolus i.v. injection of 10 μg HA22 or HA22 (R490A). Initial plasma levels reached 15,502 ± 552 and 14,857 ± 1,850 ng/mL, respectively. The decay curves are close to monoexponential. The plasma half-life of HA22 (R490A) is 18.8 minutes, not significantly different from HA22 (21.9 minutes, $P = 0.375$).

Antitumor Activity. To determine if the improved *in vitro* cytotoxic activity was translated into increased antitumor activity, HA22 (R490A) and HA22 were compared using tumor

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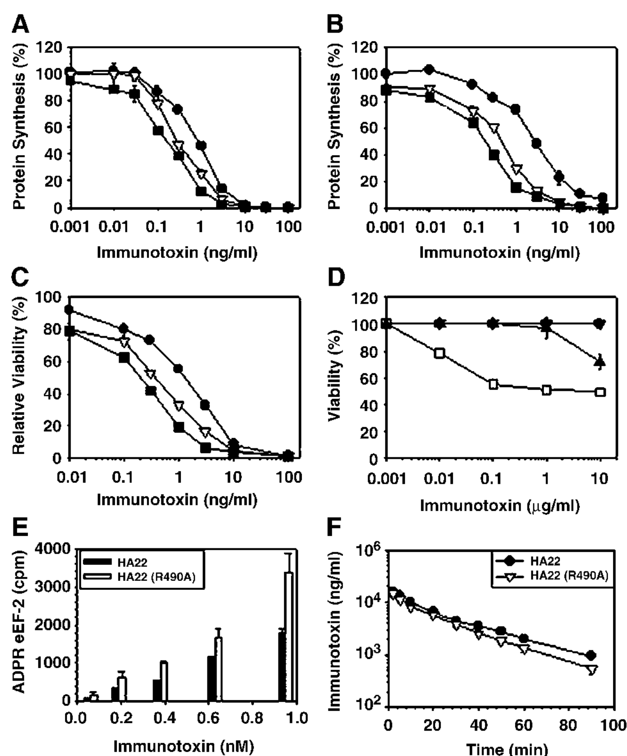

Fig. 2 Inhibition of protein synthesis (*A* and *B*) and cell viability (*C*) on CD22-positive cells. Inhibition of protein synthesis was determined as percentage of [^{3}H]leucine incorporation in cells after 20 hours of treatment with indicated concentrations of immunotoxins. *A*, CA46 cells; *B,* Daudi cells. Fifty percent inhibition of protein synthesis is halfway between the level of incorporation in the absence of toxin and that in the presence of 10 μg/mL cycloheximide. *C*, CA46 cells were incubated with immunotoxin for 40 hours before WST-8 was added for 4 hours. Formazan production was measured at $A_{450\ nm}$ and $A_{650\ nm}$. Points, mean of triplicate values; bars, SD. In *A*-*C*, ●, BL22; ▽, HA22; ■, HA22 (R490A). *D*, viability of HA22 (R490A) on HUVEC. HUVEC 3,000 cells/well incubated with various concentrations of immunotoxins for 72 hours. Cell viability was determined by a WST assay as described in Materials and Methods. Results are given as %viability of incubation without immunotoxin; points, mean of triplicate values; bars, SD. ●, BL22; ▽, HA22; ■, HA22 (R490A); ◇, HB21Fv-PE40; ▲, LMB-7. *E,* comparison of ADP-ribosylation activity between HA22 and HA22 (R490A). ADP-ribosylation assays were done as described (16) in duplicate. *F*, pharmacokinetics of HA22 (R490A) in mice. Normal female Swiss mice were injected i.v. with 10 μg HA22 (●) and HA22 (R490A; ▽). Blood samples were drawn at different times. The concentration of each immunotoxin in the circulation was determined by ELISA.

xenografts of CA46 cells growing in SCID mice. CA46 cells (1×10^7) were implanted into the flanks of SCID mice on day 0. On day 6, when the tumors reached ~100 mm^3 in size, the animals were injected i.v. with either 300 μg/kg (n = 10) or 150 μg/kg (n = 8) HA22 or HA22 (R490A) every other day ×3. As shown in Fig. 3, treatment with HA22 (R490A) or HA22 decreased tumor size compared with controls. By day 10, tumors in mice receiving 300 μg/kg of HA22 (R490A) fell to 85 ± 28.5 mm^3 in size, whereas in mice treated with 300 μg/kg of HA22 the tumors were 126 ± 31.2 mm^3 in size. Treatment with 150 μg/kg HA22 (R490A) resulted in tumors averaging 302 ± 143.6 mm^3 on day 18, whereas tumors treated with HA22 were significantly larger, averaging 592 ± 189 mm^3 (Fig. 3*A*). Antitumor activity was also dependent on dose; 150 μg/kg was less effective than 300 μg/kg for both immunotoxins. Without treatment, CA46 tumors grew rapidly. A significant difference in tumor size (Wilcoxon test, $P < 0.001$) was found between mice that received HA22 and mice that received HA22 (R490A) at 150 μg/kg on treatment day 12 (P = 0.0007), on day 14 (P = 0.0008), and on day 16 (P = 0.0008); and with 300 μg/kg treatment on day 14 (P = 0.0009; Fig. 3*C*). To show that the inhibition of tumor growth by HA22 and HA22 (R490A) is specific, we also treated mice with CA46 tumors with SS1P (R490A) that targets the mesothelin antigen. SS1P (R490A) did not inhibit the growth of CA46 tumors (data not shown).

The animal toxicity at each of these dose levels was evaluated by weighing the mice. There were no deaths but some weight loss. At 300 μg/kg, 2 of 10 mice treated with HA22 (R490A) and 3 of 10 mice treated with HA22 experienced mild weight loss (<5%) during the 4 days after the first injection. The mice began to regain weight 2 to 4 days after the last injection. No significant difference in body weights was seen between the HA22 (R490A)–treated group and the HA22 group. In contrast, at the 150 μg/kg dose, the weight curves of the immunotoxin-treated groups paralleled very closely that of the control (untreated) group (data not shown). We conclude that HA22 (R490A) has a more potent antitumor activity than HA22.

Immmunotoxin SS1P. Mesothelin is an antigen that is highly expressed on pancreatic and ovarian cancers and mesotheliomas. SS1P is an immunotoxin that binds to mesothelin and kills mesothelin-expressing cells (13). To determine if the R490A mutation would also increase the cytotoxic activity of an immunotoxin target in an epithelial cancer, we introduced the R490A mutation into SS1P to produce SS1P (R490A). The data in Table 3 shows that SS1P (490A) was significantly more active than SS1P on two mesothelin-expressing cell lines with an ~2-fold increase in activity.

ADP-Ribosylation Studies. The mechanism by which immunotoxins kill cells has been intensively studied. Many steps have been identified beginning with binding to a cellular receptor and ending in ADP ribosylation and inactivation of elongation factor 2 (17). Because R490 is located in that portion of domain III that is involved in the ADP ribosylation of EF2, we did ADP-ribosylation assays using [^{14}C]NAD and wheat germ abstracts. The results of these assays are shown in Fig. 2*E*. Using various concentrations of immunotoxin, there is an ~2-fold increase in ADP-ribosylation activity with the R490A mutation.

Table 2 Nonspecific toxicity in mice of HA22 and HA22 (R490A)

Dose (mg/kg)	BL22	HA22 (death/total)	HA22 (R490A)
0.75	0/10	0/5	
1.0	1/14	0/10	0/15
1.25	14/14	4/10	5/15
1.50	9/9	7/10	10/15
1.75		9/10	15/15

NOTE. On day 0, female Swiss mice (5-6 weeks, 18-22 g weight) were treated by tail vein single injection with various amounts of immunotoxins in 0.2 mL PBS containing 0.2% human serum albumin. Animal mortality was observed for 2 weeks.

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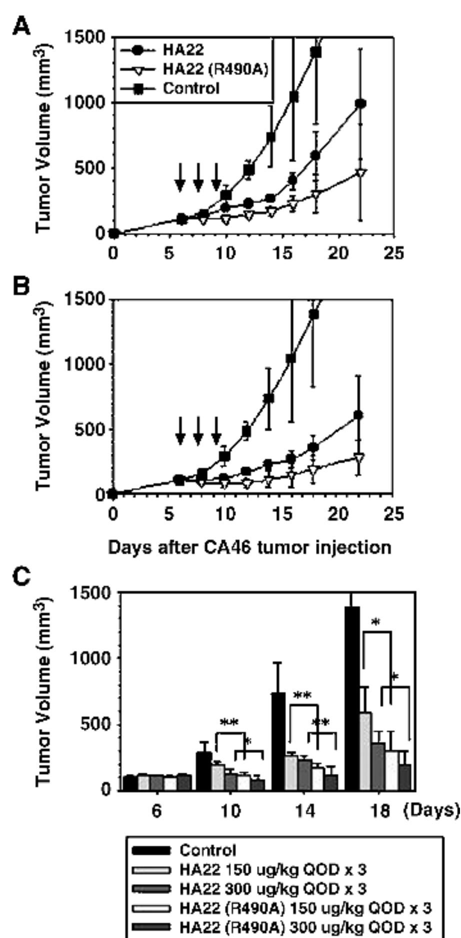

Fig. 3 Antitumor activities of HA22 and HA22 (R490A). CA46 cells were inoculated s.c. in SCID mice on day 0. *A*, on day 6, when tumors >100 mm^3 developed, groups of 8 or 10 mice were either observed (■) or began treatment with i.v. injections of HA22 (●) or HA22 (R490A; ▽) diluted in 0.2 mL PBS/0.2% human serum albumin. Therapy was given once every other day ×3 (on days 6, 8, and 10; *arrows*) at 150 μg/kg. *B*, the antitumor response of mice treated with HA22 (●) at 300 μg/kg every other day ×3 was contrasted with that of mice that were untreated (■) or treated with HA22 (R490A) 300 μg/kg every other day ×3 (▽). No death was observed at these doses. Points, mean; bars, SD. *C*, comparisons between HA22 and HA22 (R490A) at each dose were statistically significant. *$P < 0.05$; **$P < 0.001$ (Wilcoxon's rank sum test).

DISCUSSION

HA22 is a recombinant immunotoxin that has a high affinity for CD22 and is very active in killing cells from B-cell malignancies that express CD22 (8). The initial goal of the present study was to make a mutant of HA22 that is resistant to proteolytic digestion and, therefore, would have increased antitumor activity either because of less intracellular proteolytic degradation or decreased degradation in the circulation. Previous studies had shown that destruction of a proteolytic digestion site by deletion of Arg^{490} in domain III of native PE resulted in an increase in the half-life of PE in the circulation of mice. We reasoned that this increase in half-life could lead to an increase in antitumor activity. To minimize any changes in protein structure that a mutation at position 490 might introduce, we mutated R490 to alanine rather than delete R490 as was previously done with native PE (11).

We found that mutation of Arg^{490} to alanine in the toxin portion of two different recombinant immunotoxins, HA22 and SS1P, resulted in a 2-fold to 3-fold increase in cytotoxic activity and with HA22 a corresponding increase in antitumor activity. This enhanced antitumor activity occurs without any change in nonspecific toxicity in normal mice or in the tumor-bearing animals. The mutation is in the catalytic domain (domain III) of the toxin. Arg^{490} is part of a flexible loop on the surface of domain III of PE and this loop was shown to be more susceptible to proteolytic digestion than other regions of the molecule (11). ADP-ribosylation assays show that the R490A mutant has an increase in ADP-ribosylation activity probably accounting for the increased cytotoxic activity of immunotoxins containing this mutation. Several amino acids have been shown to be important for the activity of domain III of PE. These include H426, H440, W466, Y470, Y481, and E553. Because R490 is not in the active site cleft, it is unlikely to interfere sterically in NAD binding or to be directly involved in the chemical catalytic process. However, it could affect binding of EF2 to domain III. Residue 490 was chosen for mutation to alanine for two reasons. One is that alanine destroys a recognition site for trypsin and related proteases. The second is that it does not alter the main chain conformation and does not alter protein structure by imposing extreme electrostatic or steric effects. This strategy seemed to be successful because we were able to use a protein renaturation method to produce HA22 (R490A) with the same yield as the parental molecular HA22 immunotoxin.

Our initial biological characterization of HA22 (R490A) consisted of evaluating its cytotoxic activity on three CD22-positive cell lines. We found an increase in cytotoxic activity measured by a protein synthesis inhibition assay, ADP-ribosylation activity assay, and a cell viability assay. This increase in cytotoxic activity was not dependent on the nature of the target antigen because the activity of an immunotoxin (SS1P) containing an Fv targeting the mesothelin antigen was also increased by the R490A mutation (Table 3). After internalization, there are a series of steps required for a fragment of PE38 to react to the cytosol, inactivate EF2, and cause cell death. These include proteolytic processing, reduction of a disulfide bond, transport to the endoplasmic reticulum, and inactivation of elongation factor 2. Any of these steps could be affected by the R490A mutation.

Several factors control the lifetimes of immunotoxins in the circulation, including binding to tissues, filtration in the kidney,

Table 3 Cytotoxicity of SS1P and SS1P (R490A)

	IC_{50} (ng/mL)	
Immunotoxin	A431/K5	A1847
SS1P	0.67 ± 0.01 (0.74 ± 0.07)*	3.85 ± 0.39 (4.24 ± 2.45)*
SS1P (R490A)	0.48 ± 0.01 (0.46 ± 0.06)*	1.19 ± 0.18 (2.38 ± 0.61)*

NOTE. Cytotoxicity assays were done by measuring incorporation of [^{3}H]leucine in cells after 20 hours of treatment with indicated concentrations of immunotoxins. IC_{50} is the concentration that causes 50% inhibition of protein synthesis. Mean values of three experiments ± SD are shown.

*Cytotoxic effects were tested in WST-8 assay and expressed as a relative viability (%untreated control), mean ± SD.

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and inactivation or degradation of proteins by proteolysis. Previous studies had shown that elimination of a protease recognition site in domain III of native PE prolonged its half-life in the circulation of mice, and this was attributed to increased protease resistance resulting in decreased degradation in the circulation. We have now extended these studies to recombinant immunotoxins in which the binding domain of native PE is replaced by the Fv portion of an antibody. The antibody portion is human specific so that in mice there are no specific binding sites, and degradation of the immunotoxin is entirely by nonspecific pathways. In contrast to the results with native PE in which removal of a protease site resulted in an increase in half-life in the circulation, there was a very small and not significant decrease in the half-life of HA22 (R490A) compared with HA22 (R490).

The toxic side effects of immunotoxins in animals and humans are of two types. One side effect arises from the targeted killing of normal cells that have the same antigen as the tumor cells. The best solution to overcome this toxicity is to find a different target antigen that is not expressed on normal cells. The second type of toxicity arises from undefined nonspecific binding to normal cells. The liver is particularly vulnerable because it is susceptible to apoptosis induced by toxic substances, it has a high blood content, and its capillaries are fenestrated allowing immediate access of the high concentrations of immunotoxins that are in the blood just after injection. We hypothesized that the protease-sensitive site near Arg^{490} in domain III could contribute to the toxicity of HA22 in mice because plasmin and thrombin are associated with the surface of liver cells and endothelial cells (18, 19) and, therefore, HA22 might bind to protease recognition sites on those cells. However, there was very little change in the nonspecific toxicity of immunotoxins with the R490A mutation in mice. One key parameter in considering the clinical potential for HA22 (R490A) is its therapeutic window or the ratio of the maximum tolerated dose to the therapeutic dose. Initial toxicity evaluation in mice showed that HA22 (R490A) and HA22 were well tolerated, with no signs of toxicity at doses up to 1 mg/kg. At 1.75 mg/kg, both HA22 (R490A) and HA22 were toxic.

We evaluated the antitumor activity of HA22 and HA22 (R490A) by assessing their ability to decrease the size of CA46 tumors in SCID mice. We chose this model because the s.c. tumors are structurally similar to human soft tissue lymphomas and because this model produces quantitative, rapid, and reproducible results in terms of tumor reduction rather than depending on development of terminal disease for measuring antitumor activity in a disseminated model (20). Figure 3 shows that treatment with HA22 (R490A) significantly retarded the growth of CA46 tumors in SCID mice compared with HA22 treatment (Wilcoxon test, $P < 0.001$). This is consistent with the results obtained *in vitro* on the CD22-positive cell lines.

In conclusion, we have improved the cytotoxicity of HA22 on the CD22-positive cell lines by 2-fold without decreasing its nonspecific toxicity or antitumor activity in mice. These studies show that it is possible to use protein engineering to increase the efficacy of recombinant immunotoxins.

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