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Identification of Residues That Stabilize the Single-chain Fv of Monoclonal Antibodies B3*

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B3(Fv)-PE38 is a recombinant single-chain immunotoxin in which the Fv portion of the B3 antibody in a single-chain form, which serves as the targeting moiety, is fused to PE38, a truncated form of *Pseudomonas* exotoxin A, which serves as the cytotoxic moiety. B3(Fv)-PE38 is specifically cytotoxic to many human cancer cell lines and is currently evaluated in a clinical trial. Monoclonal antibodies B3 (IgG1k) and B5 (IgMk) recognize related carbohydrate epitopes on human carcinoma cells. The Fv regions of these antibodies were previously cloned and expressed as the single-chain Fv-immunotoxins B3(Fv)-PE38 and B5(Fv)-PE38, respectively. The B3(Fv)-PE38 immunotoxin binds to antigen-positive cancer cells with a higher affinity than B5(Fv)-PE38 and is a more potent cytotoxic agent than B5(Fv)-PE38. However, it is less stable and rapidly aggregates upon incubation at 37 °C. The V_L domains of the two Fvs are very similar, differing by only three residues, the fourth and seventh Fr1 residues and the fifth CDR1 residue. The V_H domains of the two Fvs vary considerably. To investigate whether any of the different V_L residues may influence the stability of the B3(Fv), we constructed a chimeric immunotoxin containing the $B3V_H$ and the $B5V_L$. This chimera had an improved stability and a higher apparent antigen binding affinity and cytotoxic activity when compared with B3(Fv)-PE38. Site-specific mutagenesis was used to show that the V_L M4L mutation has an important role in stabilizing B3(Fv), although residues V_L Ser-7 and V_L Ile-28 also play a role in the increased stability. When tested in an *in vivo* model system, the chimera containing the $B3V_H$ and the $B5V_L$ had an improved antitumor activity in a human xenograft mouse model. These studies indicate that the common use of degenerate ("family-specific") primers to clone Fv fragments may introduce destabilizing mutations.

Monoclonal antibodies B3 and B5 are murine antibodies directed against $Lewis^Y$-related carbohydrate antigens, which are abundant on the surface of many carcinomas (1). The B3 IgG or its fragments are currently used as the targeting moiety of immunotoxins that are being developed as anticancer agents. Both conventional whole IgG conjugates and single-chain recombinant immunotoxins have been prepared (1–4). The single-chain Fv[1] immunotoxin of B3 is unstable at 37 °C; it undergoes inactivation mainly by aggregation, especially upon incubation in PBS or in cell culture medium. In contrast, the B5(Fv)-PE38 immunotoxin is less susceptible to inactivation under those conditions, but it has lower apparent antigen binding affinity and cytotoxicity (5). We reasoned that we might be able to combine the advantages of each Fv by chimerization of their variable domains, since the Fvs of monoclonal antibody B3 and B5 bind the same carbohydrate antigen and are homologous in sequence (particularly in the V_L domain, where 109 of 112 residues are identical). Therefore, we sought to gain insight on the possible involvement of individual residues of the light chain on the stability and the binding affinity of the B3(Fv). In this paper we have characterized the specific cytotoxicity, stability, and binding properties of single-chain Fv immunotoxins whose Fvs are B3-B5 chimeras or B3(Fv)s carrying point mutations in the V_L domain. We found that the chimera with $B3V_H$ and $B5V_L$ was the most stable and potent of all the molecules tested in the *in vitro* assays, and we have also compared it with the parental B3(Fv)-PE38 molecule in an *in vivo* antitumor activity assay.

MATERIALS AND METHODS

Cloning of DNA Fragments Encoding the Heavy and Light Fv Segments of Monoclonal Antibodies B1 and B5—The B5 V_H and V_L DNA fragments were PCR-amplified independently, and the resulting PCR products were used as "primers" in a "domain shuffling" scheme where they replaced the corresponding B3 V_H or V_L or both, generating scFv-toxin expression plasmids having $B3V_H$-$B5V_L$, $B5V_H$-$B3V_L$, and B5Fv as described (5) (Fig. 1). Site-specific mutagenesis (6), with pULI7 uracil-containing single-stranded DNA as template and the oligodeoxynucleotide 5′ AAGTAAACTGAATGGAG(T/A)TTGGGTCA(A/T)CAGCACATCGCTTCC 3′ as a primer was used to prepare plasmids expressing B3(Fv)PE38 derivatives in carrying V_L mutations M4L and S7T in combination or separately.

Expression and Purification of Recombinant Proteins—Plasmids encoding B3(Fv)-PE38, $B3V_H$-$B5V_L$-PE38, $B5V_H$-$B3V_L$-PE38, or B5(Fv)-PE38 were expressed in *Escherichia coli*. The single-chain immunotoxins were obtained by solubilization and refolding of inclusion body protein as described (7). Properly refolded proteins were separated from contaminating proteins and aggregates by sequential ion exchange chromatography on Q-Sepharose and Mono Q (Pharmacia Biotech Inc.) followed by size exclusion chromatography on a TSK G3000SW (TosoHaas) column.

Cytotoxicity of Recombinant Immunotoxins—The cytotoxic activity of immunotoxins was tested by determination of their ability to inhibit the incorporation of [^{3}H]leucine into cell protein, reflecting inhibition of protein synthesis in cultured cells as described (3).

Stability Assays—The stability of the immunotoxins in PBS at 37 °C was determined by incubation at 0.2 mg/ml in PBS for 1, 2, or 4 h, followed by analytical chromatography on a TSK G3000SW (TosoHaas) column, to separate the monomers from the aggregates. Cytotoxic activities of aliquots of these immunotoxins were determined as described

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[1] The abbreviations used are: Fv, portion of immunoglobulin molecules consisting of the light and heavy chain variable domains; V, variable; L, light; H, heavy; CDR, complementary determining region; Fr, immunoglobulin variable framework region; HSA, human serum albumin; PBS, phosphate-buffered saline; PE, *Pseudomonas* exotoxin A; PCR, polymerase chain reaction.

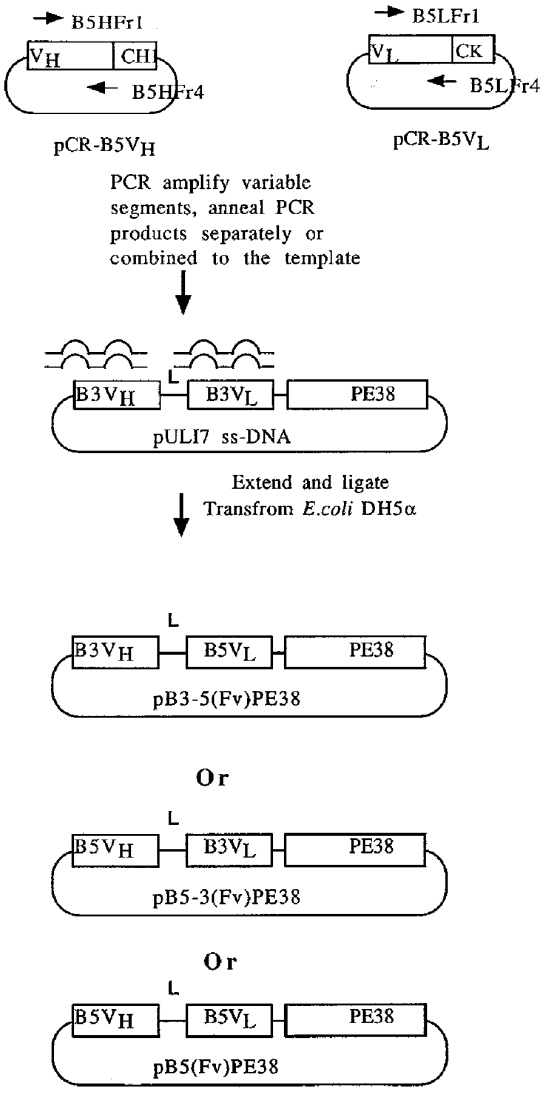

FIG. 1. **Scheme of construction of plasmids for expression of B3(Fv)PE38 and B3-B5 chimeric single-chain immunotoxins.** *L* indicates the $(Gly_4\text{-}Ser)_3$ linker that connects the V_H to the V_L in the single-chain Fv configuration. PE38 is a truncated form of *Pseudomonas* exotoxin A, which lacks the toxins' own cell binding domain I (3). The sequences of the PCR primers B5HFr1, B5HFr4, B5LFr1, and B5LFr4 are published (5).

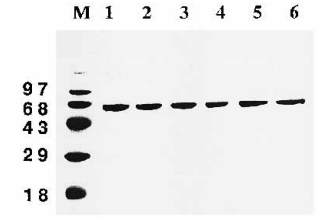

FIG. 2. **Recombinant immunotoxins.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified immunotoxins. *Lane M*, molecular mass standard indicated on the *left* in kDa. *Lane 1*, B3(Fv)-PE38; *lane 2*, $B3V_H$-$B5V_L$-PE38; *lane 3*, $B5V_H$-$B3V_L$-PE38; *lane 4*, B3(Fv)-PE38 V_L M4L; *lane 5*, B3(Fv)-PE38 V_L S7T; *lane 6*, B3(Fv)-PE38 V_L M4L/S7T. 5 μg of each protein were loaded on a 12% gel.

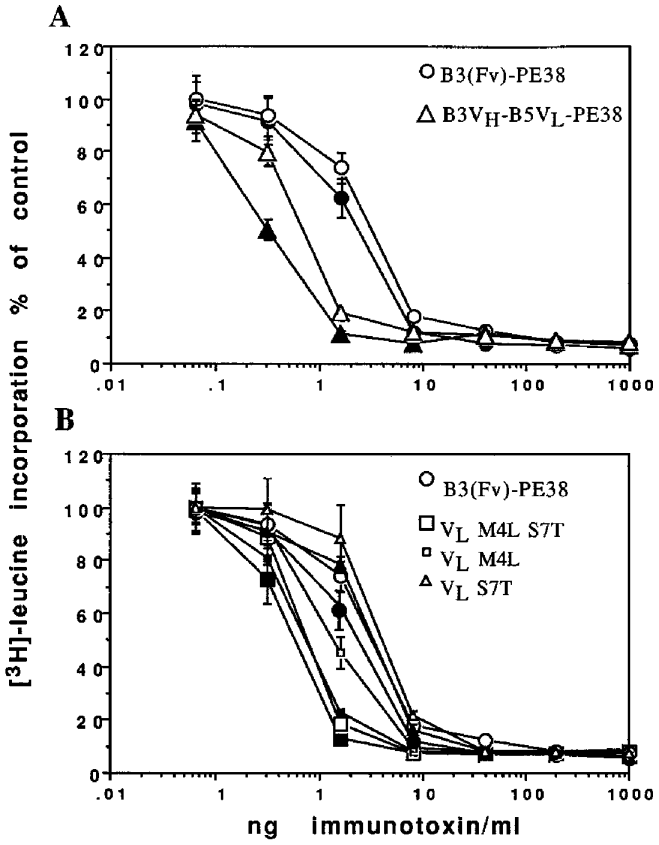

FIG. 3. **Specific cytotoxicity of immunotoxins.** Cytotoxicity toward A431 cells was measured by the inhibition of incorporation of [^{3}H]leucine into cell protein, following 2 h (*open symbols*) or 20 h (*solid symbols*) of incubation of the cells with serial dilutions in PBS, 0.2% bovine serum albumin of B3(Fv)-PE38 and $B3V_H$-$B5V_L$-PE38 (*A*), or B3(Fv)-PE38 derivatives V_L M4L/S7T, V_L M4L, V_L S7T immunotoxins (*B*). *Error bars* represent the S.E. of the data.

above, and compared with the activities of the immunotoxins that were not incubated at 37 °C.

The stability of the immunotoxins in human serum at 37 °C was determined by incubation at 0.02 mg/ml in serum for 1, 2, or 4 h. Cytotoxic activities of aliquots of these immunotoxins were determined as described above and compared with the activities of the immunotoxins that were not incubated at 37 °C.

Fluorescence Measurements—Solutions of immunotoxins at 10 μg/ml were prepared in PBS containing 0, 0.5, 1, or 2 M urea. The intrinsic tryptophan fluorescence (8) (excitation at 295 nM) of each solution was determined at 23 °C in a Perkin-Elmer LS50B luminescence spectrometer using a 1-cm path length.

Toxicity and Pharmacokinetics in Mice—Female BALB/c mice (6–8 weeks old, ~20 g) were used for all the *in vivo* experiments. The single-dose mouse LD_{50} was determined by a single intravenous injection of different doses of B3(Fv)-PE38 or $B3V_H$-$B5V_L$-PE38 diluted in 200 μl of PBS-HSA. Mice were monitored for weight loss or death for at least 2 weeks postinjection. For pharmacokinetics the mice were given a single intravenous dose of 10 μg B3(Fv)-PE38 or $B3V_H$-$B5V_L$-PE38 diluted in 200 μl of PBS-HSA. Blood samples were collected at 2, 5, 10, 20, 30, 60, 120, and 240 min postinjection. The concentration of toxin was determined by incubating serial dilutions of the serum with A431 cells and measuring its ability to inhibit protein synthesis. A standard curve, obtained by incubating serial dilutions of the injected toxins on A431 cells, was used to determine the toxin concentration in each serum sample.

Antitumor Activity—Athymic (Nu/Nu) mice were injected subcutaneously on day 0 with 3×10^6 A431 cells suspended in 0.2 ml of PBS. By day 4, tumors were about 30–40 mm^3 in size. Mice were treated on days 4, 6, and 8 by intravenous injections of different doses of immunotoxins diluted in PBS-HSA. Tumors were measured with a caliper at 2-day intervals, and the tumor volumes were calculated using the following formula: volume = length × $width^2$ × 0.4.

RESULTS

Cloning of the Variable Regions of B3 and B3-B5 Chimera as Single-chain Immunotoxins—DNA encoding the variable regions of the heavy and light chains of B5 mRNA was prepared from B5 hybridoma cells as described (5). To generate single-chain immunotoxins in which the Fv of B5 in a single-chain

TABLE I
Cytotoxicity of B3(Fv)-PE38, B3VH-B5VL-PE38, and B5(Fv)-PE38 toward various cell lines

Cytotoxicity data are given as IC_{50} values, the concentration of immunotoxin that causes a 50% inhibition of protein synthesis following its incubation on the cells for 20 h. Expression level estimation of the B3 antigen is based on immunofluorescence. +++, strong; +, weak; −, not detected.

Cell line[a]	Source	B3 antigen expression	Cytotoxicity IC_{50} ng/ml		
			B3(Fv)-PE38	$B3V_H$-$B5V_L$-PE38	B5(Fv)PE38[b]
A431	Epidermoid carcinoma	+++	2.6	0.3	20
MCF7	Breast carcinoma	+++	4.0	1.0	22
LnCap	Prostate carcinoma	+	21	11	210
KB 3–1	Cervical carcinoma	−	>1000	>1000	>1000
HUT102	T-cell leukemia	−	>1000	>1000	>1000
L929	Mouse fibroblast	−	>1000	>1000	>1000

[a] All the cell lines except L929 are of human origin.
[b] Values for B5(Fv)-PE38 are from Ref. 5.

form, is fused to PE38, the V_H and V_L fragments were PCR-amplified using phosphorylated primers to enable the ligation of extended PCR products. The PCR products were purified and annealed to uracil-containing single-stranded DNA that was obtained by rescue of the phagemid pULI7, which encodes the single-chain immunotoxin B3(Fv)-PE38 with the helper phage M13KO7. The primer sequences, the extension of the template-primer, ligation, transformation, and analysis of clones were described (5). This procedure resulted in the generation of plasmids for expression in *E. coli* in which either the V_H or the V_L domain of B3 was replaced by the corresponding domain from B5 (Fig. 1), namely $B3V_H$-$B5V_L$-PE38 and $B5V_H$-$B3V_L$-PE38.

Expression, Purification, and Cytotoxic Activity of B3(Fv)-PE38 and B3-B5 Chimeras—Cultures of BL21(λDE3) (9) transformed with each expression plasmid were used to produce immunotoxins. Following isopropyl-1-thio-β-D-galactopyranoside induction, the overproduced proteins accumulated in inclusion bodies, which were isolated, and the recombinant protein was solubilized, reduced, and refolded as described previously (3). Active immunotoxins were recovered by ion exchange and size exclusion chromatography, as described under "Materials and Methods." Fig. 2 shows that the purified monomeric proteins were over 95% pure. Next, the cytotoxic activity of B3(Fv)-PE38 and of $B3V_H$-$B5V_L$-PE38 was assessed by measuring the incorporation of [^{3}H]leucine by various human carcinoma cell lines after treatment with serial dilutions of the immunotoxin as described previously (3). Activities of the immunotoxins were compared following 2 or 20 h of incubation on the cells. As shown in Fig. 3*A*, when tested on A431 cells, which strongly bind B3 IgG, B3(Fv)-PE38 has an IC_{50} of 2.8 and 2.6 ng/ml following 2 or 20 h of incubation, respectively. $B3V_H$-$B5V_L$-PE38 has IC_{50} values of 1.0 and 0.3 ng/ml following 2 and 20 h of incubation, respectively. B5(Fv)-PE38 has an IC_{50} of 120 and 20 ng/ml following 2 or 20 h of incubation, respectively (5). $B5V_H$-$B3V_L$-PE38 also had low activity, with IC_{50} values of 200 and 120 ng/ml following 2 or 20 h of incubation, respectively (data not shown). This data shows $B3V_H$-$B5V_L$-PE38 is the most active immunotoxin, and the fact that its activity increased 3-fold, from 1.0 to 0.3 ng/ml when the incubation with cells was extended from 2 to 20 h suggests it is quite stable.

To test the specificity of the immunotoxins, the same cytotoxic assay was repeated on additional cell lines, which differ in their level of B3 antigen expression (1, 10). As shown in Table I, B3(Fv)-PE38, $B3V_H$-$B5V_L$-PE38, and B5(Fv)-PE38 had the same spectrum of recognition of the cancer cell lines tested, although they had different levels of specific cytotoxic activity. Also the cytotoxic activity of each correlates with its binding affinity.

Stability of Immunotoxins—The stabilities of B3(Fv)-PE38 and $B3V_H$-$B5V_L$-PE38 in PBS were tested by determination of their respective levels of aggregation and inactivation at 37 °C as described under "Materials and Methods." As shown in Fig. 4 and Table II, both immunotoxins were mainly monomeric before the incubation. After 1 h of incubation in PBS at 37 °C, about 30% of $B3V_H$-$B5V_L$-PE38 was aggregated, whereas B3(Fv)-PE38 was 78% aggregated. After 2 h of incubation in PBS at 37 °C, 50% of $B3V_H$-$B5V_L$-PE38 was aggregated, whereas B3(Fv)-PE38 was about 90% aggregated. After 4 h of incubation in PBS at 37 °C, $B3V_H$-$B5V_L$-PE38 was about 60% aggregated, whereas B3(Fv)-PE38 was about 95% aggregated. The aggregates were recovered in the volume excluded from the column and are multimeric forms larger than 300 kDa (Fig. 4). These aggregates have very little cytotoxic activity (data not shown).

Fig. 5 shows results from cytotoxicity assays that were performed with aliquots of the immunotoxins that had been incubated in PBS as 37 °C. Before treatment, B3(Fv)-PE38 had an IC_{50} of 2.2 ng/ml, and it retained 23, 10, and 5% of its cytotoxic activity following 1, 2, and 4 h of incubation in PBS at 37 °C, respectively. The $B3V_H$-$B5V_L$-PE38 chimera had an IC_{50} of 0.4 ng/ml, and it retained 78, 56, and 35% of its cytotoxic activity following 1, 2, and 4 h, respectively, of incubation in PBS at 37 °C. When incubated in human serum, B3(Fv)-PE38 retained 50, 25, and 12% of its cytotoxic activity following 1, 2, and 4 h at 37 °C, respectively, and $B3V_H$-$B5V_L$-PE38 retained 80, 66, and 43% at these time points. Table II compares the percentage of monomeric immunotoxin with the percentage of cytotoxic activity remaining following incubation in PBS at 37 °C as well as the percentage of active immunotoxin surviving after incubation in human serum at 37 °C. It demonstrates that upon incubation in PBS, the residual cytotoxic activity correlates strongly with the relative amount of immunotoxin monomer that survived the 37 °C incubation and that $B3V_H$-$B5V_L$-PE38 is more stable than B3(Fv)-PE38 with about a 4-fold longer half-life. The inactivation rate of the immunotoxins in human serum is slower than in PBS as we have observed previously with other immunotoxins (11).[2] However, the 4:1 ratio between the half-life of the more stable $B3V_H$-$B5V_L$-PE38 and the less stable B3(Fv)-PE38 is maintained.

Intrinsic tryptophan fluorescence was also used to determine the relative stability of B3(Fv)-PE38 and $B3V_H$-$B5V_L$-PE38 to denaturation by urea. In PBS, both immunotoxins have an emission peak at 330 nm and a second partially overlapping peak at 343 nm. The 330-nm peak remains unchanged up to 2 M urea and probably reflects the emission of the PE38 part of the molecule, which requires higher urea concentration for denaturation.[2] However, the peak at 343 nm increases with urea concentration. The relative fluorescence change is shown in Fig. 6; the peak at 343 nm increased more for B3(Fv)-PE38

[2] I. Benhar and I. Pastan, unpublished observations.

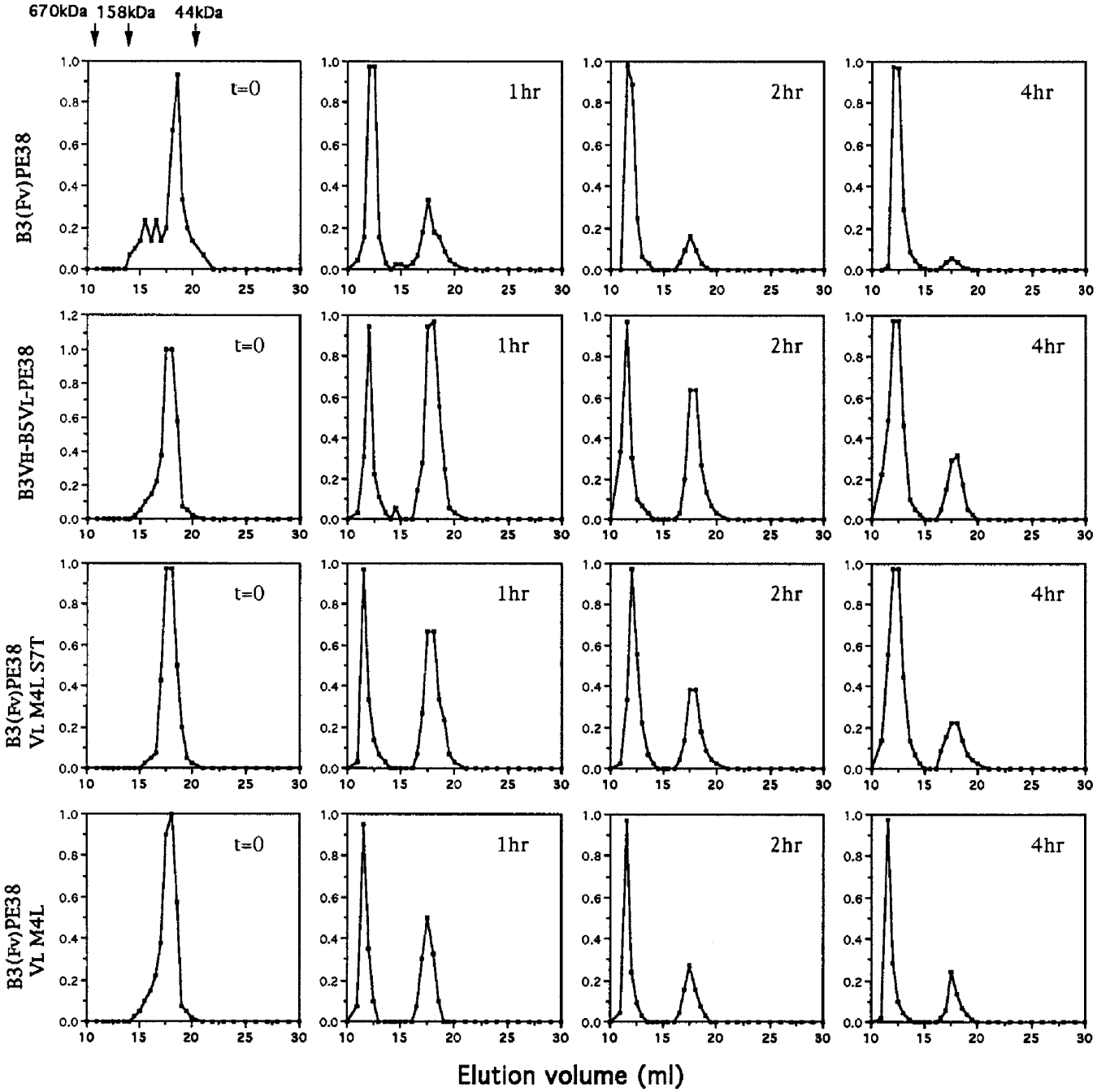

FIG. 4. **Stability of immunotoxins.** B3(Fv)PE38, $B3V_H$-$B5V_L$-PE38, and B3(Fv)PE38:V_L M4L/S7T were diluted in PBS to 0.2 mg/ml and incubated at 37 °C for 1, 2, or 4 h. The molecular forms of the immunotoxins were then analyzed by size exclusion chromatography at 4 °C as described under "Materials and Methods." The monomer peak elutes at 18–20 ml, while the aggregates elute at 11–13 ml. The elution positions of gel filtration molecular weight standards (Bio-Rad) are indicated above the *left uppermost chromatogram*.

than for $B3V_H$-$B5V_L$-PE38, indicating that the former undergoes a conformational change, which is reflected by the fluorescence change under a lower denaturant concentration than the latter.

Role of Specific Residues in the Light Chain Fv—Alignment of the amino acid sequences of the B3 and B5 V_L domains (Fig. 7), reveals that they are very similar, with 109/112 identical residues; they differ only at positions 4 and 7, which are in Fr1 and at position 28, which is the fifth residue in CDR1. To identify the residue or residues that are responsible for the stabilization of the chimeric $B3V_H$-$B5V_L$-PE38 immunotoxin, we performed a molecular dissection in which we analyzed the properties of B3(Fv)-PE38 derivatives in which V_L residues 4 and 7 were mutated to the corresponding B5 residues (Fig. 8). We did not mutate the CDR residue to minimize the risk of altering the binding specificity of B3(Fv). Plasmids expressing B3(Fv)-PE38 derivatives carrying V_L mutations M4L and S7T together or separately were prepared by site-specific mutagenesis using uracil-containing pULI7 single-stranded DNA as template and oligonucleotide primers. The plasmids were expressed in *E. coli*, and the immunotoxins were purified (Fig. 2).

The yield of purified B3(Fv)-PE38 V_L M4L/S7T and B3(Fv)-PE38 V_L M4L was 8–10% (8–10 mg of active monomeric immunotoxin was recovered from 100 mg of recombinant protein added to the refolding buffer). The yield of $B3V_H$-$B5V_L$-PE38 was also 8–10%. The yield of B3(Fv)-PE38 and B3(Fv)-PE38 V_L S7T was 2–4% (data not shown). We have previously found that the yield of active monomeric recombinant immunotoxin

TABLE II
Stability of immunotoxins in PBS and in human serum at 37 °C

	Incubation time (h)			
	0	1	2	4
	M^a/%A^b (%S)^c	M/%A (%S)	M/%A (% S)	M/%A (%S)
B3(Fv)-PE38	100/100 (100)	27/23 (50)	10/10 (25)	5/7 (12)
$B3V_H$-$B5V_L$-PE38	100/100 (100)	68/78 (80)	52/56 (66)	37/35 (43)
B3(Fv)-PE38 V_L M4L S7T	100/100 (100)	60/55 (63)	44/28 (38)	25/15 (33)
B3(Fv)-PE38 V_L M4L	100/100 (100)	45/50 (66)	36/25 (45)	19/20 (33)

[a] M, percentage of monomeric immunotoxin remaining after incubation at 37 °C for the indicated period was calculated by comparing the area under the monomer and aggregate peaks of the original fast protein liquid chromatography A_{280} recorder graphs that were used to prepare Fig. 4.

[b] % A: percentage of cytotoxic activity of immunotoxins aliquots incubated in PBS at 37 °C for the indicated period (calculated from Fig. 5).

[c] % S, percentage of cytotoxic activity of immunotoxins aliquots incubated in human serum at 37 °C for the indicated period.

directly correlates with its stability (12).[2] B3(Fv)-PE38 V_L M4L/S7T, B3(Fv)-PE38 V_L M4L and B3(Fv)-PE38 V_L S7T were analyzed for stability and specific cytotoxicity just as was done with B3(Fv)-PE38 and $B3V_H$-$B5V_L$-PE38.

As shown in Fig. 3*B*, B3(Fv)PE38 V_L M4L/S7T was the most potent of the three, with IC_{50} values of 1.2 and 0.6 ng/ml following 2 or 20 h, respectively, of incubation on A431 cells. Its cytotoxic activity was almost indistinguishable from that of the chimeric $B3V_H$-$B5V_L$-PE38 (Fig. 3*A*). B3(Fv)PE38 V_L M4L had IC_{50} values of 1.8 and 1.2 ng/ml following 2 and 20 h, respectively, of incubation on A431 cells. B3(Fv)PE38 V_L S7T had the lowest activity, with IC_{50} values of 6.0 and 5.0 ng/ml following 2 and 20 h, respectively, of incubation on A431 cells. Thus the immunotoxin with V_L S7T was much less potent than the parental B3(Fv)-PE38 immunotoxin. This mutant was not analyzed further.

As shown in Figs. 4 and 5 and Table II, both the immunotoxin with the V_L M4L/S7T mutation and the V_L M4L mutation alone aggregated in PBS at 37 °C to a lesser extent than B3(Fv)PE38 and were also more resistant to loss of cytotoxicity at 37 °C than B3(Fv)-PE38. B3(Fv)-PE38 VL M4L/S7T was 40, 56, and 75% aggregated following 1, 2, and 4 h in PBS at 37 °C, respectively, and retained 55, 28, and 15% of its cytotoxic activity at these time points. B3(Fv)-PE38 V_L M4L was 55, 64, and 81% aggregated following 1, 2, and 4 h, respectively, in PBS at 37 °C, and retained 50, 25, and 20% of its cytotoxic activity at these time points. When incubated in human serum, B3(Fv)-PE38 V_L M4L/S7T retained 63, 38, and 33% of its cytotoxic activity following 1, 2, and 4 h at 37 °C, respectively, and B3(Fv)-PE38 V_L M4L retained 66, 45, and 33% of its activity at these time points. When the intrinsic tryptophan fluorescence of PE38 V_L M4L/S7T and of PE38 V_L M4L was determined, the spectra obtained were similar to the spectra obtained for B3(Fv)-PE38 and $B3V_H$-$B5V_L$-PE38, with a peak at 330 nm that does not change and a second peak at 343 nm that increased with increasing urea concentration. As shown in Fig. 8, the increases at 343 nm measured for B3(Fv)-PE38 V_L M4L/S7T and B3(Fv)-PE38 V_L M4L were similar to each other and were higher than the increase observed for $B3V_H$-$B5V_L$-PE38 but were lower than the increase observed for B3(Fv)-PE38, indicating that the sensitivity of PE38 V_L M4L/S7T and PE38 V_L M4L to undergo a conformational change (which is reflected by the fluorescence change) following exposure to urea is greater than that of $B3V_H$-$B5V_L$-PE38 but smaller than that of the parental molecule B3(Fv)-PE38. Taken together, these data show that while more stable than B3(Fv)-PE38 and having similar stabilities to each other, both mutated immunotoxins V_L M4L/S7T and V_L M4L are somewhat less stable than the chimeric $B3V_H$-$B5V_L$-PE38. These results further suggest that most of the stabilizing effect of $B5V_L$ on the Fv immunotoxin stems from its having a leucine rather than a methionine at position 4.

Toxicity and Pharmacokinetics of Immunotoxins—Since the *in vitro* assays demonstrated that $B3V_H$-$B5V_L$-PE38 was the most stable and most potent of the immunotoxins tested, it was chosen for further characterization *in vivo*, where it was compared with our prototype anti-Le^Y immunotoxin B3(Fv)-PE38. The toxicity of the immunotoxins was evaluated by intravenous injections of different doses of immunotoxin into BALB/c mice. The mice were observed for 7–10 days postinjection. As shown in Table III, the LD_{50} values of the immunotoxins were similar, with B3(Fv)-PE38 having an LD_{50} of about 0.5 mg/kg and $B3V_H$-$B5V_L$-PE38 having an LD_{50} of about 0.6 mg/kg. This value is in agreement with the published LD_{50} of B3(Fv)-PE38 (0.5 mg/kg) (11). The pharmacokinetics of B3(Fv)-PE38 and $B3V_H$-$B5V_L$-PE38 were determined by measuring the immunotoxin levels in blood samples drawn from mice at various time points following an intravenous injection of each immunotoxin. The immunotoxin levels in the samples were determined by the ability of the serum or its dilutions to inhibit protein synthesis on A431 cells in comparison with standard curves obtained with the immunotoxins themselves. As shown in Fig. 9, both immunotoxins had a similar pharmacokinetic behavior when compared with each other, with a $t_{1/2}$of about 22 min. This value is in agreement with published pharmacokinetics of B3(Fv)-PE38 (11).

Antitumor Activity of Immunotoxins—The *in vivo* potency of the immunotoxins was evaluated by assessing their ability to cause regressions of established human carcinoma xenografts in nude mice. Tumors were induced by subcutaneous injection of 3×10^6 A431 cells on day 0. Treatment was initiated on day 4 when the tumors averaged 30–40 mm^3 in volume, and it consisted of three intravenous injections on days 4, 6, and 8 of various doses of immunotoxin. Control mice were treated with PBS-HSA only. As shown in Fig. 10, both immunotoxins had an antitumor effect that was dose-dependent. Mice treated with B3(Fv)-PE38 had complete regressions of the tumors at a dose of 0.1 mg/kg given intravenously every other day × 3. At 0.05 mg/kg × 3, the tumors showed complete regressions until day 16, but tumor growth resumed thereafter. In mice treated with 0.025 mg/kg × 3, tumor growth was arrested for the duration of the treatment and resumed after its completion. This is in agreement with published data (11). $B3V_H$-$B5V_L$-PE38 had about 2-fold better antitumor activity; tumors showed complete regressions at doses of 0.1 and 0.05 mg/kg intravenously every other day × 3. At 0.025 mg/kg intravenously, the tumors were in complete regression until day 14, and tumor growth resumed thereafter. In mice treated with 0.0125 mg/kg, tumor growth was arrested for the duration of the treatment and resumed after its completion. Control animals developed large tumors and were euthanized on day 18, when the tumor size was about 1.0 cm^3. These data show that $B3V_H$-$B5V_L$-PE38 is a more potent immunotoxin *in vivo* because it has a 2-fold better therapeutic index.

DISCUSSION

We have cloned DNA fragments encoding the variable domains of the anticarbohydrate monoclonal antibodies B3 and B5 as single-chain Fv immunotoxins. We employed the method of "variable domain shuffling" (5), which allowed us to obtain Fv chimeras having V_H and V_L segments from the two different antibodies. The activities of the immunotoxins varied, with $B3V_H$-$B5V_L$-PE38 being the most potent, with an IC_{50} of 0.3 ng/ml and $B5V_H$-$B3V_L$-PE38 being the least potent, with an IC_{50} of 120 ng/ml following 20 h of incubation on A431 cells.

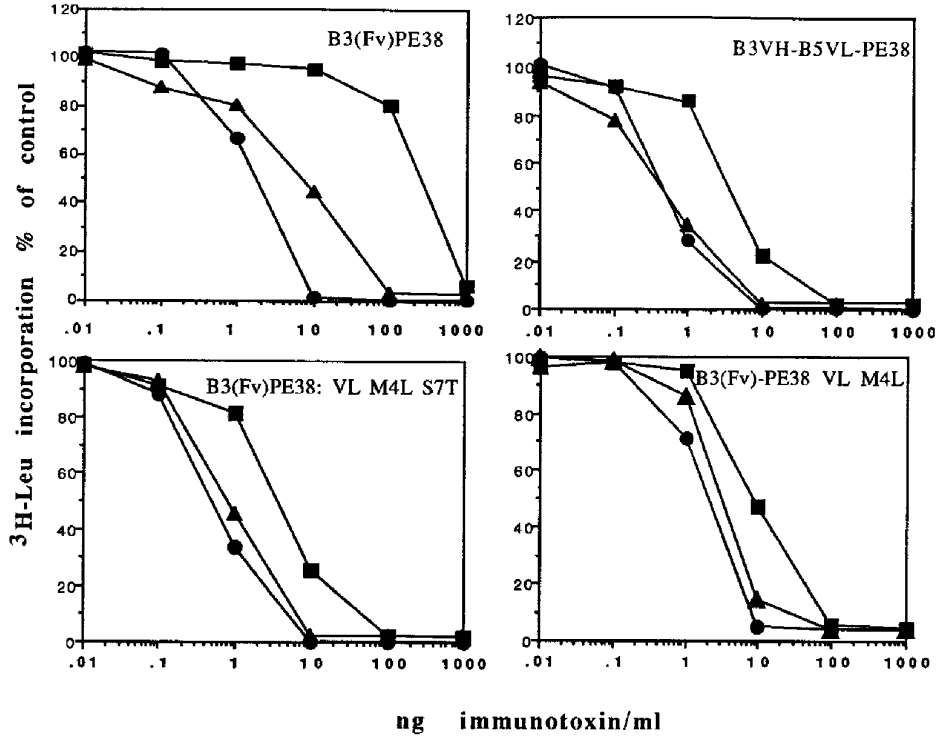

FIG. 5. **Cytotoxic activity of immunotoxins following incubation in PBS at 37 °C.** A431 epidermoid carcinoma cells were incubated with aliquots of the immunotoxins, which were diluted in PBS, 0.2% bovine serum albumin following incubation at 37 °C. [^{3}H]Leucine was added 20 h after the addition of immunotoxins. ●, t = 0 hours; ▲, 1 h in PBS at 37 °C; 2 h in PBS at 37 °C.

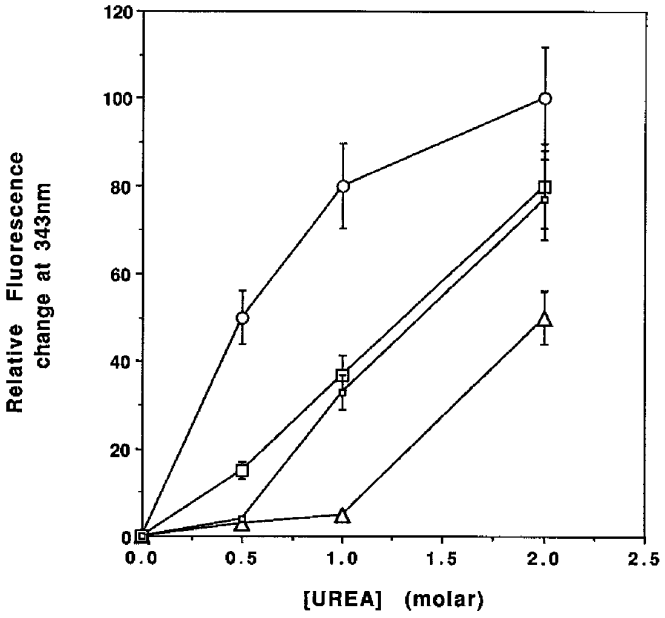

FIG. 6. **Intrinsic tryptophan fluorescence of B3(Fv)-PE38 (○), B3V$_H$-B5V$_L$-PE38 (△), B3(Fv)-PE38 V$_L$ M4L/S7T (*large square*), and B3(Fv)-PE38 (*small square*) in urea.** Samples of immunotoxins were diluted to 10 μg/ml in PBS containing between 0 and 2 M urea. Fluorescence emission between 320 and 380 nm was determined at 23 °C with excitation at 295 nm. The relative changes at 343 nm are plotted.

```
                                                CDR1
B3V_L   DVLMTQSPLSLPVSLGDQASISC RSSQIIVHSNGNTYLE
B5V_L   ...L..T.................. ....S...........

                          CDR2
B3V_L   WYLQKPGQSPKLLIY KVSNRFS
B5V_L   ..............  .......

                                                CDR3
B3V_L   GVPDRFSGSGSGTDFTLKISRVEAEDLGVYYC FQGSHVPFT FGSGTKLEIK
B5V_L   ..................................  .........  ..........
```

FIG. 7. **Alignment of B3 and B5 V$_L$ amino acid sequences.** The amino acid sequence (in single-letter code) B3V$_L$ is shown in the *upper line*, with B5V$_L$ *below* it. Identical residues are identified by *dots*.

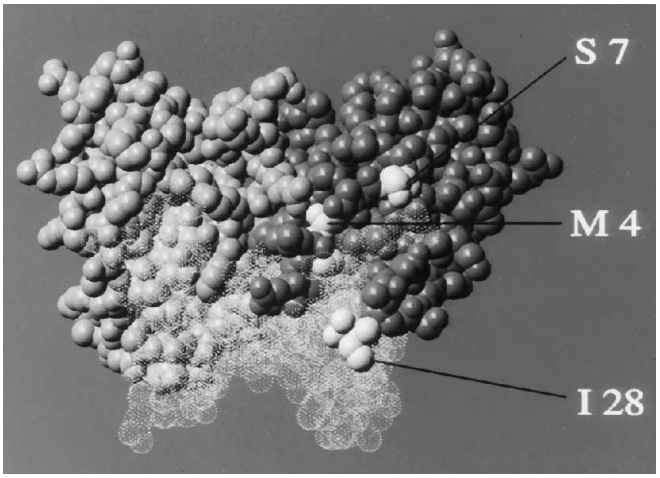

FIG. 8. **Structural model of B3(Fv).** The V$_H$ domain is shown on the *left side* of the molecule in *light gray*, and the V$_L$ domain is shown on the *right side* of the molecule in *dark gray*. The CDRs of both domains are semitransparent. Residues that differ between B3V$_L$ and B5V$_L$ are in *white* and are labeled according to their positions in V$_L$.

The similar spectrum of recognition of cell lines that differ in the level of B3 antigen expression (Table I) suggests that there is no change in specificity following chimerization of B3 and B5. We have shown previously that the parental monoclonal antibodies recognize the same carbohydrate antigen (5). Since the B3V$_H$-B5V$_L$-PE38 chimera was the most stable and potent of the immunotoxins tested here, it was chosen for *in vivo* characterization in comparison with B3(Fv)-PE38. When tested in mice, the chimera did not differ significantly from the B3(Fv)-PE38 immunotoxin in pharmacokinetics or toxicity (Fig. 9, Table III). However, it had a better antitumor activity in a human xenograft nude mouse model (Fig. 10), where it was 2-fold more potent than B3(Fv)-PE38. The improved antitumor activity correlates with the 3-fold difference in cytotoxicity between the two immunotoxins when tested on A431 cells, which were also used to establish the tumors in the mice.

TABLE III
Toxicity of B3(Fv)-PE38 and $B3V_H$-$B5V_L$-PE38 in mice

Groups of four or two Balb/c mice were injected intravenously with 200 μl of diluent or with increasing doses of B3(Fv)-PE38 or $B3V_H$-$B5V_L$-PE38.

Days postinjection	Diluent	0.25 mg/kg	0.5 mg/kg	0.75 mg/kg
B3(Fv)-PE38				
2	2/2[a]	2/2	4/4	1/2
4	2/2	2/2	4/4	1/2
7	2/2	2/2	4/4	0/2
14	2/2	2/2	4/4	0/2
$B3V_H$-$B5V_L$-PE38				
2	2/2	2/2	2/4	1/2
4	2/2	2/2	2/4	0/4
7	2/2	2/2	2/4	0/4
14	2/2	2/2	2/4	0/4

[a] Number of mice alive of mice injected.

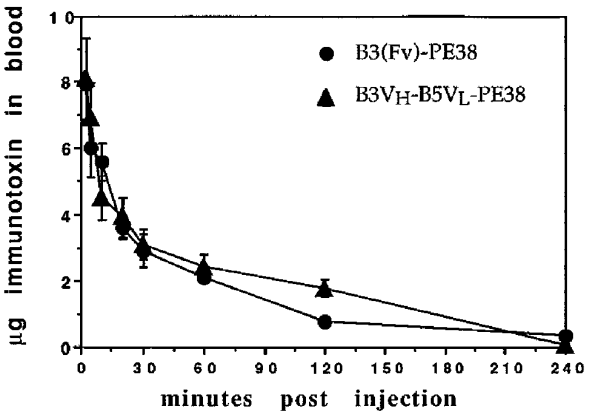

FIG. 9. **Blood levels of B3(Fv)-PE38 and $B3V_H$-$B5V_L$–PE38 in mice.** Female Balb/c mice were injected intravenously with 10 μg of immunotoxin. Mice were bled at different times, and the immunotoxin level was measured by a cell-killing assay, in which the ability of serum dilutions to inhibit protein synthesis by A431 cells was tested. Results are from three mice for each time point ± S.E.

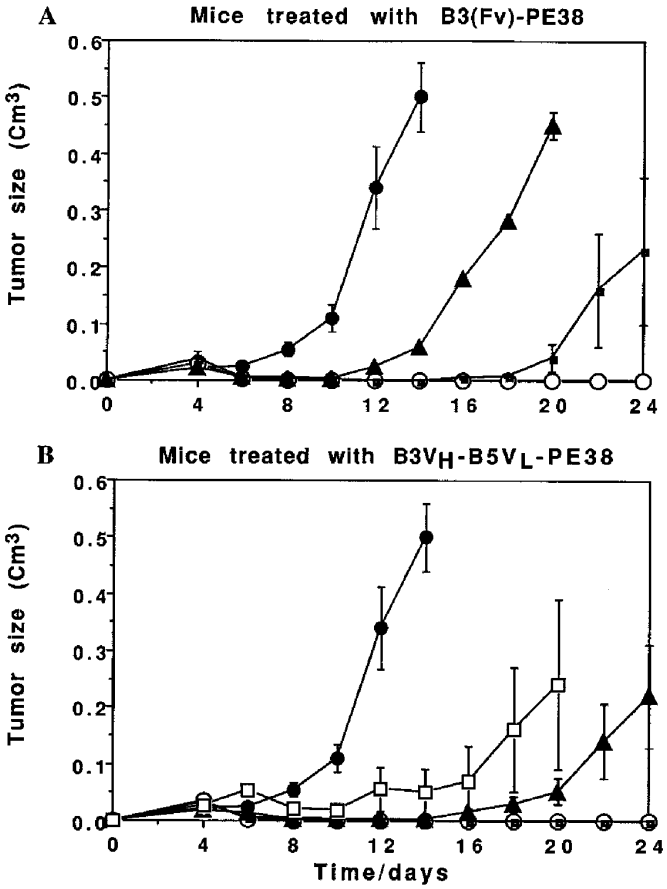

FIG. 10. **Antitumor effect of B3(Fv)-PE38 and $B3V_H$-$B5V_L$-PE38 in a nude mouse model.** Groups of five mice were injected subcutaneously with 3×10^6 cells on day 0 and were treated by intravenous injections of B3(Fv)-PE38 (*A*) or $B3V_H$-$B5V_L$-PE38 (*B*) diluted in PBS containing 0.2% human serum albumin on days 4, 6, and 8 (indicated by *vertical arrows*) when the tumors were established. Control mice were treated with PBS-HSA. *Error bars* represent the S.E. of the data. ●, control; ○, 0.1 mg/kg; ■, 0.05 mg/kg; ▲, 0.025 mg/kg; □, 0.0125 mg/kg.

The higher cytotoxic activities (lower IC_{50} values) of the $B5V_H$-$B3V_L$-PE38 chimera and the B3(Fv)-PE38 V_L M4L/S7T and B3(Fv)-PE38 V_L M4L mutants and their higher apparent antigen binding affinity (not shown) may be explained by the fact that both are more stable than the wild type B3(Fv)-PE38. This improved stability is evident from their slower aggregation and loss of cytotoxic activity upon incubation in PBS or in human serum at 37 °C; very little B3(Fv)-PE38 monomer survives a 4-h-long incubation, whereas the stabilized variants (the chimera and the mutants) retain significant cytotoxic activity. This accounts for the fact that there is very little difference in the cytotoxic activity of B3(Fv)-PE38 when incubated with A431 cells for 2 or 20 h, whereas with the stabilized variants there is a 2–3-fold increase upon a 20-h incubation with A431 cells. Furthermore, the intrinsic tryptophan fluorescence data (Fig. 6) provides additional evidence that B3(Fv)-PE38 undergoes a conformational change (unfolding; Ref. 8) under less severe chaotropic conditions than the chimera or the mutants, reflecting its inferior stability.

Site-specific mutagenesis followed by stability and cytotoxicity assays were used to identify which of the three V_L residues that differ between B3 and B5 (Fig. 7) are responsible for the stabilizing effect. Since the $B3V_H$-$B5V_L$-PE38 and B3(Fv)-PE38 V_L M4L/S7T (which differs from the chimera only at the fifth CDR1 residue) had similar characteristics in the cytotoxicity and stability assays, we conclude that the CDR residue does not play a major role in stability. Although, judging from the differences in stability between the $B3V_H$-$B5V_L$-PE38 chimera and the B3(Fv)-PE38 variants carrying $B5V_L$ residues at positions 4 and 7 or position 4 alone, the CDR residue may also contribute to the Fv immunotoxins' stability. Yasui *et al.* (13) have recently reported that mutations in CDR residues can influence the stability of Fvs analysis of B3(Fv)-PE38 derivatives carrying mutations V_L M4L or V_L S7T separately showed that replacing V_L methionine 4 with leucine stabilized the immunotoxin almost as much as the $B3V_H$-$B5V_L$-PE38 combination, whereas replacing V_L serine 7 with threonine had no stabilizing effect and was, in fact, less active than B3(Fv)-PE38 (Fig. 3).

Examination of a structural model of B3(Fv) (Fig. 8) (10), reveals that the side chain of V_L serine 7 is exposed to the solvent and does not appear to interact directly with any other part of the molecule. The bulkier side chain of a threonine at the same position would probably also be exposed to solvent. V_L methionine 4 is a buried residue (Fig. 7) as would probably be a leucine at the same position. Methionine 4 does not appear to interact directly with any of the V_H residues, and the stabilizing effect resulting from its replacement with leucine would have to be explained by an effect on the independent folding of the V_L domain. Creamer *et. al.* (14) have observed that there is a diminished entropy loss when leucine replaces methionine and is folded into the buried part of the protein. Eriksson *et al.* (15) have observed that replacing leucine at buried positions of lysozyme with other hydrophobic residues that have smaller side chains (including methionine) destabilized the protein.

Leucine is somewhat more hydrophobic than methionine, so it may be favored in a buried position (16–18). In the studies cited above, there is a small but significant difference in the stability of the protein, and the methionine-containing variant is less stable than the leucine-containing variant (14, 15). The high tendency of B3(Fv)s to aggregate may be improved by a small but favorable alteration in its refolding thermodynamics due to V_L M4L replacement.

B3(Fv)-PE38 is less stable than B5(Fv)-PE38 (5), as were the $B3V_H$-$B5V_L$-PE38 chimera, B3(Fv)-PE38 V_L M4L/S7T and B3(Fv)-PE38 V_L M4L (not shown). It is thus clear that part of the stability difference results from differences in the V_H domains. We have identified the $B3V_H$ residue, which is responsible for its relative instability. But that residue cannot be mutated without a severe loss in binding affinity, and this has important implications on the mechanism of antigen binding by the B3 antibody.[3] Finally, we wish to emphasize that the state of the art in cloning antibody fragments in *E. coli* is the use of "family-specific primers" for the PCR amplification of the variable region segments from cDNA (19–22). Our data demonstrates that one must be cautious when such techniques are used, because the N termini of the obtained clones may not match the original protein sequence, and this alteration can lead to Fvs with altered stabilities and affinities.

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[3] U. Brinkmann, I. Benhar, K. Webber, Y. Reiter, and I. Pastan, manuscript in preparation.

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