[CANCER RESEARCH 61, 5070–5077, July 1, 2001]

Lowering the Isoelectric Point of the Fv Portion of Recombinant Immunotoxins Leads to Decreased Nonspecific Animal Toxicity without Affecting Antitumor Activity

Masanori Onda, Satoshi Nagata, Yasuo Tsutsumi, James J. Vincent, Qing-cheng Wang, Robert J. Kreitman, Byungkook Lee, and Ira Pastan[1]

Laboratory of Molecular Biology, Division of Basic Sciences, National Cancer Institute, NIH, Bethesda, Maryland 20892-4255

ABSTRACT

Recombinant immunotoxins are genetically engineered proteins in which the Fv portion of an antibody is fused to a toxin. Our laboratory uses a 38-kDa form of *Pseudomonas* exotoxin A termed PE38 for this purpose. Clinical studies with immunotoxins targeting CD25 and CD22 have shown that dose-limiting side effects are attributable to liver damage and other inflammatory toxicities. We recently showed that mutating exposed surface neutral residues to acidic residues in the framework region of the Fv portion of an immunotoxin targeting CD25 [anti-Tac-(scFv)-PE38] lowered its isoelectric point (pI) and decreased its toxicity in mice without impairing its cytotoxic or antitumor activities. We have now extended these studies and made mutations that change basic residues to neutral or acidic residues. Initially the pI of the mutant Fv (M1) of anti-Tac(scFv)-PE38 was decreased further. Subsequently, mutations were made in two other immunotoxins, SS1(dsFv)-PE38 targeting ovarian cancer and B3(dsFv)-PE38 targeting colon and breast cancers. We have found that all these mutant molecules fully retained specific target cell cytotoxicity and antitumor activity but were considerably less toxic to mice. Therefore, lowering the pI of the Fv may be a general approach to diminish the nonspecific toxicity of recombinant immunotoxins and other Fv fusion proteins without losing antitumor activity.

INTRODUCTION

Recombinant toxins are chimeric proteins in which a cell-targeting moiety is fused to a toxin (1). If the cell-targeting moiety is the Fv portion of an antibody, the molecule is termed a recombinant IT[2] (2). The toxin moiety is genetically altered so that it cannot bind to the toxin receptor present on most normal cells. Recombinant ITs selectively kill cells, which are recognized by the antigen-binding domain. Fv fragments are the smallest functional modules of antibodies. When used to construct ITs, Fv fragments are probably better therapeutic reagents than whole IgGs, because their small size facilitates better tumor penetration (3). Initially Fvs were stabilized by making recombinant molecules in which the V_H and V_L domains are connected by a peptide linker so that the antigen-binding domain site is regenerated in a single protein (scFv; Refs. 4 and 5). However many Fvs could not be stabilized by this approach. Our laboratory developed an alternative method to stabilize the Fv moiety in recombinant ITs. In this approach, the Fv is stabilized by a disulfide bond (dsFv) that is engineered between framework regions of the two Fv domains, and the toxin is fused to either of the Fv domains (6). One striking difference between scFv ITs and dsFv ITs is that dsFv ITs are more stable. Also, dsFv ITs can often be produced by the refolding of inclusion bodies with higher yields than with the corresponding scFv ITs (7). For the toxin moiety, our laboratory uses a 38-kDa mutant form of PE (8). PE is a 66-kDa protein composed of three domains: a cell binding domain, a translocation domain, and an ADP-ribosylating domain. Recombinant ITs are made by deleting the cell-binding domain of PE and replacing it with the Fv portion of an antibody.

During the past several years, we have made a variety of recombinant ITs using antibodies recognizing different antigens on cancer cells. Several of these have been tested in Phase I trials in patients with cancer. B3(dsFv)-PE38 (LMB-9) is a disulfide-bonded IT targeted at epithelial cancers that express $Lewis^Y$ (9). SS1(dsFv)-PE38 is an IT targeted at ovarian cancers and other epithelial cancers expressing the protein mesothelin (10, 11). Anti-Tac(scFv)-PE38(LMB-2) is a single chain IT directed at CD25-expressing hematological malignancies and RFB4(dsFv)-PE38(BL-22) is directed at CD22-expressing hematological malignancies. Anti-Tac(scFv)-PE38 and RFB4(dsFv)-PE38 both have shown good antitumor activity in patients (12–14). One of the dose limiting toxicities of recombinant ITs is liver damage attributable to cytokine release as well as other inflammatory toxicities. Liver damage attributable to TNF-α release is also a dose-limiting toxicity in mice given anti-Tac(scFv)-PE38 (15). Several approaches to reduce nonspecific toxicity are being pursued using anti-Tac(scFv)-PE38 as the initial molecule for these studies. We used molecular modeling and site-directed mutagenesis to lower the pI of the Fv of anti-Tac without decreasing its binding activity. The IT containing this mutated Fv is termed M1(scFv)-PE38. The toxicity in mice of M1(scFv)-PE38 is >3-fold lower than that of anti-Tac(scFv)-PE38. The pI of the Fv of anti-Tac Fv is 10.21 and has been lowered to 6.82 in M1 Fv, yet its ability to kill CD25-positive target cells is undiminished (16).

In the current study, we have made additional modifications in the M1 Fv by mutating basic residues, whereas previously the only mutations were the conversion from neutral to acidic amino acids. Furthermore, we lowered the pI of two other ITs. One of these is SS1(dsFv)-PE38, which is targeted at an antigen (mesothelin) expressed on ovarian cancers. The other is B3(dsFv)-PE38 (LMB-9) that is targeted at the $Lewis^Y$ antigen present on many epithelial cancers. For clinical purposes, it is preferable to stabilize the Fv portion of an IT by replacing the peptide linking V_H and V_L with a disulfide bond that is introduced in the framework region. Therefore, in the current study, we first converted M1(scFv)-PE38 into M1(dsFv)-PE38. The other two ITs are already linked with a disulfide bond. Our results show that animal toxicity is markedly diminished by lowering the pI of the Fv, and antitumor activity is fully retained.

MATERIALS AND METHODS

Calculated pI Value. The pI of each IT was calculated using a program in the Genetics Computer Group (Madison, WI) package.[3] In the Fv portion,

Received 1/3/01; accepted 5/1/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom requests for reprints should be addressed, at Laboratory of Molecular Biology, National Cancer Institute, Building 37, Room 4E16, 37 Convent Drive, MSC 4255, NIH, Bethesda MD 20892-4255; Phone: (301) 496-4797; Fax: (301) 402-1344; E-mail: pasta@helix.nih.gov.

[2] The abbreviations used are: IT, immunotoxin; V_H, variable heavy; V_L, variable light; PE, *Pseudomonas* exotoxin A; scFv, single-chain Fv; dsFv, disulfide-stabilized Fv; pI, isoelectric point; TNF, tumor necrosis factor; CR, complete regression; ALT, alanine aminotransferase.

[3] Available at Internet address: http://molbio.info.nih.gov/molbio/gcglite/protform.html.

cysteines have no charge, because they are disulfide bonded. These were converted to serine for the pI calculation.

Mutagenesis. Mutagenesis of M1(dsFv)-PE38 was done by Kunkel's method (17) with some modifications. CJ236 cells were transformed with pOND9-1 and pOND9-2. The transformants were grown in 2× YT medium containing 100 μg/ml ampicillin at 37°C. At an OD600 of 0.36, the cells were infected with the helper phage M13 at a multiplicity of infection of 5. After incubation at 37°C/110 rpm for 1 h, the culture was maintained at 37°C/300 rpm for another 6 h. The bacterial cells were then precipitated by centrifugation, and the phage from the supernatant was precipitated with polyethylene glycol. The single-stranded uracil-containing DNA from the purified phage was extracted with phenol/chloroform and precipitated with sodium chloride and ethanol. This ssDNA codes for the sense strand of M1(dsFv)-PE38. The following primers were used for M1 mutagenesis: M1 VL K18Q, 5′-GGCACTGCAGGTTATGGTGACTTGCTCCCCTGGAGATGCAGACAT-3′; M1 VL K45Q, 5′-GGATGTGGTATAAATCCATAGCTGGGGAGAAGTGCCTGGCTT-3′; M1 VL R77N, 5′-GGCAGCATCTTCAGCCTCCATATTCGAAATTGTGAGAGAGTAATCGGT-3′; M1 VL K103-107E, GTTAGCAGCCGAATTCTATTCGAGTTCCAGCTCGGTCCCGCAACCGAACGTGAG-3′; M1 VH K13, 5′-CATCTTCACTGAGGCCCCAGGTTCTGCGAGCTCAGCCCCAGA-3′; and M1 VH K73D, 5′-GCTCAGTTGCATGTAGGCAGTACTGGAGGAATCGTCTGCAGTCAA-3′. The primers were phosphorylated using polynucleotide kinase and T4 DNA ligase buffer from Boehringer Mannheim (Indianapolis, IN). These phosphorylated primers were used to introduce mutations in the uracil template of pOND9-1 and pOND9-2 using Bio-Rad (Richmond, CA) Muta-Gene kit. The product at the end of the mutagenesis reaction was used to directly transform DH5α competent cells. Mutations in the clones were confirmed by automated DNA sequencing.

To introduce cysteines into the framework regions of M1(scFv)-PE38 for making disulfide bond Fv (dsFv), the following primers were used same way: M1 VL D100C, 5′-GCCGCCCTCGGGACCTGAATTCTATTTGAGCTCCAGCTTGGTCCCGCAACCGAACGTGAGTGGGTA-3′; and M1 VH G44C, 5′-ATATCCAATCCATTCCAGACACTGTTCAGGCCTCTGTTTTACCCAGTGCAT-3′.

Mutagenesis of SS1(dsFv)-PE38 was also done by Kunkel's method. After making the uracil template of pSC7-4 and pSC7-7, the following phosphorylated primers were used for SS1(dsFv)-PE38 mutagenesis: SS1 VL R7D, 5′-CATGATTGCTGGATCCTGAGTGAGCTC-3′; SS1 VL K18Q, 5′-GTCATGGTGACCTGCTCCCCGGGAGATGCAG-3′; SS1 VL G60D, 5′-CCACTGCCACTGAAGCGATCGGGGACTCCAGAA-3′; SS1 VL A80E, 5′-CTGGCAGTAATACGTAGCATCATCTTCCTCCTCCACGCTGC-3′; SS1 VL K108E, 5′-CCGAATTCATTATTCTATTTCAAGCTTTGTCCCGCA; SS1 VH Q1EK13Q, 5′-AAGCGCCAGGCTGCTCGAGCTCAGGCCCAGACTGCTGCAGTTGTACCTCCATATGTATATCTC-3′; and SS1 VH S75S82BS84 5′-TGCAGAGTCTTCATTTGTCAGATTGAGGAGGTCCATGTAGGCAGTACTTTGTGACTTGTCTAC-3′.

Mutagenesis of B3(dsFv)-PE38 was also done by Kunkel's method. After making the uracil template of pUli 39-1 and pYR38-2, the following phosphorylated primers were used for B3(dsFv)-PE38 mutagenesis: B3 VL L3E, 5′-CAATGGAGACTGGGTCATCTCCACGTCCATATGTATATCTCCTTC-3′; and B3 VL K103D, 5′-CTCGGGACCTCCGGAAGCATCTATTTCCAGATCTGTCCCACAGCCGAACGT-3′.

Production of Recombinant IT. The components of ITs were expressed in *Escherichia coli* BL21(λDE3) and accumulated in inclusion bodies, as previously described for other ITs (7). Inclusion bodies were solubilized in GuCl, reduced with dithioerythritol and refolded by dilution in a refolding buffer containing arginine to prevent aggregation, and oxidized and reduced with glutathione to facilitate redox shuffling. Active monomeric protein was purified from the refolding solution by ion exchange and size exclusion chromatography. Protein concentrations were determined by Bradford Assay (Coomassie Plus; Pierce, Rockford, IL).

Isoelectric Focusing. The recombinant ITs and standard markers with pIs ranging from 4.5 to 9.6 were analyzed using the Ready Gel System (IEF Ready Gel; Bio-Rad, Richmond, CA). These gels were stained by Coomasie Blue R-250 to reveal the protein bands.

Cytotoxicity Assay. The specific cytotoxicity of each IT was assessed by protein synthesis inhibition assays (inhibition of incorporation of tritium-labeled leucine into cellular protein) in 96-well plates, as previously described (18). The activity of the molecule is defined by the IC_{50}, the toxin concentration that reduced incorporation of radioactivity by 50% compared with cells that were not treated with toxin.

Nonspecific Toxicity Assay. Groups of five female NIH Swiss mice were given single injections i.v. through the tail vein of escalating doses of ITs, as previously described (16). Animal mortality was observed over 2 weeks. The LD_{50} was calculated with the Trimmed Spearman-Karbar statistical method, from the Ecological Exposure Research Division of the United States Environmental Protection Agency (19, 20).

Analysis of Liver Enzymes. Liver damage was assessed by measuring plasma enzyme activity of ALT measured by ANILYTICS Inc. (Gaithersburg, MD).

Stability Assay. Stability of the ITs after serum treatment was determined by incubation at 0.01 mg/ml in 50% mice sera in DPBS containing Ca^{2+} and Mg^{2+} at 37°C for up to 18 h. Mice serum was taken from NIH Swiss mice 6–8 weeks of age. At each time point, the samples were frozen at −80°C. At the end of the experiment, the samples were thawed and tested for cytotoxic activity.

Antitumor Activity of ITs in Nude Mice. The antitumor activity of ITs was determined in nude mice bearing human cancer cells that have appropriate antigen expression. Cells (3×10^6) were injected s.c. into nude mice on day 0. Tumors ~0.05 cm^3 in size developed in animals by day 4 after tumor implantation. Starting on day 4, animals were treated with i.v. injections of each of the ITs diluted in 0.2 ml of PBS/0.2% HSA. Therapy was given once every other day (on days 4, 6, and 8), and each treatment group consisted of five animals. Tumors were measured with a caliper every 2 or 3 days, and the volume of the tumor was calculated by using the formula: tumor volume (cm^3) = length × $(\text{width})^2$ × 0.4.

Pharmacokinetics. NIH Swiss mice were injected i.v. with 4 μg of anti-Tac(dsFv)-PE38, M1(dsFv)-PE38, M16(dsFv)-PE38, SS1(dsFv)-PE38, or St6(dsFv)-PE38, or 10 μg of B3(dsFv)-PE38 or Mt9(dsFv)-PE38. Blood samples were drawn at different times. The level of IT in blood was measured by a bioassay in which serum samples were incubated with human cancer antigen-positive cells, and the ability of the serum sample to inhibit protein synthesis was measured. A standard curve, obtained by incubating serial dilutions of the injected toxins on antigen-positive cells, was used to determine the toxin concentration in each serum sample. Pharmacokinetic parameters were calculated using an exponential curve-fitting program, RSTRIP (micro-Math Scientific Software, Salt Lake City, UT).

Statistical Analysis. Values are expressed as mean ± SD. For comparison between the two experimental groups, Student's *t* test was used. $P < 0.05$ was considered statistically significant. The relationship was calculated using nonlinear least-square regression.

RESULTS

Conversion of M1(scFv)-PE38 to M1(dsFv)-PE38. Previously, we have used a mouse model to investigate the basis of toxicity of ITs and found that M1(scFv)-PE38, in which the pI of the Fv of anti-Tac was lowered from 10.21 to 6.82 by selective mutation of surface residues, showed a 3-fold decrease in animal toxicity and hepatic necrosis (16). For clinical purposes, it is preferable to stabilize the Fv portion of the IT by replacing the peptide linking V_H and V_L with a disulfide bond that is introduced in the framework region. Therefore, we first converted M1(scFv)-PE38 into M1(dsFv)-PE38 and found that M1(dsFv)-PE38 had the same toxicity in mice as its single-chain counterpart (LD_{50}, 1.22 mg/kg; Table 1).

Identification of Mutation Sites in the Framework Region of Fv. In our previous study, the nonspecific toxicity of anti-Tac(scFv)-PE38 was reduced by mutating neutral residues to acidic residues (16). In this study, we extended this work by mutating basic and neutral residues in the framework regions to acidic or neutral residues. To identify residues that were candidates for mutation, we first calculated the frequencies at which various amino acids occur at each position of the Fv using the Kabat database, and we ranked them according to their frequency (Fig. 1; Ref. 21). We also created models based on other Fv crystal structures to determine which residues were exposed at the surface and which residues interacted with others. With this information, we devised the

Table 1 *IT mutations, pIs, and LD_{50}s*

IT	Mutations of Fv	pI of Fv[a]	pI of IT[a]	LD_{50} (mg/kg)[b]	95% confidence of LD_{50}[b]
M1(dsFv)-PE38[c]	(V_H: Q1E, H3Q, Q5V, Q6E, G42E) + (V_L: Q1D, V3E, S70D)	7.76	5.27	1.22	1.14–1.31
M16(dsFv)-PE38	dsM1 + (V_H: K13E, K73D) + (V_L: K18Q, K45Q, R77N, K103E, K107E)	4.42	4.69	2.52	2.22–2.85
SS1(dsFv)-PE38	None	9.46	5.61	0.75	0.59–0.95
St6(dsFv)-PE38	(V_H: Q1E, K13Q, S75Q, S84N, S(82B)N) + (V_L: S7D, K18Q, G60D, A80E, K107E)	4.67	4.94	1.85	1.60–2.20
B3(dsFv)-PE38	None	8.96	5.26	0.97	0.84–1.10
Mt9(dsFv)-PE38	(V_L: L3E, K74T, R77S, K103D, K107D)	4.74	4.83	2.29	1.93–2.72

[a] pI of Fv and pI of entire IT was calculated using a program in the Genetics Computer Group (Madison, WI).
[b] Groups of >five female NIH Swiss mice were given injections i.v. once through the tail vein. The LD_{50} is calculated with the trimmed Spearman-Karbar statistical method.
[c] M1(dsFv)-PE38 is a mutant form of anti-Tac(dsFv)-PE38 (16).

following procedure to identify the positions which should be changed. (*a*) Align the V_L and V_H sequences with the Kabat database frequency table (Fig. 1); (*b*) exclude CDR residues; (*c*) exclude acidic residues; (*d*) exclude residues with 100% conservation (according to the frequency table); (*e*) exclude positively charged residues at positions where neutral or acidic amino acids do not occur and exclude neutral residues at positions where acidic amino acids do not occur; (*f*) exclude residues in which <30% of the surface area was exposed; (*g*) exclude residues which interact with others; (*h*) for the remaining candidate residues, choose an acidic or neutral residue with the highest occupancy as a mutation candidate; and (*i*) allow mutations in the COOH termini of both chains.

Using the above procedure, we developed candidates for mutation for M1, SS1, and B3 as shown in Table 1 and Fig. 1. We examined the effects of the change in pI on the electrostatic potential of the surface of the Fvs using models built previously (16). Fig. 2 shows the electrostatic potential mapped to the molecular surface of the three mutant models, with *red* designating negative and *blue* designating positive. It is evident that large areas of positive potential are decreased in the mutants with lower pI. Because most of the Fv is now acidic, this would lead to decreased interactions with negatively

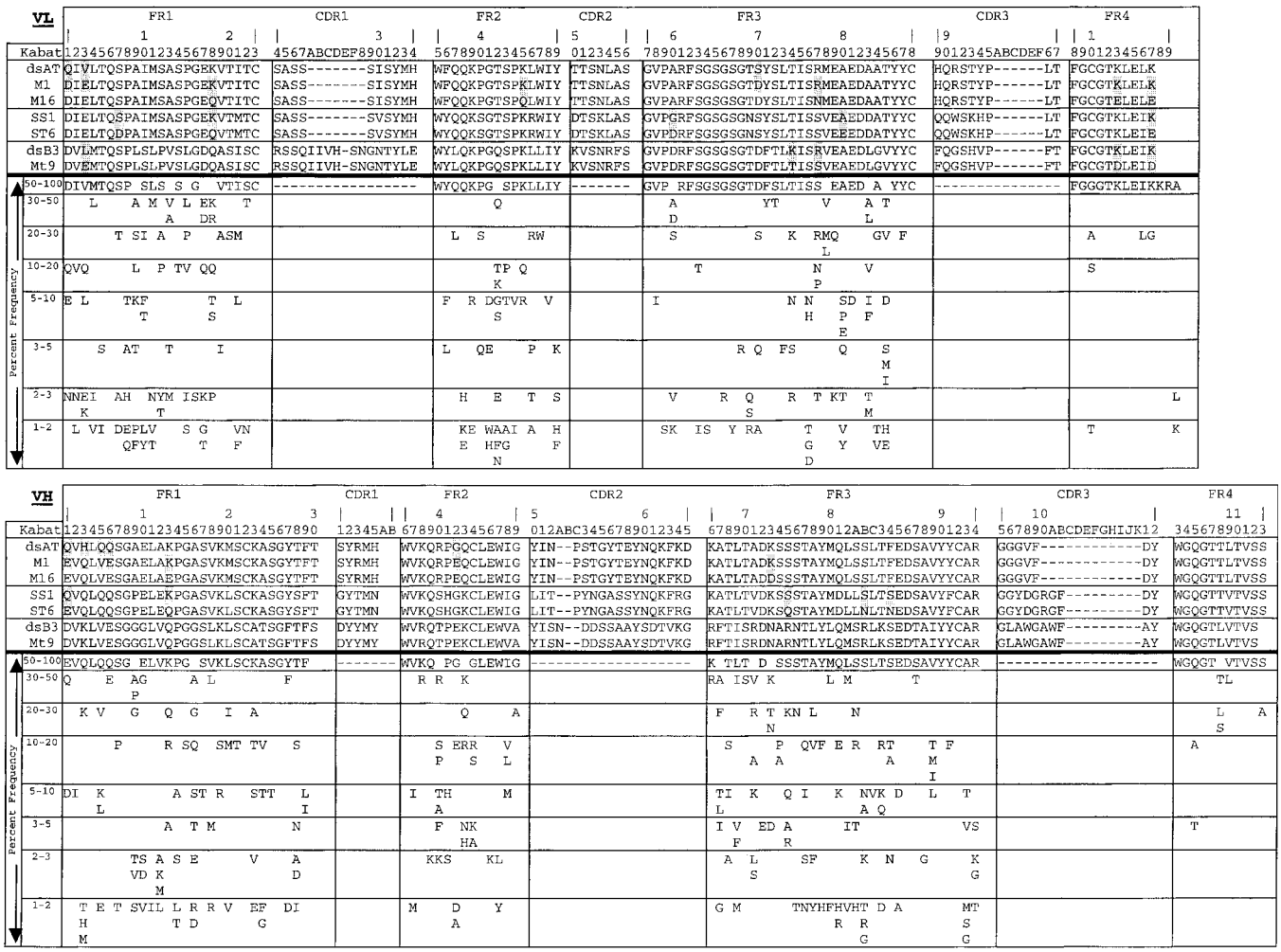

Fig. 1. Sequences of Fvs aligned with frequency table. *Top*, the first three sequences are aligned V_L sequences of anti-Tac ITs that were constructed in our laboratory. Numbering is according to Kabat. The next two sequences are the V_L sequences of anti-mesothelin ITs. The next sequences are the anti-Lewis^Y ITs. After this is a table of amino acids sorted into bins according to their frequency in the Kabat database for each position. *Bottom*, corresponding alignments for V_Hs. Residues shaded in *gray* indicate mutations made between sequences.

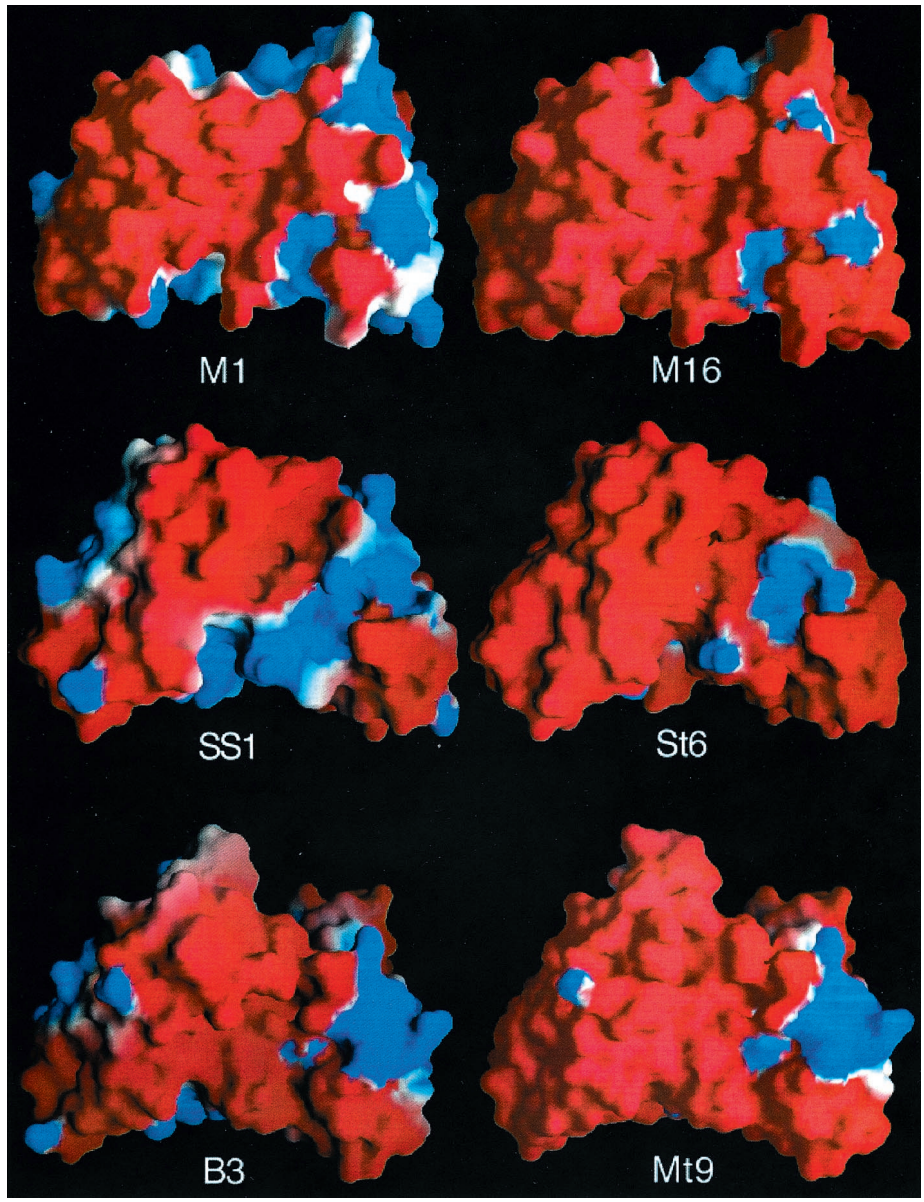

Fig. 2. Electrostatic potential mapped to molecular surface. Three Fv mutant pairs are shown: M1 and M16; SS1 and St6; and B3 and Mt9. *Red*, negative electrostatic potential (−1 kT); *blue*, positive electrostatic potential (+1 kT). The antigen binding site is located at the *top center* in all models. The images were produced using GRASP (30).

charged residues on the cell surface. The figure also shows regions of remaining positive charge, which mainly represent residues that were not selected for mutation.

Expression and Purification of Disulfide Bond-stabilized Fv Recombinant ITs. All of the mutations were confirmed by DNA sequencing and the recombinant proteins were expressed in *E. coli*, where they all accumulated in inclusion bodies. All ITs were purified by our standard method, which consisted of ion-exchange and size-exclusion chromatography using renatured inclusion body protein. Each of six ITs (three mutant and three wild-type) eluted as a monomer upon TSK gel-filtration chromatography and migrated as a single, major band of about 62 kDa in SDS-PAGE (Fig. 3).

Isoelectric Focusing of ITs. The ITs along with pI marker proteins were subjected to acrylamide gel IEF to compare their pIs. The pIs of the proteins were determined from the slope of pI markers that covered a range of pI 4.5 to 9.6. The pI of M1(dsFv)-PE38, M16(dsFv)-PE38, SS1(dsFv)-PE38, St6(dsFv)-PE38, B3(dsFv)-PE38, and Mt9(dsFv)-PE38 were 5.0, 4.8, 5.2, 5.0, 5.1, and 4.9, respectively (Fig. 4). These pIs were well correlated with the calculated pIs. In several cases, more than one band was noted, possibly because of the decrease of net charge that would result from deamidation.

Cytotoxicity of Mutants with Lowered pIs. The data in Table 2 show the activity of each of the mutant molecules tested on the appropriate antigen-positive target cells. Each of the mutant acidic molecules had the same activity on their respective target cells as did the parent molecule. For example, on ATAC4 cells, the IC_{50} of M1(dsFv)-PE38 and M16(dsFv)-PE38 was 0.04 ng/ml, and on HUT102 cells it was 0.1 ng/ml. Therefore, by lowering the pI of the Fv, there was no loss of specific cytotoxic activity on the target cell.

Nonspecific Toxicity of ITs. Each of these molecules was then evaluated for its nonspecific toxicity in mice (Table 1). Groups of five or more mice were injected once with varying doses of IT and observed for 2 weeks. LD_{50}s were calculated with the trimmed Spearman-Karbar statistical method. Almost all of the deaths occurred within 72 h after treatment. In all cases, the molecules with lower pIs were less toxic than wild-type molecules. When mutations were introduced into M1(dsFv)-PE38 (pI 5.27) to produce M16(dsFv)-PE38 (pI 4.69), the single-dose toxicity in mice was greatly diminished; the LD_{50} rose from 1.22 mg/kg to 2.52 mg/kg. St6(dsFv)-PE38 has a pI of 4.94. The animal toxicity (LD_{50}) of St6(dsFv)-PE38 is 1.85 mg/kg. Thus it is less toxic than the starting molecule SS1(dsFv)-PE38 (LD_{50}, 0.75 mg/kg). Mt9(dsFv)-PE38 has a pI of 4.83 compared

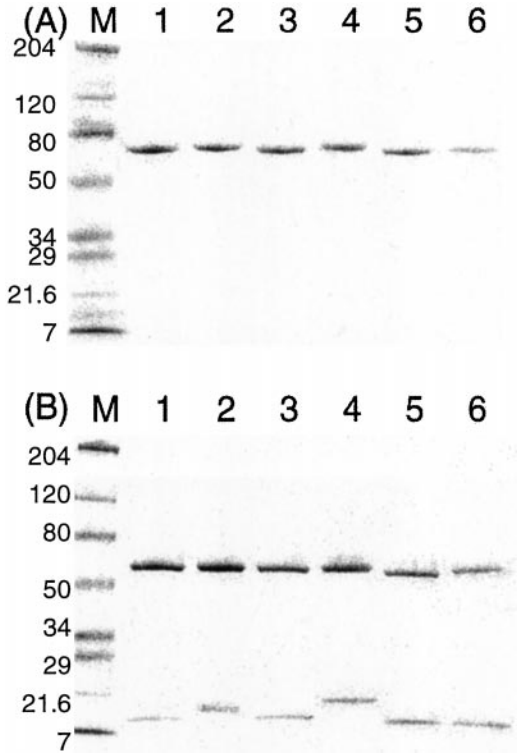

Fig. 3. PAGE of purified recombinant ITs. The purified proteins were run on 4–20% gradient SDS polyacrylamide electrophoresis gels under nonreducing conditions (*A*), and under reducing conditions (*B*). The gels were stained with Coomasie Blue. *Lane 1*, M1(dsFv)-PE38; *Lane 2*, M16(dsFv)-PE38; *Lane 3*, SS1(dsFv)-PE38; *Lane 4*, St6(dsFv)-PE38; *Lane 5*, B3(dsFv)-PE38; and *Lane 6*, Mt9(dsFv)-PE38; *M*, molecular mass standards are (*top to bottom*) 204, 120, 80, 50, 34, 29, 21.6, and 7 kDa, respectively.

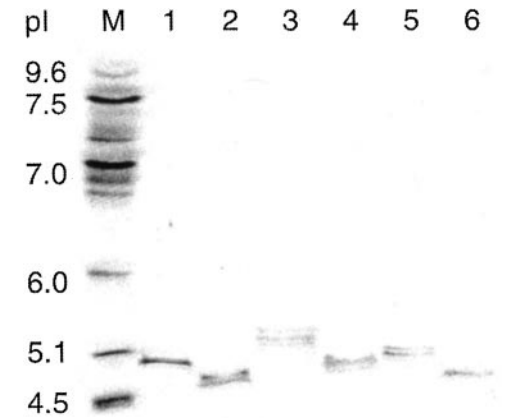

Fig. 4. Isoelectric focusing of ITs. *M*, pI standard marker; *Lane 1*, M1(dsFv)-PE38; *Lane 2*, M16(dsFv)-PE38; *Lane 3*, SS1(dsFv)-PE38; *Lane 4*, St6(dsFv)-PE38; *Lane 5*, B3(dsFv)-PE38; and *Lane 6*, Mt9(dsFv)-PE38, respectively.

with pI 5.23 for B3(dsFv)-PE38. The Fv of Mt9(dsFv)-PE38 is 2.29 mg/kg, whereas the LD_{50} of B3(dsFv)-PE38 is 0.97 mg/kg. The values are all shown in Table 1. A plot of the pI of each of the ITs against animal toxicity is shown in Fig. 7. It is evident that there is a strong correlation between the pI of the IT and the LD_{50} (toxicity) in mice ($r = -0.9691$; $P = 0.0014$). It is also clear that there is a good correlation between the pI of the Fv and toxicity in mice (data not shown).

Liver Toxicity of ITs. To determine the cause of death of the mice, we collected blood samples 3 h and 24 h after the administration of 18 μg of M1(dsFv)-PE38, M16(dsFv)-PE38, SS1(dsFv)-PE38, St6(dsFv)-PE38, B3(dsFv)-PE38, or Mt9(dsFv)-PE38. As shown in Table 3, there is a large increase in the level of plasma ALT after the administration of M1(dsFv)-PE38, SS1(dsFv)-PE38, and B3(dsFv)-PE38. However there is little change in the plasma level of ALT after the injection of the same dose of the molecules with lower pIs.

Stability of ITs. Changes in the framework region could affect the stability of the ITs. Therefore, we examined stability by incubating each IT at 37°C for 6 and 18 h. M1(dsFv)-PE38 has good stability at 37°C. As shown in Table 4, after 18 h in PBS/50% mice serum at 37°C, M1(dsFv)-PE38 retained >60% of its cytotoxic activity. M16(dsFv)-PE38 has a stability similar to M1(dsFv)-PE38. SS1(dsFv)-PE38 retained >70% of cytotoxic activity after 18 h in PBS/50% mice serum at 37°C, and St6(dsFv)-PE38 was similarly stable. B3(dsFv)-PE38 retained ~50% of its cytotoxic activity, and Mt9(dsFv)-PE38 also retained >60% of its cytotoxic activity after 18 h. Thus the framework mutations had little effect on stability of the dsFv ITs.

Antitumor Activity of ITs. To evaluate the antitumor activity of the anti-Tac ITs, ATAC4 tumor cells were implanted s.c. on day 0 in mice, and i.v. therapy was initiated on day 4 using increasing doses of M1(dsFv)-PE38 or M16(dsFv)-PE38. Each agent was given every other day for three doses. Typical tumor regression results are shown in Fig. 5 for mice treated with increasing doses of M16(dsFv)-PE38. Table 5 shows the toxicity at each dose level and a summary of tumor responses. The data confirm that M16(dsFv)-PE38 is much less toxic

Table 2 *Cytotoxic activity of wild-type IT and lower pI mutants*

Cells	Type	IC_{50} (ng/ml)	
A. Anti-Tac ITs			
		M16(dsFv)-PE38	M1(dsFv)-PE38
ATAC4	Epidermoid	0.04 ± 0.01	0.04 ± 0.01
HUT102	ATL	0.1 ± 0.02	0.1 ± 0.02
Raji	Burkitt lymphoma	>1000	>1000
OHS	Osteosarcoma	>1000	>1000
B. Anti-mesothelin ITs			
		SS1(dsFv)-PE38	St6(dsFv)-PE38
A431-K5	Epidermoid	0.63 ± 0.06	0.53 ± 0.07
C. Anti-Lewis^Y ITs			
		B3(dsFv)-PE38	Mt9(dsFv)-PE38
A431	Epidermoid	0.43 ± 0.05	0.51 ± 0.04

Table 3 *Plasma ALT levels in mice treated with ITs*[a]

IT	Plasma ALT (U/L)[b] after injection of ITs	
	3 h	24 h
M1(dsFv)-PE38	30 ± 5.7	256 ± 248
M16(dsFv)-PE38	28 ± 20	61 ± 43
SS1(dsFv)-PE38	19 ± 4.2	4762 ± 2058
St6(dsFv)-PE38	28 ± 25	127 ± 56
B3(dsFv)-PE38	37 ± 19	6338 ± 1934
Mt9(dsFv)-PE38	38 ± 35	113 ± 12

[a] Data are expressed as the mean ± SD ($n = 4$).
[b] Normal range of plasma ALT is <150 units/liter.

Table 4 *Stability of wild-type and lowered pI mutants*

ITs	IC_{50} (ng/ml)[a] after incubation in PBS/50% mice serum		
	0 (h)	6 (h)	18 (h)
M1(dsFv)-PE38	0.06 ± 0.01	0.06 ± 0.02	0.07 ± 0.02
M16(dsFv)-PE38	0.05 ± 0.01	0.06 ± 0.02	0.09 ± 0.02
SS1(dsFv)-PE38	0.63 ± 0.10	0.66 ± 0.10	0.78 ± 0.10
St6(dsFv)-PE38	0.53 ± 0.10	0.60 ± 0.10	0.68 ± 0.10
B3(dsFv)-PE38	0.45 ± 0.10	0.59 ± 0.07	0.65 ± 0.10
Mt9(dsFv)-PE38	0.50 ± 0.08	0.64 ± 0.05	0.70 ± 0.10

[a] Data are expressed as the mean ± SD ($n = 3$).

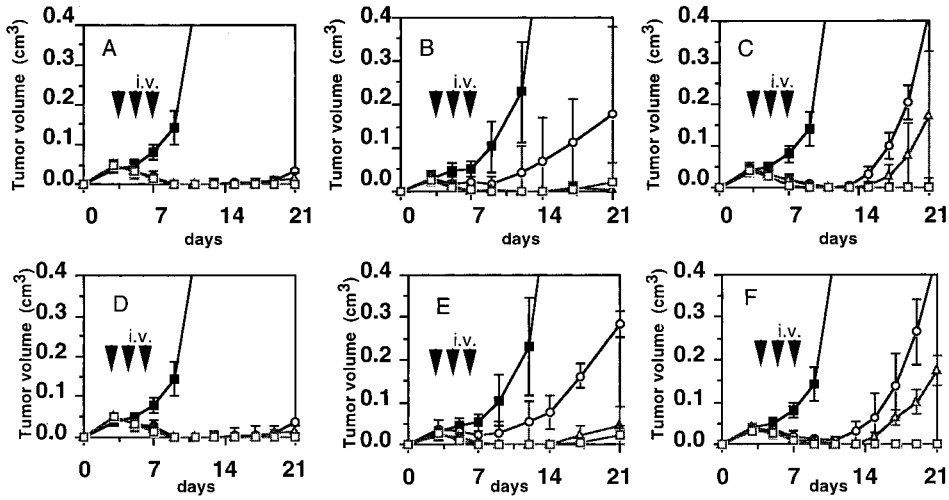

Fig. 5. Antitumor activities of M1(dsFv)-PE38 (*A*), SSl(dsFv)-PE38 (*B*), B3(dsFv)-PE38 (*C*), M16(dsFv)-PE38 (*D*), St6(dsFv)-PE38 (*E*), and Mt9(dsFv)-PE38 (*F*) in nude mice bearing human cancer cells that have antigen expression (ATAC4 cells for *A* and *D*, A431-K5 cells for *B* and *E*, and A431 cells for *C* and *F*). Groups of five animals were injected s.c. with 3×10^6 human cancer cells on day 0. Tumors ~0.05 cm^3 in size developed in animals by day 4 after tumor implantation. Starting on day 4, animals were treated with i.v. injections of each IT diluted in 0.2 ml of PBS/0.2% HSA. Therapy was given once every other day (on days 4, 6, and 8; *arrowhead*). Control groups received carrier alone. No death or toxicity was observed at these doses. ■, control (*A* and *D*); ○, 0.075 mg/kg; △, 0.15 mg/kg; □, 0.3 mg/kg; (*B* and *E*); ○, 0.2 mg/kg; △, 0.4 mg/kg; □, 0.6 mg/kg; (*C* and *F*); ○, 0.1 mg/kg; △, 0.15 mg/kg; □, 0.2 mg/kg.

to mice than M1(dsFv)-PE38. With M1(dsFv)-PE38, 3 of 10 mice died at 0.75 mg/kg × 3. In contrast, there were no deaths with M16(dsFv)-PE38 at 0.75 mg/kg × 3. Thus, the three-dose toxicity results in nude mice confirm the one-dose study in normal mice, showing that M16(dsFv)-PE38 is much less toxic than M1(dsFv)-PE38. The effect of the two ITs on tumor response is also shown in Table 5. The molecules were equally active, producing 4 of 5 and 3 of 5 CRs at 0.075 mg/kg × 3 doses, 7 of 10 and 8 of 10 CRs at 0.15 mg/kg × 3 doses, 9 of 10 and 9 of 10 CRs at 0.3 mg/kg × 3 doses, and 10 of 10 and 9 of 10 CRs at 0.45 mg/kg × 3 doses. At the dose levels where M1(dsFv)-PE38 caused death, M16(dsFv)-PE38 produced 10 of 10 CRs (0.75 mg/kg × 3). These data clearly show the usefulness of being able to give higher doses of ITs.

To evaluate the antitumor activity of anti-mesothelin ITs, A431-K5 cells were injected s.c. into mice. The method of treatment is the same as with the anti-Tac ITs. Typical tumor regression results are illustrated in Fig. 5 for mice treated with increasing doses of St6(dsFv)-PE38. The data in Table 5 also show the toxicity at each dose level and a summary of tumor responses. The data confirm that SS1(dsFv)-PE38 is much more toxic to mice than St6(dsFv)-PE38. We observed that 2 of 10 mice died at 0.4 mg/kg × 3, and 3 of 5 died at 0.6 mg/kg × 3. In contrast, there were no deaths with St6(dsFv)-PE38 at these doses. The effect of the two ITs on tumor response is also shown in Table 5. The molecules were equally active at 0.2 mg/kg × 3, producing 3 of 10 and 2 of 10 CRs. At the dose levels where SS1(dsFv)-PE38 caused death, St6(dsFv)-PE38 produced 3 of 10 CRs (0.4 mg/kg × 3) and 6 of 10 CRs (0.6 mg/kg × 3).

Finally, to measure the antitumor activity of anti-Lewis^Y ITs, mice were implanted on day 0 with A431 tumor cells, and i.v. therapy was initiated on day 4 using increasing doses of Mt9(dsFv)-PE38 or B3(dsFv)-PE38. The treatment protocol was the same as with other ITs. Typical tumor regression results are illustrated in Fig. 5 for mice treated with increasing doses of Mt9(dsFv)-PE38. The data in Table 5 shows the toxicity at each dose level and a summary of tumor regressions. The molecules were equally active at the 0.15 mg/kg × 3 and 0.2 mg/kg × 3 doses, producing one of five and one of five CRs at the lower dose and five of five and five of five CRs at the higher dose, respectively.

Pharmacokinetics of ITs. To gain information on the mechanism of the reduced nonspecific toxicity of the lower pI mutants, the pharmacokinetic properties were examined. NIH Swiss mice were injected i.v. with a single dose of 5 μg of M1(dsFv)-PE38, M16(dsFv)-PE38, SS1(dsFv)-PE38, or St6(dsFv)-PE38 or a single dose of 10 μg of B3(dsFv)-PE38 or Mt9(dsFv)-PE38. Blood samples were drawn at different times after the injection of ITs. The level of IT in the blood was measured by a bioassay in which serum samples were incubated with human cancer antigen-positive cells (ATAC4 for anti-Tac ITs, A431-K5 for anti-mesothelin ITs, and A431 for anti-Lewis^Y ITs), and the ability of the serum samples to inhibit protein synthesis was measured. Results are the average of samples of four or five animals for each time point ± SE (Fig. 6).

The plasma half-life of the M1(dsFv)-PE38 (pI, 5.27) was 16.8 min (Table 6), whereas the half-life of M16(dsFv)-PE38, which has a pI of 4.69, was 9.4 min. Thus, lowering the pI of anti-Tac IT has a significant effect on half-life.

Next, we measured the plasma half-life of SS1(dsFv)-PE38 (pI, 5.61) and found it to be 23.9 min. In contrast, the half-life of

Table 5 *Effect of increasing doses of ITs on toxicity and tumor response in mice*

IT	Death/Total[a]	PR[b]	CR[b]
M1(dsFv)-PE38			
0.075 mg/kg × 3	0/5	5/5	3/5
0.15 mg/kg × 3	0/10	10/10	8/10
0.3 mg/kg × 3	0/10	10/10	9/10
0.45 mg/kg × 3	0/10	10/10	9/10
0.75 mg/kg × 3	3/10	7/7[c]	7/7[c]
M16(dsFv)-PE38			
0.075 mg/kg × 3	0/5	5/5	4/5
0.15 mg/kg × 3	0/10	10/10	7/10
0.3 mg/kg × 3	0/10	10/10	9/10
0.45 mg/kg × 3	0/10	10/10	10/10
0.75 mg/kg × 3	0/10	10/10	10/10
SS1(dsFv)-PE38			
0.2 mg/kg × 3	0/10	10/10	2/10
0.4 mg/kg × 3	2/10	8/8[c]	2/8[c]
0.6 mg/kg × 3	3/5	2/2[c]	0/2[c]
St6(dsFv)-PE38			
0.2 mg/kg × 3	0/10	10/10	3/10
0.4 mg/kg × 3	0/10	10/10	3/10
0.6 mg/kg × 3	0/10	10/10	6/10
B3(dsFv)-PE38			
0.05 mg/kg × 3	0/5	5/5	0/5
0.1 mg/kg × 3	0/5	5/5	0/5
0.15 mg/kg × 3	0/5	5/5	1/5
0.2 mg/kg × 3	0/5	5/5	5/5
Mt9(dsFv)-PE38			
0.05 mg/kg × 3	0/5	5/5	0/5
0.1 mg/kg × 3	0/5	5/5	0/5
0.15 mg/kg × 3	0/5	5/5	1/5
0.2 mg/kg × 3	0/5	5/5	5/5
PBS/0.2% HSA[d] × 3	0/50	0/50	0/50

[a] Number of mice that died divided by number injected.
[b] PR, reduction of the sum of the tumor length and width by >50% of pretreatment values. CR, no tumor for a minimum of 4 days.
[c] Dead mice could not be evaluated.
[d] HSA, human serum albumin.

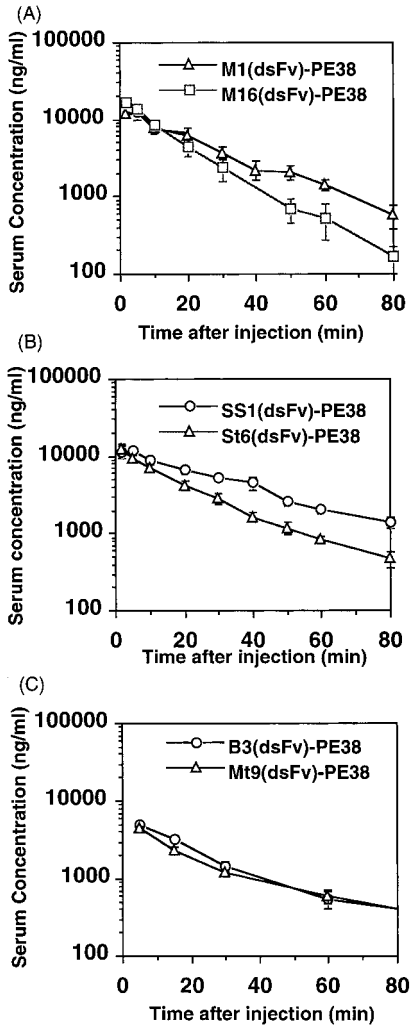

Fig. 6. Pharmacokinetics of (*A*) M1(dsFv)-PE38 and M16(dsFv)-PE38, (*B*) SS1(dsFv)-PE38 and St6(dsFv)-PE38, and (*C*) B3(dsFv)-PE38 and Mt9(dsFv)-PE38 in mice. NIH Swiss mice were injected i.v. with 5 μg of M1(dsFv)-PE38, M16(dsFv)-PE38, SS1(dsFv)-PE38, or St6(dsFv)-PE38, or 10 μg of B3(dsFv)-PE38 or Mt9(dsFv)-PE38. Blood samples were drawn at different times. The level of immunotoxin in blood was measured by a bioassay in which serum samples were incubated with human cancer antigen positive cells (ATAC4 cells for *A*, A431-K5 cells for *B*, or A431 cells for *C*), and the ability of the serum sample to inhibit protein synthesis was measured. Results are the average of 4 or 5 animals for each time point ± SE.

St6(dsFv)-PE38 (pI, 4.94) was 12.6 min. The data with the anti-mesothelin ITs also show that lowering of the pI shortens the half-life.

The plasma half-life of B3(dsFv)-PE38 (pI, 5.26) was 15.9 min, and the half-life of Mt9(dsFv)-PE38 (pI, 4.83) was 13.4 min. This data also show that lowering the pI shortens the half-life, but the effects are less dramatic. A reduction of plasma half-life is strongly associated with a reduction of the pI of the ITs ($r = 0.9610$; $P = 0.0023$).

DISCUSSION

Previously, we reported that the nonspecific toxicity of the recombinant single-chain IT, anti-Tac(scFv)-PE38, was reduced by introducing mutations in the Fv portion that lowered the pI of the Fv from 10.21 to 6.82. This was accomplished without altering residues in the complementarity determining regions, and therefore it did not affect the specific cytotoxic effect of the IT on target cells. As a consequence, tumor-bearing mice could be treated with higher doses and obtain enhanced antitumor activity (16). In the previous report, most of the changes were from neutral to acidic residues, and only one IT was studied. In this study, we examined three different ITs and changed many basic residues to neutral or acidic residues using as a guide the consensus sequence of residues in the framework regions (21). Many residues are highly conserved; and these were not changed, because such mutations might affect Fv folding or stability. We also studied ITs in which the Fvs were connected by a disulfide bond linking between V_H and V_L, because these molecules are more stable and better suited for patient use (22). We were able to improve the LD_{50} of the anti-Tac IT in mice from 0.34 mg/kg for anti-Tac(scFv)-PE38 (the IT is now in clinical trials) to 2.52 mg/kg for M16(dsFv)-PE38. If this 7-fold decrease in mouse toxicity is reproducible in humans, we should be able to greatly increase the response to ITs developed against CD25, which have already shown good antitumor activity in patients with leukemias or lymphomas (13). In this study, one complete response and many partial responses were observed. By giving a higher dose of a less toxic molecule, more complete responses may be obtained. A clinical study to investigate this hypothesis has been planned.

With each of the three IT pairs that were studied, the decrease in animal toxicity caused by a lowering of the pI was >2-fold. Fig. 7 shows that the correlation between the LD_{50} and the pI is statistically significant ($r = -0.9691$; $P = 0.0014$). We also observe that a plot of the pI of the Fv against toxicity shows a highly significant correlation (data not shown). Because the Fv portions of the various ITs have a higher pI than the toxin portion (pI 4.87), we chose to make mutations in the Fv portion to lower the pI of the whole molecule. We do not know all of the details of how the toxin causes hepatic toxicity, but it is clear that ADP ribosylation activity must be preserved (15), and it is likely that some portion of the IT must bind to Kupffer cells in a pI-dependent manner.

Recently, we analyzed the mechanism by which IT anti-Tac(scFv)-PE38 produces toxicity in mice (15). We showed that TNF-α produced by Kupffer cells plays an important role in causing hepatocyte damage. It was reported previously that charged molecules can affect the release of TNF-α by macrophages (23). It is possible that macrophages bind fewer negatively charged IT molecules than positively charged molecules and consequently release less TNF-α, which is toxic to hepatocytes.

Table 6 *Pharmacokinetics of ITs*

IT	pI of IT	$T_{1/2}$ (min)	AUC (ng min/ml)[a]
M1(dsFv)-PE38	5.27	16.8	324424
M16(dsFv)-PE38	4.69	9.4	260909
SS1(dsFv)-PE38	5.61	23.9	387350
St6(dsFv)-PE38	4.94	12.6	237591
B3(dsFv)-PE38	5.26	15.9	136927
Mt9(dsFv)-PE38	4.83	13.4	110691

[a] Calculated by RSTRIP. AUC, area under the curve.

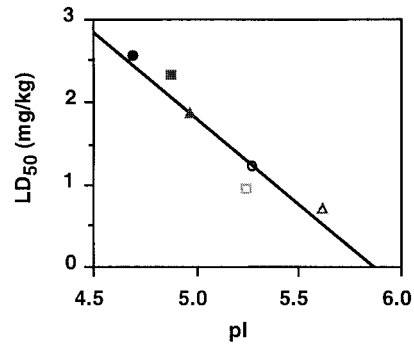

Fig. 7. Correlation between pI and mice toxicity (LD_{50}). ○, M1(dsFv)-PE38; ●, M16(dsFv)-PE38; △, SS1(dsFV)-PE38; ▲, St6(dsFv)-PE38; □, B3(dsFv)-PE38; and ■, Mt9(dsFv)-PE38. $n = 6$; $r = -0.9691$; and $P = 0.0014$.

To clarify the cause of decreasing nonspecific toxicity by lowering the pI of Fv, we studied their pharmacokinetic properties. We observed that molecules with a lower pI were more rapidly cleared from the circulation, although their antitumor activity was not altered. It is possible that tumor uptake is increased or the distribution of the IT in the tumor is more efficient when the pI is lowered, and this compensates for the more rapid clearance, which should by itself decrease antitumor activity. Because the main route of metabolism of the parental molecule anti-Tac(scFv)-PE38 is by the kidney (24), we assume all recombinant ITs are filtered through the glomerulus and degraded by tubular cells. Therefore, based on classic studies by Brenner *et al.* (25), we expected increasing the negative charge would decrease renal clearance and metabolism and increase half-life. However, it did not. It will be necessary to radiolabel the ITs and carry out biodistribution studies to resolve this dilemma. The correlation between plasma half-life and pI was highly significant statistically ($r = 0.9610$; $P = 0.0023$). Several investigators have shown that when an antibody, Fv, or Fab are anionized by the acylation of the ϵ-amino group of lysine residues or by the addition of amino acids to the COOH terminus, pharmacokinetic properties are altered. Some reports have shown that lowering the pI increased clearance from the circulation, whereas other reports showed that lowering the pI decreased clearance (26–29). Our strategy of reducing the pI by mutating specific framework residues is different from the approach used in other studies, where the properties of the proteins are grossly and often unpredictably altered. Although the modified antibodies of other studies often had a decrease in specific binding to target molecules, the molecules in the current study with lower pIs actually have the same binding activity as the parental molecules. Changing the surface residues of the Fv could also alter the immunogenicity of the IT. Clinical trials have shown that the toxin part of the IT is much more immunogenic than the Fv.[4] Therefore, alterations in the immunogenicity of the Fv, if any, would probably not be clinically significant.

In summary, we have been able to decrease the nonspecific toxicity of M1(dsFv)-PE38, SS1(dsFv)-PE38, and B3(dsFv)-PE38 in mice by >2-fold without decreasing their specific cellular toxicity or antitumor activity. The approach of lowering the pI of the Fv to reduce animal toxicity of ITs should be evaluated with other ITs as well as growth-factor toxin fusion proteins. We plan to develop M16(dsFv)-PE38 for clinical uses and to evaluate its toxicity and antitumor activity in patients with CD25-positive malignancies.

ACKNOWLEDGMENTS

We thank Dr. Kenneth Santora for careful reading of this manuscript and Robb Mann for expert editorial assistance.

REFERENCES

1. Pastan, I., and Fitzgerald, D. J. Recombinant toxins for cancer treatment. Science (Wash. DC), *254:* 1173–1177, 1991.
2. Chaudhary, V. K., Queen, C., Junghans, R. P., Waldmann, T. A., Fitzgerald, D. J., and Pastan, I. A recombinant immunotoxin consisting of 2 antibody variable domains fused to *Pseudomonas* exotoxin. Nature (Lond.), *339:* 394–397, 1989.
3. Yokota, T., Milenic, D. E., Whitlow, M., and Schlom, J. Rapid tumor penetration of a single-chain Fv and comparison with other immunoglobulin forms. Cancer Res., *52:* 3402–3408, 1992.
4. Huston, J. S., Levinson, D., Mudgett-Hunter, M., Tai, M., Novotny, J., Margolies, M. N., Ridge, R. J., Bruccoleri, R. E., Haber, E., Crea, R., and Oppermann, H. Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichea coli*. Proc. Natl. Acad. Sci. USA, *85:* 5879–5883, 1988.
5. Glockshuber, R., Malia, M., Pfitzinger, I., and Pluckthun, A. A comparison of strategies to stabilize immunoglobulin Fv-fragments. Biochemistry, *29:* 1362–1367, 1990.
6. Brinkmann, U., Reiter, Y., Jung, S. H., Lee, B., and Pastan, I. A recombinant immunotoxin containing a disulfide-stabilized Fv. Proc. Natl. Acad. Sci. USA, *90:* 7538–7542, 1993.
7. Reiter, Y., Brinkmann, U., Kreitman, R. J., Jung, S. H., Lee, B., and Pastan, I. Stabilization of the Fv fragments in recombinant immunotoxins by disulfide bonds engineered into conserved framework regions. Biochemistry, *33:* 5451–5459, 1994.
8. Hwang, J., Fitzgerald, D. J., Adhya, S., and Pastan, I. Functional domains of *Pseudomonas* exotoxin identified by deletion analysis of the gene expressed in *Escherichia coli*. Cell, *48:* 129–136, 1987.
9. Pastan, I., Lovelace, E. T., Gallo, M. G., Rutherford, A. V., Magnani, J. L., and Willingham, M. Characterization of monoclonal antibodies B1 and B3 that react with mucinous adenocarcinomas. Cancer Res., *51:* 3781–3787, 1991.
10. Chowdhury, P. S., Viner, J. L., Beers, R., and Pastan, I. Isolation of a high-affinity stable single-chain Fv specific for mesothelin from DNA-immunized mice by phage display and construction of a recombinant immunotoxin with anti-tumor activity. Proc. Natl. Acad. Sci. USA, *95:* 669–674, 1998.
11. Chowdhury, P. S., and Pastan, I. Improving antibody affinity by mimicking somatic hypermutation *in vitro*. Nat. Biotechnol., *17:* 568–572, 1999.
12. Kreitman, R. J., Wilson, W. H., Robbins, D., Margulies, I., Stetler-Stevenson, M., Waldmann, T. A., and Pastan, I. Responses in refractory hairy cell leukemia to a recombinant immunotoxin. Blood, *94:* 3340–3348, 1999.
13. Kreitman, R. J., Wilson, W. H., White, J. D., Stetler-Stevenson, M., Jaffe, E. S., Giardina, S., Waldmann, T. A., and Pastan, I. Phase I trial of recombinant immunotoxin anti-Tac(Fv)PE38 (LMB-2) in patients with hematologic malignancies. J. Clin. Oncol., *18:* 1622–1636, 2000.
14. Kreitman, R. J., Margulies, I., Stetler-Stevenson, M., Wang, Q., Fitzgerald, D. J., and Pastan, I. Cytotoxic activity of disulfide-stabilized recombinant immunotoxin RFB4(dsFv)-PE38 (BL22) toward fresh malignant cells from patients with B-cell leukemias. Clin. Cancer Res., *6:* 1476–1487, 2000.
15. Onda, M., Willingham, M., Wang, Q., Kreitman, R. J., Tsutsumi, Y., Nagata, S., and Pastan, I. Inhibition of TNF-α produced by Kupffer cells protects against the nonspecific liver toxicity of immunotoxin anti-Tac(Fv)-PE38, LMB-2. J. Immunol., *165:* 7150–7156, 2000.
16. Onda, M., Kreitman, R. J., Vasmatzis, G., Lee, B., and Pastan, I. Reduction of the nonspecific animal toxicity of anti-Tac(Fv)-PE38 by mutations in the framework regions of the Fv which lower the isoelectric point. J. Immunol., *163:* 6072–6077, 1999.
17. Kunkel, T. A. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA, *82:* 488–492, 1985.
18. Brinkmann, U., Pai, L. H., Fitzgerald, D. J., Willingham, M., and Pastan, I. B3(Fv)-PE38KDEL, a single-chain immunotoxin that causes complete regression of a human carcinoma in mice. Proc. Natl. Acad. Sci. USA, *88:* 8616–8620, 1991.
19. Hamilton, M., Russo, R., and Thurston, R. V. Trimmed Spearman-Karber method for estimating median lethal concentration in toxicity bioassays. Environ. Sci. Technol., *11:* 714–719, 1977.
20. Finney, D. J. The median lethal dose and its estimation. Arch. Toxicol., *56:* 215–218, 1985.
21. Chowdhury, P. S., Vasmatzis, G., Lee, B., and Pastan, I. Improved stability and yield of a Fv-toxin fusion protein by computer design and protein engineering of the Fv. J. Mol. Biol., *281:* 917–928, 1998.
22. Reiter, Y., Brinkmann, U., Lee, B., and Pastan, I. Engineering antibody Fv fragments for cancer detection and therapy: disulfide-stabilized Fv fragments. Nat. Biotechnol., *14:* 1239–1245, 1996.
23. Yui, S., and Yamazaki, M. Augmentation and suppression of release of tumor necrosis factor from macrophages by negatively charged phospholipids. Jpn. J. Cancer Res., *82:* 1028–1034, 1991.
24. Kreitman, R. J., and Pastan, I. Accumulation of a recombinant immunotoxin in a tumor *in vivo*: fewer than 1000 molecules per cell are sufficient for complete responses. Cancer Res., *58:* 968–975, 1998.
25. Brenner, B. M., Hostetter, T. H., and Humes, H. D. Glomerular permselectivity: barrier function based on discrimination of molecular size and charge. Am. J. Physiol, *234:* F455–F460, 1978.
26. Tenkate, C. I., Fischman, A. J., Rubin, R. H., Fucello, A. J., Riexinger, D., Wilkinson, R. A., Du, L., Khaw, B. A., and Strauss, H. W. Effect of isoelectric point on biodistribution and inflammation—imaging with indium-111-labeled IgG. Eur. J. Nucl. Med., *17:* 305–309, 1990.
27. Pavlinkova, G., Beresford, G., Booth, B. J. M., Batra, S. K., and Colcher, D. Charge-modified single chain antibody constructs of monoclonal antibody CC49: generation, characterization, pharmacokinetics, and biodistribution analysis. Nucl. Med. Biol., *26:* 27–34, 1999.
28. Kobayashi, H., Le, N., Kim, I. S., Kim, M. G., Pie, J. E., Drumm, D., Paik, D. S., Waldmann, T. A., Paik, C. H., and Carrasquillo, J. A. The pharmacokinetic characteristics of glycolated humanized anti-Tac Fabs are determined by their isoelectric points. Cancer Res., *59:* 422–430, 1999.
29. Kim, I. S., Yoo, T. M., Kobayashi, H., Kim, M., Le, N., Wang, Q., Pastan, I., Carrasquillo, J. A., and Paik, C. H. Chemical modification to reduce renal uptake of disulfide-bonded variable region fragment of anti-Tac monoclonal antibody labeled with Tc-99m. Bioconjug. Chem., *10:* 447–453, 1999.
30. Nicholls, A., Sharp, K., and Honig, B. Protein folding and association: insights from the interfacial and thermodynamic properties of hydrocarbons. Proteins, *11:* 281–296, 1991.

[4] Unpublished data.

Lowering the Isoelectric Point of the Fv Portion of Recombinant Immunotoxins Leads to Decreased Nonspecific Animal Toxicity without Affecting Antitumor Activity

Masanori Onda, Satoshi Nagata, Yasuo Tsutsumi, et al.

Cancer Res 2001;61:5070-5077.