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Immunology

Improved antitumor activity of a recombinant anti-Lewis^y immunotoxin not requiring proteolytic activation

(cancer therapy/*Pseudomonas* exotoxin/monoclonal antibody B1 Fv fragment/disulfide-stabilized Fv fragment/protein engineering)

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ABSTRACT **B1(dsFv)-PE33 is a recombinant immunotoxin composed of a mutant form of *Pseudomonas* exotoxin (PE) that does not need proteolytic activation and a disulfide-stabilized Fv fragment of the anti-Lewis^y monoclonal antibody B1, which recognizes a carbohydrate epitope on human carcinoma cells. In this molecule, amino acids 1–279 of PE are deleted and domain Ib (amino acids 365–394) is replaced by the heavy chain variable region (V_H) domain of monoclonal antibody B1. The light chain (V_L) domain is connected to the V_H domain by a disulfide bond. This recombinant toxin, termed B1(dsFv)-PE33, does not require proteolytic activation and it is smaller than other immunotoxins directed at Lewis^y, all of which require proteolytic activation. Furthermore, it is more cytotoxic to antigen-positive cell lines. B1(dsFv)-PE38 has the highest antitumor activity of anti-Lewis^y immunotoxins previously constructed. B1(dsFv)-PE33 caused complete regression of tumors when given at 12 μg/kg (200 pmol/kg) every other day for three doses, whereas B1(dsFv)-PE38 did not cause regressions at 13 μg/kg (200 pmol/kg). By bypassing the need for proteolytic activation and decreasing molecular size we have enlarged the therapeutic window for the treatment of human cancers growing in mice, so that complete remissions are observed at 2.5% of the LD_{50}.**

Recombinant Fv-immunotoxins are chimeric proteins in which a truncated toxin is fused to an Fv fragment of an antibody. The Fv region targets antigens on tumor cells and the toxin moiety kills the cell. Fv-immunotoxins have very good cytotoxic activity on human tumor cell lines and can cause complete regression of established human tumor xenografts in mice (1–3). Several Fv-immunotoxins are currently being evaluated in clinical or preclinical trials (4). Originally, the Fv fragments of the recombinant toxins were designed in a single-chain form (scFv-immunotoxins), in which the heavy and light chain variable region (V_H and V_L) domains are connected by a flexible peptide linker (5, 6). Subsequently a method was developed to stabilize the Fv fragments by an interchain disulfide bond that connects structurally conserved framework regions of the V_H and V_L domains (refs. 7–9 and reviewed by Reiter and Pastan in ref. 10). Such disulfide-stabilized dsFv-immunotoxins are much more stable than scFv-immunotoxins, and some have improved antigen-binding affinities and improved antitumor activities (11). A major advantage of using Fv fragments, which are the smallest functional modules of antibodies, in recombinant immunotoxins is that these molecules are significantly smaller than chemical conjugates made with whole antibodies. This allows them to effectively penetrate into solid tumors (12, 13).

Pseudomonas exotoxin (PE)-based recombinant immunotoxins require proteolytic activation. Domain II of the toxin is cleaved between amino acids 279 and 280, a reaction that is catalyzed by the enzyme furin (14, 15). This step could be rate limiting, because the furin concentration in cells is low and some cancer cells may be furin deficient. Therefore, we have constructed a recombinant immunotoxin that does not need proteolytic activation. Furin cleavage generates a 37-kDa carboxyl-terminal fragment of PE, amino acids 280–613 (PE37), that contains the translocating and ADP-ribosylation activity of PE (16, 17). If a functional Fv fragment could be inserted into PE37 without destroying its ADP-ribosylation activity or translocating ability and the Fv still retained its binding affinity, then the recombinant molecule should be more active than a toxin which needs to be proteolytically processed.

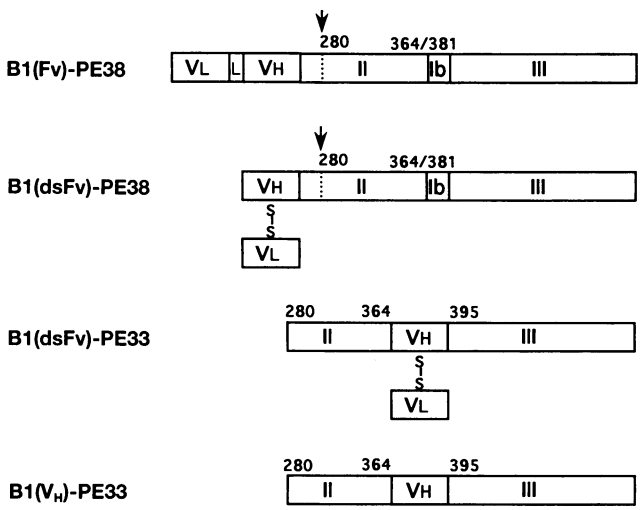

FIG. 1. Schematic of expressed proteins. Positions of amino acids that span PE sequences are numbered. The arrow marks the proteolytic site of PE for activation. S—S shows the disulfide bond linkage between the Fv fragments. L, peptide linker; II, PE domain II for translocation; Ib, PE domain Ib (function unknown); III, PE domain III for ADP-ribosylation of EF2.

Monoclonal antibody (mAb) B1 is a murine antibody directed against Lewis^y-related carbohydrate antigens, which are abundant on the surface of many carcinomas (18). mAb B1 has been used to make both single-chain and disulfide-stabilized Fv-immunotoxins (18–20). These agents are capable of causing complete regression of established xenografts in nude mice (20). To develop a recombinant immunotoxin that is small and stable and does not need proteolytic processing, we have replaced domain Ib (amino acids 365–394) of PE37 with the

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Abbreviations: mAb, monoclonal antibody; V_H and V_L, variable heavy and light chain, respectively; scFv and dsFv, single-chain and disulfide-stabilized Fv, respectively; PE, *Pseudomonas exotoxin*; Mes, 4-morpholineethanesulfonic acid.

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V_H fragment of mAb B1 and linked the V_H domain to the V_L domain with a disulfide bond (Fig. 1). We find that the resulting molecule, B1(dsFv)-PE33, is more active than any previous mAb B1-containing immunotoxin.

MATERIALS AND METHODS

Construction of Plasmids for Expression of B1(dsFv)-PE33. "Sticky feet"-directed mutagenesis (21) using uracil-containing pDF1 as a template was used to construct the expression plasmid encoding for B1(V_H)R44C-PE33, the component of the intramolecularly inserted dsFv-immunotoxin. The construction of plasmids pDF1, which encodes PE37, which starts at methionine followed by PE amino acids 281–613 (a truncated form of PE that does not require proteolytic activation), and pB1V_HR44C-PE38, which encodes the single-domain B1(V_H)R44C-PE38 immunotoxin, has been described (16, 20). The B1(V_H)R44C DNA was PCR amplified by using the plasmid pB1V_HR44C-PE38 as a template and oligo primers CT119 and 5′-phosphorylated CT120. The forward PCR primer CT119, 5′-GGCAACGACGAGGCCGGCGCGGCC-AACGGCGGTGGCGGATCCGAGGTGCAGCTGGTGG-AATCTGGA-3′, had sequences that are identical to the DNA encoding PE residues 356–364 and a peptide linker GGGGS inserted at the 5′ end of V_H, and a *Bam*HI site was created (underlined). The reverse PCR oligonucleotide primer CT120, 5′-GTCGCCGAGGAACTCCGCGCCAGTGGGCTC-GGGACCTCCGGAAGCTTTTGC-3′, had sequences that are complementary to the DNA encoding PE residues 395–403 and an Fv-toxin junction (connector) inserted at the 3′ end of V_H, and a *Hin*dIII site was created (underlined). The PCR product was purified and annealed with a uracil-containing single-stranded DNA prepared by the rescue of pDF1 phagemid with an M13K07 helper phage (Bio-Rad). The DNA was extended and ligated according to the Muta-Gene mutagenesis kit (Bio-Rad). Because the annealing efficiency of the PCR fragment to the single-stranded template and the mutagenesis efficiency were low (≈10%), the DNA pool of the mutagenesis reaction was digested with a restriction endonuclease which cuts a unique site in the domain Ib region but not in B1(V_H). This extra digestion step improved the mutagenesis efficiency to more than 50%. Correct clones were identified by DNA restriction analysis and verified by DNA sequencing. The resulting immunotoxin clone was named pB1(V_H)R44C-PE33 or pCTK104. It encodes a single-domain B1(V_H)-immunotoxin in which the V_H domain replaces the domain Ib region (amino acids 365–394) of PE37. The plasmid pB1V_LA105CSTOP encodes B1(V_L)A105C, which is a component of dsFv-immunotoxin as described previously (20).

Production of Recombinant Immunotoxin. The components of the disulfide-stabilized immunotoxins B1(V_H)R44C-PE38, B1(V_H)R44C-PE33, and B1(V_L)A105C or the single-chain immunotoxin B1(Fv)-PE38 were produced in separate *Escherichia coli* BL21(λDE3) (22) cultures harboring the corresponding expression plasmid. All recombinant proteins accumulated in inclusion bodies. Disulfide-stabilized immunotoxins were obtained by mixing equimolar amounts of solubilized and reduced inclusion bodies essentially as described (23), except that the final oxidation step was omitted and refolding was carried out at pH 9.5. Properly folded disulfide-stabilized and single-chain immunotoxins were purified by sequential ion-exchange (Q-Sepharose and Mono Q) followed by size-exclusion chromatography on a TSK G3000SW (TosoHaas) column as described (7).

Analysis of Immunotoxins. The cytotoxic activity of immunotoxins was determined by inhibition of protein synthesis as described (24). For competition assays designed to prove the specificity of the recombinant immunotoxins, we changed the medium and added 50 μg of antibody per well 30 min prior to the addition of the immunotoxin. Thermal stability of the immunotoxins was determined by incubating them at 100 μg/ml in phosphate-buffered saline (PBS; 6.7 mM sodium phosphate, pH 7.4/150 mM NaCl) at 37°C for 8 h, followed by analytical chromatography on a TSK G3000SW (TosoHaas) column to separate the monomers from larger aggregates (8). Relative binding affinities of the immunotoxins were determined by adding ^{125}I-labeled B1-IgG to 10^5 A431 cells as a tracer with various concentrations of the competitor. The binding assays were performed at 4°C for 2 h in RPMI medium 1640 containing 1% bovine serum albumin and 50 mM Mes (Sigma) as described (25).

Toxicity and Antitumor Activity in Nude Mice. The single-dose mouse LD_{50} was determined by using female BALB/c mice (6–8 weeks old, ≈20 g), which were given a single i.v. injection of different doses of B1(dsFv)PE38 or B1(dsFv)PE33 diluted in 200 μl of PBS containing 0.2% human serum albumin (PBS-HSA). Mice were followed for 2 weeks after injection. Athymic (*nu/nu*) mice, females 6–8 weeks old, ≈20 g, were injected s.c. on day 0 with 3×10^6 A431 cells suspended in RPMI medium without fetal bovine serum. By day 5, tumors were about 50–70 mm^3 in size. Mice were treated on days 5, 7, and 9 by i.v. injections of different doses of immunotoxins diluted in PBS-HSA. Tumors were measured with a caliper and the tumor volumes were calculated by using the formula volume = length × width^2 × 0.4.

RESULTS

Plasmid Constructions and Production of B1(dsFv)-PE33. Our goal was to construct an active recombinant immunotoxin that was smaller than the current generation of recombinant immunotoxins and that did not need intracellular proteolytic cleavage for activation. To do this we inserted the B1 dsFv fragment between domains II and III by replacing domain Ib of PE37, a truncated form of PE that contains only the portion of the toxin that undergoes translocation to the cytosol. As shown in Fig. 1, the V_H domain replaces amino acids 365–394 of PE37 and the V_L domain is connected to the V_H domain by a disulfide bond engineered into the framework region, with cysteines introduced at position 44 of V_H and position 105 of V_L (7). The resulting recombinant immunotoxin, termed B1(dsFv)-PE33, is 5 kDa smaller than B1(dsFv)-PE38 or B1(Fv)-PE38 (Fig. 1). In the toxin portion, Cys-287 was changed to a Ser to reduce the chance of incorrect disulfide bond formation (26). B1(V_H)R44C is inserted after amino acid 364 of PE and the insert is preceded by a small flexible peptide linker, GGGGS. Following the V_H domain is another peptide, KASGGPE, C3 connector (27), that connects the carboxyl terminus of V_H to amino acid 395 of PE.

The "sticky feet"-directed mutagenesis protocol used for the construction of B1(V_H)R44C-PE33 is described in *Materials and Methods*. Immunotoxins were expressed in *E. coli* BL21(λDE3); cultures for expressing the components of the dsFv-immunotoxin were prepared separately. The immunotoxins were purified by refolding of inclusion bodies in a redox-shuffling buffer and sequential ion-exchange and gel-filtration chromatography as described in *Materials and Methods*. The proteins obtained were more than 95% homogeneous and had the expected molecular mass of 59 kDa on SDS/PAGE as shown in lane 2 of Fig. 2. In the presence of the reducing agent 2-mercaptoethanol, the dsFv-immunotoxin, B1(dsFv)-PE33, dissociated into its two components (lane 4), B1(V_L) and B1(V_H)-PE33. The apparent molecular masses of these components are 13 kDa and 46 kDa, respectively. We also produced the single-domain B1(V_H)-PE33 immunotoxin as shown in Fig. 2. The yield of either B1(dsFv)-PE33 or B1(V_H)-PE33 was 8–10% of the total protein present in inclusion bodies.

Improved Cytotoxic Activity of B1(dsFv)-PE33 Toward B1-Antigen-Expressing Cell Lines. The cytotoxicity of B1(dsFv)-

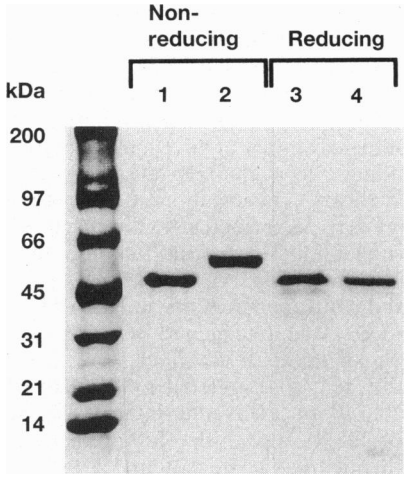

FIG. 2. Purity of B1(dsFv)-PE33 and B1(V_H)-PE33: SDS/4–20% PAGE. Lanes: 1, B1(V_H)-PE33, nonreduced; 2, B1(dsFv)-PE33, nonreduced; 3, B1(V_H)-PE33, reduced; and 4, B1(dsFv)-PE33, reduced. The left lane contains mass markers.

PE33 was determined by measuring the decrease in incorporation of [^{3}H]leucine by various human cancer cell lines after treatment with immunotoxin (24). B1(dsFv)-PE38 and B1(V_H)-PE33 (no light chain) were included for comparison. Fig. 3*A* and Table 1 show that all three proteins are cytotoxic to cells expressing B1 antigen (A431, MCF7, CRL1739, and LNCaP) but not to cells that do not bind mAb B1 (L929 and HUT102). In this assay, B1(dsFv)-PE33 had an IC_{50} of 0.25 ng/ml on A431 cells and 0.35 ng/ml on MCF7 cells. We found

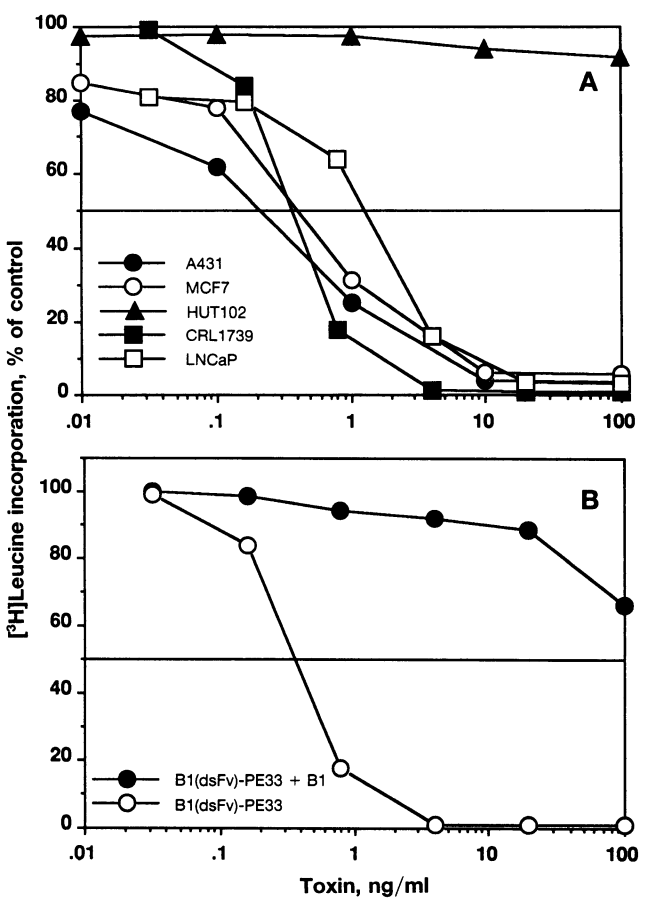

FIG. 3. (*A*) Toxicity of B1(dsFv)-PE33 for various cell lines. (*B*) mAb B1 inhibition of the cytotoxicity of B1(dsFv)-PE33 for A431 cells.

Table 1. Cytotoxicity of B1 immunotoxins toward various cell lines

Cell line*	Cancer type	Antigen expression†	IC_{50},‡ ng/ml B1(dsFv)-PE38	B1(dsFv)-PE33	B1(V_H)-PE33
A431	Epidermoid	+++	0.5	0.25	2.0
MCF7	Breast carcinoma	+++	0.9	0.35	4.0
CRL1739	Gastric	+++	0.4	0.31	ND
LNCaP	Prostate	+	7.0	1.3	ND
HUT102	T-cell leukemia	−	>1000	>1000	>1000
L929	Mouse fibroblast	−	>1000	>1000	>1000

ND, not determined.
*All the cell lines except L929 are of human origin.
†The level of antigen expression is marked +++, +, and − for strong, medium, and no detectable expression in immunofluorescence, respectively.
‡Cytotoxicity data are given as IC_{50} value, the concentration of immunotoxin that causes a 50% inhibition of protein synthesis after a 20-h incubation with immunotoxin.

that B1(dsFv)-PE33 was more active on all antigen-positive cell lines compared with B1(dsFv)-PE38, which requires proteolytic processing. To analyze whether the cytotoxicity of B1(dsFv)-PE33 was specific, competition experiments were carried out with an excess of mAb B1. Fig. 3*B* shows that the intoxication of A431 carcinoma cells by B1(dsFv)-PE33 is due to the specific binding to the B1 antigen, since its cytotoxicity was blocked by excess mAb B1. We also tested B1(V_H)-PE33, which is not associated with light chain, and found that it was only about 10-fold less cytotoxic (IC_{50} of 2 ng/ml on A431 cells) than B1(dsFv)-PE33 (Table 1), indicating the heavy chain has a major role in antigen binding. However, a related single-domain immunotoxin, B3(V_H)-PE38, which requires proteolytic processing for activation, is much less active, with an IC_{50} of 40 ng/ml on A431 cells (28).

Antigen Binding of B1(dsFv)-PE33. To determine whether the improved cytotoxicity of B1(dsFv)-PE33 is due to improved binding, we analyzed its binding affinity to antigen-positive cells (A431 cells) by competition assays, in which increasing concentrations of each immunotoxin competed for the binding of ^{125}I-labeled B1 IgG to A431 cells at 4°C. The results shown in Fig. 4 indicate that B1 IgG, B1(dsFv)-PE38, B1(dsFv)-PE33, and B1(V_H)-PE33 competed for the binding of ^{125}I-labeled B1 IgG to A431 cells by 50% at 40 nM, 2 μM, 3.5 μM, and 25 μM, respectively. Thus, the binding affinity of

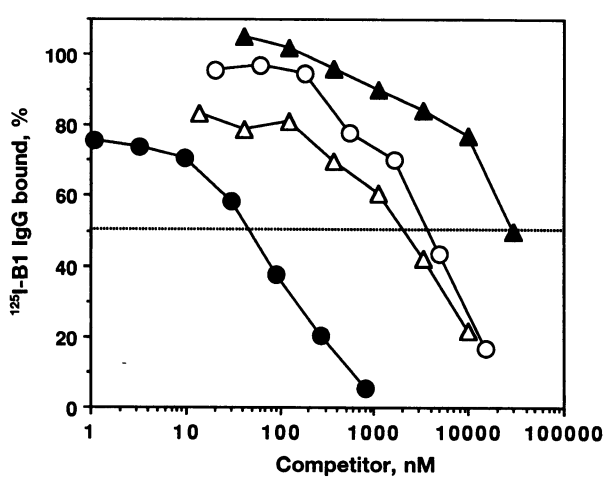

FIG. 4. Binding of B1(dsFv)-PE33, B1(dsFv)-PE38, and B1(V_H)-PE33 to A431 cells: Competitive binding analysis of the ability of purified B1(dsFv)-PE33, B1(dsFv)-PE38, and B1(V_H)-PE33 to inhibit the binding of ^{125}I-labeled B1 IgG to A431 cells overexpressing B1 antigen. ●, B1 IgG; ○, B1(dsFv)-PE33; △, B1(dsFv)-PE38; and ▲, B1(V_H)-PE33.

Table 2. Thermal stability of immunotoxins

Immunotoxin	Amount, % of control	
	Monomer	Aggregates
B1(dsFv)-PE38	100	<0.1
B1(Fv)-PE38	≈40	≈60
B1(dsFv)-PE33	100	<0.1

The thermal stability of immunotoxins to heat treatment was determined by incubation at 0.1 mg/ml in PBS at 37°C for 8 h, followed by analytical chromatography on a TSK G3000SW (TosoHaas) column, to quantitate the amount of monomers and aggregates compared to the untreated immunotoxins.

B1(dsFv)-PE33 is slightly reduced compared with B1(dsFv)-PE38. Therefore, the improved cytotoxicity cannot be due to improved binding, suggesting that elimination of the requirement for proteolytic activation is probably responsible for the improved cytotoxicity. The single-domain immunotoxin B1(V_H)-PE33 exhibited a 10-fold lower binding affinity relative to the dsFv-immunotoxins, which is consistent with its diminished cytotoxicity (Table 1).

Stability of Immunotoxin. The stability of immunotoxins at 37°C is an important factor in their usefulness as therapeutic agents. The stability of an immunotoxin is governed by its tendency to aggregate at 37°C. The thermal stability of B1(dsFv)-PE33, B1(dsFv)-PE38, and B1(Fv)-PE38 was determined by measuring the amount of aggregation and inactivation at 37°C as described in *Materials and Methods.* We found that both B1(dsFv)-PE33 and B1(dsFv)-PE38 were monomers before incubation in PBS at 37°C and remained monomeric for 8 h (Table 2). In contrast, the single-chain immunotoxin B1(Fv)-PE38 formed >60% aggregates after an 8-h incubation at 37°C (Table 2; also see ref. 20). Following the 8-h incubation at 37°C, B1(dsFv)-PE33 and B1(dsFv)-PE38 retained almost all their initial cytotoxic activity, while B1(Fv)-PE38 lost 75% of its initial cytotoxic activity (20). Thus, both B1(dsFv)-PE38 and B1(dsFv)-PE33 are extremely stable at 37°C, presumably because they do not tend to aggregate as do the scFv-immunotoxins.

Toxicity of Immunotoxins in Mice. The toxicity of single doses of the immunotoxins B1(dsFv)-PE33 and B1(dsFv)-PE38 was measured by i.v. injections of different amounts of immunotoxin into BALB/c mice. The mice were observed for 14 days after injection. The LD_{50} of both immunotoxins was found to be 0.5 mg/kg, similar to the LD_{50} determined for the B1(dsFv)-PE38 as well as other anti-Lewis^y Fv-immunotoxins (23). The results show that even though the immunotoxin is more active on target cells because it does not require proteolytic activation, it is not more toxic to mice. This toxicity in mice is presumed to be due to nonspecific uptake of the toxin moiety by the liver (29).

Improved Antitumor Activity of B1(dsFv)-PE33. To determine whether the improved cytotoxicity *in vitro* is accompanied by an increase in antitumor activity, B1(dsFv)-PE33 and B1(dsFv)-PE38 were compared by assessing their ability to cause regression of established human carcinoma xenografts in nude mice. Nude mice were injected with 3×10^6 A431 cells s.c. on day 0. Beginning 5 days later, when tumors averaged

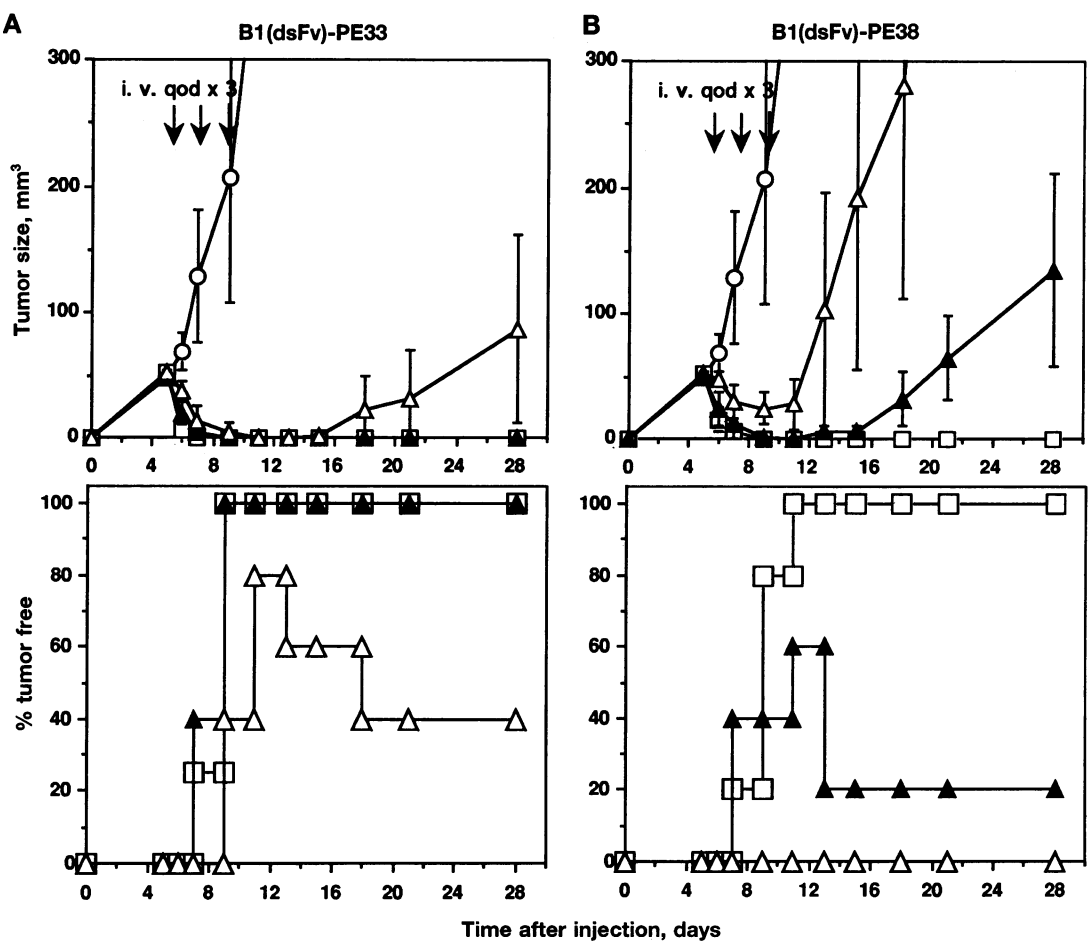

FIG. 5. Antitumor effect and durability of complete remissions due to B1(dsFv)-PE33 and B1(dsFv)-PE38 in a nude mouse model. Groups of five mice were injected s.c. with 3×10^6 A431 cells on day 0 and were treated by i.v. injections of B1(dsFv)-PE33 (*Left*) or B1(dsFv)-PE38 (*Right*) on days 5, 7, and 9 (indicated by arrows), when the tumors were established. Control mice were treated with PBS-HSA. Error bars represent the standard error of the data. ○, Control; □, 400 pmol/kg; ▲, 200 pmol/kg; and △, 100 pmol/kg.

50–70 mm^3 in volume, the mice were treated with i.v. injections on days 5, 7, and 9 of various doses of immunotoxin. Control mice were treated with PBS-HSA only. As shown in Fig. 5, both immunotoxins demonstrated significant dose-dependent antitumor activity. B1(dsFv)-PE38 caused only partial regression of A431 tumors at the 6.5 μg/kg (100 pmol/kg) dose level, whereas B1(dsFv)-PE33 at the same dose caused complete disappearance of the tumors (Fig. 5). Furthermore, the tumors treated with B1(dsFv)-PE38 at 200 pmol/kg (13 μg/kg) regressed completely after the third injection but regrew within a few days, whereas B1(dsFv)-PE33 at 200 pmol/kg (12 μg/kg) caused complete regressions that lasted over 1 month in 5 of 5 animals. These results indicate that B1(dsFv)-PE33 has significantly better antitumor activity than B1(dsFv)-PE38. Thus, the improved cytotoxicity *in vitro* results in improved antitumor activity in animals.

DISCUSSION

We have developed a recombinant immunotoxin composed of a dsFv fragment of mAb B1 and a truncated form of PE that is smaller than other recombinant PE-derived immunotoxins and does not require intracellular proteolytic activation. Another advantage is that it is more active *in vitro* and a more potent antitumor agent than immunotoxins made with the same antibody that require proteolytic processing (20).

Location for B1(dsFv) Insertion in PE33. The B1 dsFv fragment was inserted between the translocation domain and ADP-ribosylation domain of PE, replacing domain Ib. The rationale for this design is that domain Ib (amino acids 364–395) is not essential for the cytotoxic activity (30), and it can be completely deleted from immunotoxins without loss of activity. In fact, it is also possible to delete a portion of domain II (amino acids 343–364) without loss of activity. In addition, analysis of the proposed structure of B1(dsFv)-PE33 by using computer graphics (N. Kurochkina, C.-T.K. and I.P., unpublished results) indicates that domain Ib should be a good location for insertion of the dsFv fragment, because the complementarity-determining regions of the Fv should be "free" to interact with antigen. The results in Fig. 4 show that the presence of B1(dsFv) in this region only minimally affected antigen binding. In another study, we have inserted a dsFv fragment of mAb e23, which binds to the erbB2 antigen, near the carboxyl terminus of PE35 at amino acid 607; this location was found to significantly decrease antigen binding of e23(dsFv) to its antigen (31). It is necessary to investigate whether the domain Ib location is useful for the insertion of other dsFvs.

Improved Antitumor Activity of B1(dsFv)-PE33. To compare the antitumor activity of B1(dsFv)-PE33 with B1(dsFv)-PE38, we used the A431 human epidermoid carcinoma model to evaluate the ability of both immunotoxins to cause complete regression of tumors. B1(dsFv)-PE38 is very potent in antitumor activity (20). We found that when the nude mice were treated with three doses of 200 pmol/kg, given every other day, B1(dsFv)-PE38 caused significant tumor regressions but did not produce cures. In contrast, B1(dsFv)-PE33 caused complete remissions and cures in all animals at the same dose of 200 pmol/kg (Fig. 5). Thus, B1(dsFv)-PE33 has better antitumor activity than B1(dsFv)-PE38. Since both B1(dsFv)-PE33 and B1(dsFv)-PE38 have the same toxicity in mice, the PE33 immunotoxin has a larger therapeutic window. The effective dose causing complete remissions in nude mice is 2.5% of the LD_{50}. This makes B1(dsFv)-PE33 a good candidate for clinical development. The improved antitumor activity of B1(dsFv)-PE33 over B1(dsFv)-PE38 is a consequence of better cytotoxicity *in vitro* due to lack of requirement for proteolytic activation and possibly smaller size for better tumor penetration. Since the efficiency of proteolytic activation can vary in different types of cells, the type of recombinant immunotoxin described here may be more useful than the previous generation of molecules, which require proteolytic activation.

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