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Immunology

A recombinant immunotoxin containing a disulfide-stabilized Fv fragment

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ABSTRACT **B3(dsFv)-PE38KDEL is a recombinant immunotoxin composed of the Fv region of monoclonal antibody B3 connected to a truncated form of *Pseudomonas* exotoxin (PE38KDEL), in which the unstable Fv heterodimer (composed of heavy- and light-chain variable regions) is held together and stabilized by a disulfide bond [termed disulfide-stabilized Fv (dsFv)]. A computer modeled structure of the B3(Fv), made by mutating and energy minimizing the amino acid sequence and structure of McPC603, enabled us to identify positions in conserved framework regions that "hypothetically" could be used for disulfide stabilization without changing the structure or affecting antigen binding. This prediction was evaluated experimentally by constructing a disulfide-linked two-chain dsFv-immunotoxin that was produced in *Escherichia coli*. The activity and specificity of this immunotoxin was indistinguishable from its single-chain Fv (scFv) counterpart, indicating that, as in B3(scFv), the structure of the binding region is retained in B3(dsFv). Because we introduced the stabilizing disulfide bond in between two framework residues in a position that is conserved in most Fv molecules, this method of linkage between the heavy- and light-chain variable regions should be generally applicable to construct immunotoxins and dsFv molecules using other antibodies. Furthermore, the finding that B3(dsFv) was much more stable at 37°C in human plasma than B3(scFv) indicates that dsFvs are possibly more versatile for therapeutic application than scFvs.**

Fv fragments of immunoglobulins are the smallest functional modules of antibodies required for high-affinity binding of antigen. Their small size makes them potentially more useful than whole antibodies for clinical applications like imaging tumors and directing recombinant immunotoxins to tumors (1–8), because size strongly influences tumor and tissue penetration. Fv fragments are heterodimers of the heavy-chain variable domain (V_H) and the light-chain variable domain (V_L). Whereas the heterodimers of whole IgG (H chain/L chain) or Fab fragments (Fd fragment/L chain) are connected by a disulfide bond, the Fv fragments are not and, therefore, unmodified Fv fragments are unstable (9). This instability has been overcome by making recombinant Fv molecules that have V_H and V_L connected by a peptide linker (2, 3). Such single-chain Fv molecules (scFvs) can fully retain specificity and affinity and have been shown to be useful for imaging tumors (4) and to make recombinant immunotoxins for tumor therapy (5–8). However, some scFvs have a reduced affinity for antigen and the peptide linker can interfere with binding (10, 11). Another way to stabilize Fvs would be to connect V_H and V_L by a disulfide bond. One previous study (9) indicated that this is possible. But in that approach "custom disulfides" were placed in complementarity-determining regions (CDRs) of an antibody whose structure was known (12) in a manner that did not or had only small effect on ligand binding. This strategy cannot be used to stabilize other Fvs with unknown structures because disulfide-bridging CDRs is very likely to interfere with antigen binding. In contrast to that, we attempted to identify residues in conserved framework regions of V_H and V_L that when mutated to cysteines can stabilize the Fvs by a disulfide bond without interfering with antigen binding. This should then be generally applicable to stabilize Fvs. To analyze such a disulfide-stabilized Fv (dsFv), we chose to make a dsFv-immunotoxin derived from monoclonal antibody (mAb) B3, which reacts with specific carbohydrates present on many human cancers (13). We have made B3(scFv)-immunotoxins that are specifically cytotoxic to cells containing the B3 antigen and cause complete regression of human cancer xenografts in nude mice (14). The analysis of the specific Fv-mediated toxicity of scFv- and dsFv-immunotoxins enables us to directly compare the binding and specificity of those Fv derivatives.

Here we describe the computer modeling and identification of residues in the conserved framework regions of V_H and V_L of the mAb B3 that can be mutated to cysteines and form a dsFv without interfering with antigen binding. The experimental proof of this prediction is an active immunotoxin containing such a dsFv.

MATERIALS AND METHODS

Computer Analyses. The initial model of the B3(Fv) structure was obtained from the structure of the variable domain of McPC603 (refs. 12 and 15; Protein Data Bank Entry IMCP) by deletion and mutation of appropriate residues using the molecular graphics program GEMM (unpublished in-house software). The structure of this model and those of various mutants were refined by a series of the adopted basis set Newton Raphson (ABNR) energy minimization procedure using the molecular dynamics simulation program CHARMM (16) Version 22. Details of this procedure will be presented elsewhere.

Plasmid Constructions. Uracil-containing single-stranded DNA from the F^+ origin present in our expression plasmids was obtained by cotransfection with M13 helper phage and was used as template for site-directed mutagenesis as described (17). The complete nucleotide sequence of B3(Fv) has been described (8). The mutagenic oligonucleotides were 5′-TATGCGACCCACTCGAGACACTTCTCTGGAGTCT-3′ to change Arg-44 of B3(V_H) to cysteine, 5′-TTTCCAGCTTTGTCCCACAGCCGAACGTGAATGG-3′ to replace Ser-105 of B3(V_L) with cysteine, and 5′-CCGCCACCACCGGATCCGCGAATTCATTAGGAGACAGTGACCAGAGTC-3′ to introduce stop codons followed by an *Eco*RI site at the 3′ end of the B3(V_H) gene. Restriction sites (*Xho* I and *Eco*RI) introduced into these oligonucleotides to

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Abbreviations: mAb, monoclonal antibody; H, heavy; L, light; V, variable; scFv and dsFv, single-chain and disulfide-stabilized Fv, respectively; CDR, complementarity-determining region; IB, inclusion body.

facilitate identification of mutated clones or subcloning are underlined. The oligonucleotides 5′-TCGGTTGGAAACTTTGCAGATCAGGAGCTTTGGAGAC-3′, 5′-TCGGTTGGAAACGCAGTAGATCAGAAGCTTTGGAGAC-3′, 5′-AGTAAGCAAACCAGGCGCACCAGGCCAGTCCTCTTGCGCAGTAATATATGGC-3′, and 5′-AGTAAGCAAAACAGGCTCCCCAGGCCAGTCCTCTTGCGCAGTAATATATGGC-3′ were used to introduce cysteines at positions L54, L55, H103, and H105 of B3(Fv), which correspond to the positions L55, L56, H106, and H108 of the described McPC603 dsFv (ref. 9, see Table 2). All mutated clones were confirmed by DNA sequencing.

Expression of Recombinant Protein. B3(Fv)-PE38KDEL, [Cys^{H44},Cys^{L105}]B3(Fv)-PE38KDEL, [Cys^{105}]B3(V_L)-PE38KDEL, and [Cys^{44}]B3(V_H) were produced in separate *Escherichia coli* BL21 λDE3 cultures containing pULI9, pULI37, pULI39, or pYR38-2, respectively, as described (8).

Refolding and Purification of B3(dsFv)-PE38KDEL. Inclusion bodies (IBs) were purified, solubilized, reduced, and diluted into redox-shuffling and aggregation-preventing additive-containing refolding buffer as described for single-chain immunotoxins (18). The following changes were made when preparing dsFv-immunotoxins: V_H and V_L-toxin IBs were solubilized separately and combined in 2:1 molar ratio in the refolding solution. In addition, refolding of these molecules required a "final oxidation" step, in which a 10-fold excess of oxidized glutathione was added after 24 h of refolding in "standard" redox-shuffle buffer (18). Properly folded B3(dsFv)-immunotoxins were separated from contaminating proteins and aggregates by ion-exchange (Mono Q) and size-exclusion chromatography (8).

Cytotoxicity Assays. Inhibition of protein synthesis by *Pseudomonas* exotoxin-derived immunotoxins was determined as described (8). For competition experiments, excess mAb B3 or HB21 (to a final concentration of 1 mg/ml) was added 15 min before addition of toxin. A high concentration of mAb B3 is necessary for competition because of the large amount of B3 antigen present on carcinoma cells (8, 13, 19).

Stability Assays. The stability of Fv-immunotoxins was determined by incubating them at 10 μg/ml at 37°C in human serum. Active immunotoxin remaining after incubation was determined by cytotoxicity assays on A431 cells.

RESULTS

Design of a Disulfide Connection Between V_H and V_L of mAb B3 That Does Not Affect the Structure of the Binding Site. Because the structure of mAb B3 is not known, we generated a model of B3(Fv) from the structure of mAb McPC603 by replacing or deleting appropriate amino acids. mAb McPC603 was selected because it has the highest overall (L+H) sequence identity and similarity among all published mouse antibody structures. A total of 44 (including two deletions) and 40 (including one deletion) amino acids of the V_H and V_L domains, respectively, of McPC603 had to be changed. This structure was then energy-minimized using CHARMM in stages. Parts of the molecule were varied as follows: first only the hydrogen atoms, then the deleted regions, then all the mutated residues, and finally, the whole molecule.

Three criteria were used to select possible positions for disulfide connections between V_H and V_L (Fig. 1). (*i*) The disulfide should connect amino acids in structurally conserved framework regions of V_H and V_L so that the disulfide stabilization works not only for B3(Fv) but also for other Fvs. (*ii*) The distance between V_H and V_L should be small enough to enable the formation of a disulfide without generating strain on the Fv structure. (*iii*) The disulfide should be at a sufficient distance from the CDRs to avoid interference with antigen binding. These criteria were met by two potential disulfide bridges. One possibility was to replace Arg-44 of B3(V_H) and Ser-105 of B3(V_L) by cysteines to generate a

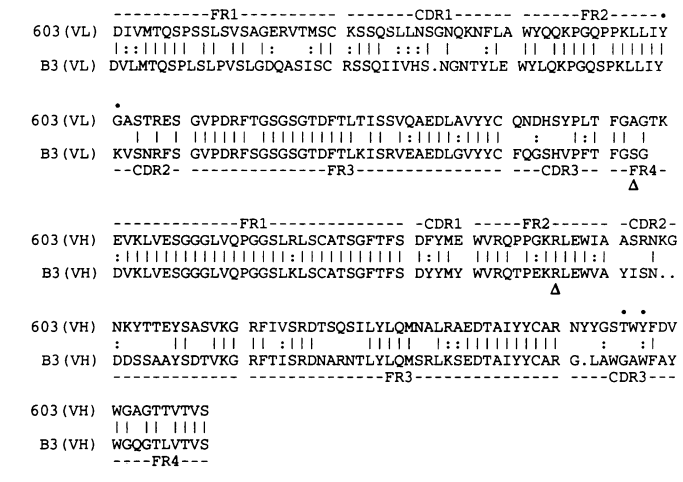

FIG. 1. Design of a disulfide connection between V_H and V_L of mAb B3 that does not affect the overall structure or binding site. Comparison of the variable regions of mAb B3 and mAb McPC603. ●, Positions of cysteine replacements in previously designed disulfide-stabilized McPC603(Fv) where CDRs are affected (9); △, positions of cysteine replacements in framework regions of B3(Fv) (Arg-44 → Cys in V_H and Ser-105 → Cys in V_L) to stabilize the Fv without affecting CDRs. The assignment of framework regions 1–4 (FR1–4) and CDR1–3 is according to Kabat *et al.* (20).

disulfide bond between those positions. The other was to change Gln-111 of B3(V_H) and Ser-48 of B3(V_L) to cysteines. These two pairs are related to one another by the pseudo-two-fold symmetry that approximately relates the V_H and V_L structures. In each case, one of the residues involved in the putative disulfide bond (V_H position 111 and V_L position 105) is flanked on both sides by a highly conserved glycine residue that can help absorb local distortions to the structure caused by the introduction of the disulfide bond. We energy-minimized models for both possibilities as well as one in which both disulfide bonds are present. The V_H44–V_L105 connection between V_H position 44 and V_L position 105 was chosen for further study because the energy-refined model structure with this connection had a slightly better disulfide bond geometry than that with the connection between V_H position 111 and V_L position 48. With some other antibodies this latter choice may be preferable over the former. Details of how the modeling was accomplished will be published elsewhere (S.-H.J., I.P., and B.L., unpublished results).

Construction of Plasmids for Expression of B3(dsFv)-Immunotoxins. The parent plasmid for the generation of plasmids for expression of ds(Fv)-immunotoxins, in which Arg-44 in V_H (Arg-H44) and Ser-105 in V_L (Ser-L105) were replaced by cysteines, encodes the single-chain immunotoxin [Tyr^{H95}]B3(Fv)-PE38KDEL (Fig. 2). In this molecule the V_H and V_L domain of mAb B3 are held together by a $(Gly_4\text{-}Ser)_3$ peptide linker [B3(scFv)] and then fused to the PE38KDEL gene encoding the translocation and ADP-ribosylation of *Pseudomonas* exotoxin (PE) (8, 21). [Tyr^{H95}]B3(Fv)-PE38KDEL is identical to B3(Fv)-PE38KDEL (8) except for a change of Ser-95 of B3(V_H) [V_H position 91 according to Kabat *et al.* (20)] to tyrosine. This tyrosine residue is conserved in the framework of most murine V_H domains and fills a cavity in the V_H–V_L interface, probably contributing to V_H–V_L domain interactions. We have compared the properties of B3(Fv)-PE38KDEL and [Tyr^{H95}]B3(Fv)-PE38KDEL, including ability to be renatured, behavior during purification, and cytotoxic activity toward carcinoma cell lines, and found them to be indistinguishable (unpublished data). The plasmids for expression of the components of ds(Fv)-immunotoxins, [Cys^{44}]B3(V_H) and [Cys^{105}]B3(V_L)-PE38KDEL, were made by site-directed mutagenesis using uridine-containing single-

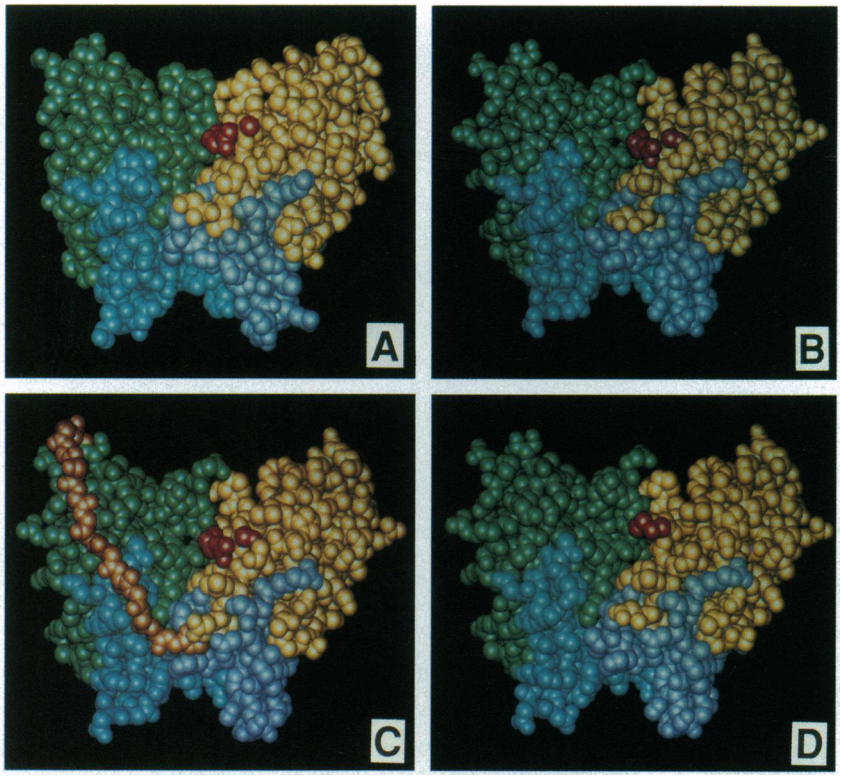

FIG. 2. Structure models of the V regions of McPC603, B3(Fv), B3(scFv), and B3(dsFv). (*A*) Crystal structure model of the V regions of McPC603. (*B*) Putative structure of B3(Fv), obtained by mutating the McPC603 sequence to that of B3 and energy minimization. (*C*) B3(scFv) containing a peptide linker between the C terminus of B3(V_H) and the N terminus of B3(V_L). (*D*) B3(dsFv) in which Arg-H44 and Ser-L105 were changed to cysteine to form a disulfide bond between V_H and V_L. Framework regions are green (V_H) and yellow (V_L); the CDRs are dark blue (V_H) and light blue (V_L). The $(Gly_4\text{-}Ser)_3$ peptide linker in B3(scFv) (*C*) is brown. Arg-H44 and Ala-L106 of McPC603 (*A*), the corresponding Arg-H44 and Ser-L105 of B3 (*B* and *C*), and the disulfide bond between Cys-H44 and Cys-L105 in B3(dsFv) are red.

stranded DNA derived from the F^+ origin in pULI28 as template to mutate Arg-44 in B3(V_H) and Ser-105 in B3(V_L) to cysteines. The final plasmids pYR38-2 for expression of [Cys^{44}]B3(V_H) and pULI39 for [Cys^{105}]B3(V_L)-PE38KDEL were made by subcloning from the mutagenized plasmids. Details of the cloning strategy are shown in Fig. 3.

Expression in IBs, Refolding, and Purification. To produce recombinant B3(dsFv)-immunotoxins, separate *Escherichia coli* BL21 (λDE3) cultures containing either the [Cys^{44}]B3(V_H) encoding plasmid pYR38-2 or the [Cys^{105}]B3(V_L)-PE38KDEL encoding plasmid pULI39 were induced with isopropyl β-D-thiogalactoside upon which the recombinant proteins accumulated to 20–30% of the total protein in intracellular IBs (Fig. 4). Active immunotoxins were prepared as described for the preparation of single-chain immunotoxins (18) with two modifications. (*i*) Instead of adding only one solubilized and reduced protein [e.g., B3(Fv)PE38KDEL] to the refolding solution, we prepared IBs containing [Cys^{44}]V_H and [Cys^{105}]V_L-toxin separately and mixed them in a 2:1 molar ratio to a final total protein concentration of 100 μg/ml in the refolding buffer. We found that a 2- to 5-fold excess of V_H over the V_L-toxin gave the best yield of renatured immunotoxin.

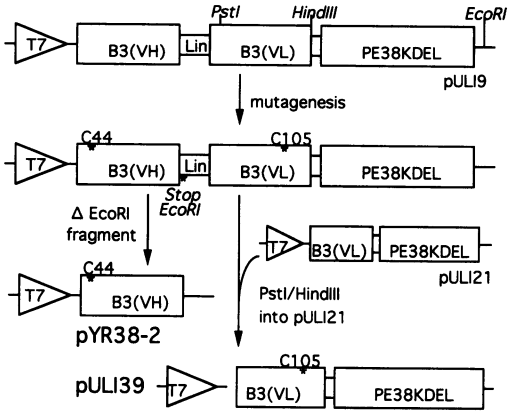

FIG. 3. Plasmids for expression of B3(dsFv)-immunotoxins. Single-stranded uracil-containing DNA of pULI28 was the template to mutate Arg-44 of B3(V_H) and Ser-105 of B3(V_L) to cysteine by Kunkel mutagenesis (17). The expression plasmid pYR38-2 for [Cys^{44}]B3(V_H) was generated by deletion of a V_L-PE38KDEL encoding an *Eco*RI fragment. pULI39 encoding [Cys^{105}]B3(V_L)-PE38KDEL was constructed by subcloning a [Cys^{105}]V_L-containing *Pst* I–*Hin*dIII fragment into pULI21 (14), which encodes B3(V_L)-PE38KDEL.

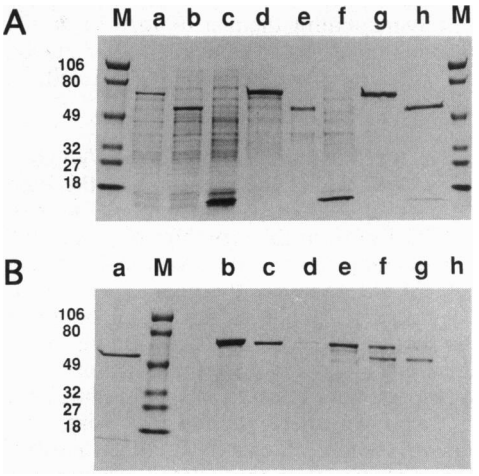

FIG. 4. Expression and purification of B3(dsFv)-PE38KDEL. (*A*) Reducing SDS/PAGE. Lanes: M, molecular mass standard; a, *E. coli* expressing B3(Fv)-PE38KDEL, total cells; b, [Cys^{105}]B3(V_L), total cells; c, [Cys^{44}]B3(V_H), total cells; d, IBs containing B3(Fv)-PE38KDEL; e, [Cys^{105}]B3(V_L)-PE38KDEL IBs; f, [Cys^{44}]B3(V_H) IBs; g, purified B3(Fv)-PE38KDEL; h, purified B3(dsFv)-PE38-KDEL. (*B*) SDS/PAGE. Lanes: M, molecular mass standard; a, B3(dsFv)-PE38KDEL, reduced; b, B3(Fv)-PE38KDEL, nonreduced; c, B3(dsFv)-PE38KDEL after ion-exchange and size-exclusion chromatography, nonreduced; d–h, profile of protein peak nonreduced and eluted from a Mono Q column after refolding of IBs. Properly folded (dsFv)-PE38KDEL (upper band) comes slightly earlier than monomeric [Cys^{105}]B3(V_L)-PE38KDEL (lower band) and can be separated by repeated chromatography.

Equal molar addition of V_H and V_L-toxin into the renaturation solution or a >5-fold excess of V_H resulted in a reduction of the yield of active monomeric immunotoxin; with too much V_H we observed increased aggregation. (*ii*) A final oxidation step was done in which excess oxidized glutathione was added to the refolding solution after the redox-shuffling was completed. This oxidation increased the yield of properly folded functional protein by at least 5-fold, probably because the disulfide bond connecting V_H and V_L is exposed on the surface of the Fv, is accessible to the slight reducing conditions in the refolding buffer, and would remain reduced without final oxidation.

To recover active immunotoxins after refolding, we adapted the purification scheme established for scFv-immunotoxins (8, 14, 18). Properly folded dsFv-immunotoxins have to be separated not only from aggregates, which separate easily, but also from "single-domain" V_L-toxins, which have a chromatographic behavior close to dsFv-immunotoxins (14). Fig. 4*B* shows that after refolding of B3(dsFv)-PE38KDEL, the Mono Q "monomer peak" contains two proteins; the dsFv-immunotoxin elutes slightly earlier than the V_L-toxin. We purified B3(dsFv)-PE38KDEL to near homogeneity by consecutive cycles of chromatography, pooling early fractions, rechromatographing peak fractions, and discarding late fractions (see Fig. 4*B*). Despite significant losses of active dsFv-immunotoxin (discarded late fractions still contain dsFv protein), this procedure is efficient enough to obtain >8 mg of pure dsFv-immunotoxin from bacterial V_H and V_L-toxin cultures (each at 1 liter).

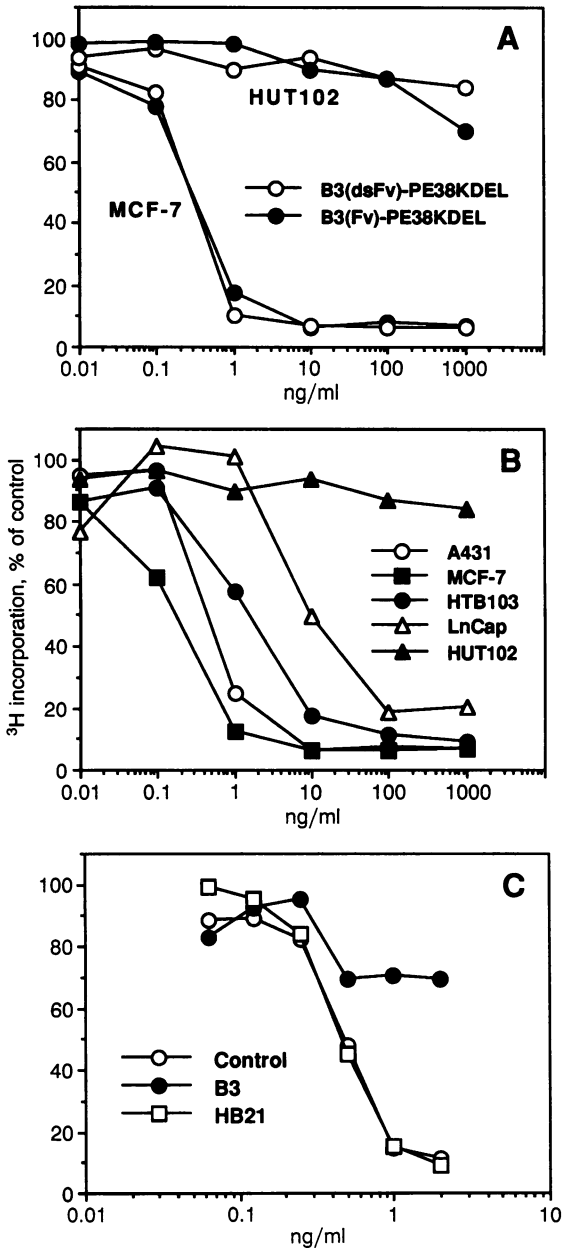

FIG. 5. Specific cytotoxicity of B3(dsFv)-PE38KDEL and B3(Fv)-PE38KDEL toward different carcinoma cell lines. (*A*) Comparison of cytotoxicity of B3(Fv)-PE38KDEL and B3(dsFv)-PE38KDEL toward B3-antigen-expressing A431 cells and B3-negative HUT102 cells. (*B*) Cytotoxicity of B3(dsFv)-PE38KDEL toward various cell lines. (*C*) Competition of cytotoxicity toward A431 cells by addition of excess mAb B3.

Specific Toxicity of B3(dsFv)-PE38KDEL Toward B3-Antigen-Expressing Carcinoma Cell Lines. A comparison of Fv-mediated specific cytotoxicity of a single-chain immunotoxin B3(Fv)-PE38KDEL and the corresponding disulfide-stabilized B3(dsFv)-PE38KDEL shows that both proteins recognize the same spectrum of cells and are equally active (Fig. 5 and Tables 1 and 2). B3(dsFv)-PE38KDEL like B3-(Fv)-PE38KDEL only is cytotoxic to B3-antigen-expressing cells and has no effect on cells that do not bind mAb B3 (e.g., HUT102). This cytotoxicity can be competed by mAb B3 but not HB21, an antibody to the human transferrin receptor (Fig. 5*C*). The finding that the specificity and activity of scFv- and dsFv-immunotoxins are indistinguishable indicates that the binding region is conserved equally well in the B3(dsFv) and in the linker-stabilized molecule.

Stability of B3(Fv)- and B3(dsFv)-PE38KDEL in Human Serum. Because dsFv- and scFv-immunotoxins have equal activity toward cultured carcinoma cells, B3(dsFv)-PE38KDEL should also be useful for cancer treatment like its scFv counterpart, B3(Fv)-PE38KDEL (8). One factor that contributes to the therapeutic usefulness of immunotoxins is their stability. Previous comparisons (9) indicated dsFvs might be more stable than scFvs, although those data cannot be generalized for other than the analyzed McPC603 (see *Introduction* and *Discussion*). Table 3 shows a comparison of the stability of scFv- and dsFv-immunotoxins in human serum. The scFv-toxin B3(Fv)-PE38KDEL is stable for 1–2 h and then begins to loose activity. In marked contrast, the dsFv-toxin B3(dsFv)-PE38KDEL retains full cytotoxic activity for >24 h.

DISCUSSION

We have made a recombinant immunotoxin, B3(dsFv)-PE38KDEL, that is composed of the Fv region of mAb B3 fused to a truncated form of *Pseudomonas* exotoxin. In contrast to previously described single-chain immunotoxins such as B3(Fv)-PE38KDEL (8), OVB3(Fv)-PE40 (22), anti-Tac(Fv)-PE40 (6), and e23(Fv)-PE38KDEL (7) in which V_H

Table 1. Cytotoxicity of recombinant B3-immunotoxins toward various cell lines

Cell line	Cancer type	B3 antigen	Cytotoxicity IC_{50}, ng/ml B3(Fv)	Cytotoxicity IC_{50}, ng/ml B3(dsFv)
MCF7	Breast	+++	0.25	0.25
A431	Epidermoid	+++	0.3	0.35
LNCaP	Prostate	+	9	8.5
HTB103	Gastric	+	3.5	3.5
HUT102	Leukemia	−	>1000	>1000

Cytotoxicity data are given as IC_{50} values, the concentration of immunotoxin that causes a 50% inhibition of protein synthesis after a 16-h incubation with immunotoxin. Immunotoxins tested were B3(Fv)-PE38KDEL [B3(Fv)] and B3(dsFv)-PE38KDEL [B3(dsFv)]. −, Negative; +, weak; +++, strong.

Table 2. Placement of the disulfide bond connecting V_H and V_L at various positions of B3(Fv)

Cell line	Cytotoxicity IC_{50}, ng/ml				
	B3(Fv)	B3(dsFv)	B3(scdsFv) H44-L105	B3(scdsFv) H105-L55	B3(scdsFv) H103-L56
A431	0.3	0.3	0.4	80	250
MCF7	0.25	0.25	0.3	90	200

Activity of various immunotoxins toward carcinoma cell lines was determined as described in Table 1. B3(scdsFv)-PE38KDEL [B3(scdsFv)] molecules are single-chain immunotoxins that in addition to the $(Gly_4\text{-}Ser)_3$ linker have cysteines introduced in V_H and V_L to form an interchain disulfide. H44-L105 corresponds to B3(dsFv)-PE38KDEL, except that in B3(dsFv) the linker peptide is deleted. H105-L54 and H103-L55 are the positions where cysteine residues were introduced in the custom made H108-L55 and H106-L56 disulfide-bonded McPC603 (Fv) (9).

and V_L domains are connected by a peptide linker, B3(dsFv)-PE38KDEL contains a Fv region held together and stabilized by a disulfide bond. A computer-modeled structure of B3(Fv) was generated by "mutation" and energy minimization of the x-ray structure of McPC603 and used to find positions in conserved V_H and V_L framework regions that were likely to stabilize the Fv when connected to each other by a disulfide bond, without interfering with antigen binding. We evaluated this prediction experimentally by constructing a disulfide-linked two-chain dsFv-immunotoxin, B3(dsFv)-PE38KDEL. This dsFv-immunotoxin was purified after refolding from bacterial IBs containing [Cys^{44}]B3(V_H) and [Cys^{105}]B3(V_L)-PE38KDEL. The activity and specificity of this dsFv-immunotoxin were the same as its scFv counterpart, indicating the structure of the binding region is retained in the dsFv as well as in the scFv of B3.

Stabilization of the Fv by a disulfide bond between V_H and V_L has been described before for McPC603 (9). That approach placed the disulfide bond connecting the V_H and V_L into CDRs, but only a minor influence on antigen binding was observed. Because the structure of the antigen–antibody complex of that particular antibody was known (12), the disulfide bond could be placed at a position in the CDR that would not interfere with antigen binding. It seemed very unlikely that this stabilization strategy could be generalized for other Fvs, especially for those whose structures are not known. To examine this point directly, we introduced the "McPC603 disulfide bonds" into the corresponding positions of B3(Fv)-immunotoxins. When these molecules were tested, they had very little cytotoxic activity because of this alteration in the antigen binding site (Table 2). Altering CDRs of other Fvs by disulfide bonds would almost certainly also decrease their antigen binding ability.

Table 3. Stability of B3(Fv)-PE38KDEL and B3(dsFv)-PE38KDEL in human serum

Sample	% activity left							
	0	0.5	1	2	4	8	12	24
scFV in serum 1	100	100	87	50	31	14	14	1
scFv in serum 2	100	88	58	35	20	6	4	1
dsFv in serum 1	100	100	100	100	100	100	100	100
dsFv in serum 2	100	100	100	100	100	100	100	100

Each type of immunotoxin at 10 μg was incubated with human serum at 37°C for the times shown (in hours) and then assayed for cytotoxic activity on A431 cells. Serum 1 and serum 2 are from different donors.

In contrast, it is likely that stabilization of Fvs by a disulfide bond using the positions that we describe here for B3(dsFv) (consequently between the corresponding positions in other Fvs) can be used for Fvs with unknown structures, because the positions for introduction of cysteine residues were chosen in conserved framework regions and at a sufficient distance from CDRs. Inspection of the crystal structures of human antibodies indicates that recombinant human Fvs, which due to advanced PCR techniques have recently become more available (23), can probably also be stabilized at the corresponding framework positions. As a result, we believe that this strategy of disulfide stabilization of Fvs will be at least as versatile as and possibly more versatile than the "linker" strategy (2, 3). It should be possible to test this hypothesis by making other dsFv-immunotoxins as well as dsFv molecules by themselves.

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