Stabilization of the Fv Fragments in Recombinant Immunotoxins by Disulfide Bonds Engineered into Conserved Framework Regions†

Yoram Reiter, Ulrich Brinkmann, Robert J. Kreitman, Sun-Hee Jung, Byungkook Lee, and Ira Pastan*

Laboratory of Molecular Biology, Division of Cancer Biology, Diagnosis and Centers, National Cancer Institute, National Institutes of Health, Building 37, Room 4E16, Bethesda, Maryland 20892

Received December 13, 1993; Revised Manuscript Received February 28, 1994®

ABSTRACT: Disulfide-stabilized Fv's (dsFv's) are recombinant Fv fragments of antibodies in which the unstable variable heavy (V_H) and variable light (V_L) heterodimers are stabilized by disulfide bonds engineered at specific sites that lie between structurally conserved framework positions of V_H and V_L. We have recently described one example of a recombinant immunotoxin, B3(dsFv)–PE38KDEL, that is composed of such a dsFv connected to a truncated form of *Pseudomonas* exotoxin [Brinkmann, U., Reiter, Y., Jung, S.-H., Lee, B., & Pastan, I. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90,* 7538–7542]. This disulfide-stabilized immunotoxin has the same cytotoxic activity and specificity as its single-chain immunotoxin counterpart. To determine whether the stabilization of Fv's by disulfides at these positions is generally applicable, we made and analyzed two other dsFv-containing immunotoxins. One is made from the e23 antibody, which binds to the carcinoma-associated antigen erbB2; the other is made from the anti-Tac antibody, which binds to the p55 subunit of the IL-2 receptor. Comparison of the specificity and activity of these immunotoxins with those of their scFv counterparts revealed that e23(dsFv)–PE38KDEL was considerably more active than e23(Fv)–PE38KDEL, whereas anti-Tac(dsFv)–PE38KDEL was only somewhat more active than its single-chain counterpart. These results suggest that dsFv's have at least the same binding properties as scFv's, and in some cases they may have better binding. Thus, it should be feasible to use the positions we have identified in the conserved framework region to disulfide-stabilize many different Fv's. Furthermore, we have optimized the design of the immunotoxin and the purification scheme, so that the yields of dsFv–immunotoxins are consistently higher than those of scFv–toxins and one can obtain up to 70 mg of pure active immunotoxin from 1 L of bacterial culture. This increased yield is mainly due to a decreased tendency of properly folded dsFv–immunotoxins to aggregate. Because dsFv–immunotoxins have equal or improved activity, they are easier to produce with high yields and are more stable than scFv–immunotoxins; dsFv–immunotoxins (and dsFv's alone) might be more useful than scFv's in clinical and other applications that require large amounts of stable recombinant Fv's.

Fv fragments of antibodies are heterodimers composed of a heavy-chain variable domain (V_H)[1] and a light-chain variable domain (V_L). They are the smallest functional modules of antibodies required for high-affinity binding of antigen (Yokota et al., 1992; Huston et al., 1988; Bird et al., 1988; Milenic et al., 1991; Pastan & FitzGerald, 1991; Chaudhary et al., 1989; Batra et al., 1992; Brinkmann et al., 1991). The heterodimers of whole IgG or Fab fragments are connected by a disulfide bond. However, Fv fragments are not connected by a disulfide bond and, thus, by themselves are unstable (Glockshuber et al., 1990). This instability of Fv's can be overcome by making recombinant Fv molecules in which the V_H and V_L domains are connected by a peptide linker, such that the antigen combining site is regenerated in a single protein (Huston et al., 1988; Bird et al., 1988). Such single-chain Fv's (scFv's) can retain specificity and affinity and have been used for tumor imaging (Milenic et al., 1991) and for making recombinant immunotoxins for tumor therapy (Pastan & FitzGerald, 1991; Chaudhary et al., 1989; Batra et al., 1992; Brinkmann et al., 1991). Because of their small size, which facilitates tumor penetration, scFv's are potentially more useful than whole antibodies for the diagnosis and/or therapy of diseases such as cancer, where target antigens are expressed on the surface of malignant cells.

We have made several scFv–immunotoxins that are specifically cytotoxic to tumor cells and cause complete regression of human cancer xenografts in nude mice (Pastan & FitzGerald, 1991; Chaudhary et al., 1989; Batra et al., 1992; Brinkmann et al., 1991). These immunotoxins contain the scFv connected to a truncated form of *Pseudomonas* exotoxin (PE38KDEL), which contains the translocation and ADP-ribosylation domains and an altered carboxyl-terminal sequence, KDEL, that increases cytotoxic activity, probably by increasing delivery of the active toxin fragment to the endoplasmic reticulum (Seetharam et al., 1991). Examples of active single-chain immunotoxins are B3(Fv)–PE38KDEL, directed against a carbohydrate antigen present on many human tumors (Brinkmann et al., 1991; Seetharam et al., 1991), e23(Fv)–PE38KDEL, directed against the erbB2 carcinoma-associated antigen (Batra et al., 1992), and anti-Tac(Fv)–PE38 and its KDEL derivative, directed against the Tac antigen, the p55 subunit of the IL-2 receptor, which is highly expressed in leukemias and lymphomas (Chaudhary et al., 1989).

Some scFv's, for example, e23(Fv), have a lower affinity for antigen than the Fab counterpart, which could be due to the fact that the peptide linker somehow interferes with binding (Batra et al., 1992; Pantoliano et al., 1991; Stemmer et al.,

† Y.R. is supported by a grant from the Rothschild Foundation.

* Author to whom correspondence should be addressed. Telephone (301) 496-4797. Fax (301) 402-1344.

® Abstract published in *Advance ACS Abstracts,* April 1, 1994.

[1] Abbreviations: MAb, monoclonal antibody; H, heavy; L, light; V, variable; scFv and dsFv, single-chain and disulfide-stabilized Fv's, respectively; CDR, complementary determining region; IB, inclusion body; PE, *Pseudomonas* exotoxin.

1993). An alternative approach for stabilizing Fv fragments is to connect the V_H and V_L domains by a disulfide bond (Glockshuber et al., 1990; Brinkmann et al., 1993b). To accomplish this goal, we recently identified residues in conserved framework regions of the V_H and V_L domains which, when mutated to cysteines, can form a disulfide bond, stabilize the Fv, and maintain good antigen binding (Brinkmann et al., 1993b). The positions for disulfide replacement were identified using a structural model of MAb B3 derived from the crystal structure of MAb McPC603. Among all of the published mouse antibody structures, MAb McPC603 has an amino acid sequence that most resembles MAb B3.

The criteria that were used to select possible positions of a site for a disulfide connection between V_H and V_L were that the disulfide should connect amino acids in structurally conserved regions of the Fv, the distance between the V_H and V_L should be small enough to enable the formation of a disulfide bond without the generation of strain in the Fv molecule, and the disulfide should be distant enough from the CDRs to not interfere with antigen binding. Using the position predicted to be most favorable for disulfide stabilization, an immunotoxin B3(dsFv)–PE38KDEL composed of a disulfide-stabilized Fv and a truncated form of *Pseudomonas* exotoxin was constructed and shown to be indistinguishable in specific cytotoxic activity *in vitro*, as well as *in vivo*, from its scFv counterpart, indicating that the antigen binding of the scFv and dsFv is equal (Brinkmann et al., 1993b). Thus, in B3(dsFv), disulfide stabilization was achieved without interfering with antigen binding. Because the cysteines introduced to form the disulfide bond are in the framework regions of the Fv at positions that are structurally conserved in most antibodies, we reasoned that disulfide stabilization of Fv's using these sites should be generally applicable to stabilize other Fv's.

Here we present two additional examples of active immunotoxins that contain disulfide-stabilized Fv's. These are anti-Tac(dsFv)–PE38KDEL and e23(dsFv)–PE38KDEL. In addition, we show that the toxin moiety in dsFv–immunotoxins can be fused to either V_H or V_L. However, the V_H–toxin fusion is preferred because immunotoxins containing the V_H–toxin are made readily and can be purified with greater yields. The finding that three out of three different Fv's (B3, anti-Tac, and e23) analyzed could be stabilized by disulfides formed at the same position indicates that our approach is generally applicable to form stable Fv-containing immunotoxins, as well as Fv molecules not linked to a toxin.

EXPERIMENTAL PROCEDURES

Design of a Disulfide Bond between V_H and V_L of MAb B3, e23, and anti-Tac. The positions of disulfides for the stabilization of B3(Fv) were identified using a computer-modeled structure of the B3(Fv), generated by mutating and energy minimizing the amino acid sequence and structure of McPC603, as described previously (Brinkmann et al., 1993b; Jung et al., 1993). The amino acid sequences of e23(Fv) and anti-Tac(Fv) were simply aligned with that of B3(Fv) to determine the positions to insert cysteine residues. The assignments of the framework regions and CDRs are according to Kabat et al. (1991).

Plasmid Construction. Uracil-containing single-stranded DNA from the F+ origin present in our expression plasmids was obtained by cotransfection of *Escherichia coli* CJ236 (Bio-Rad, Richmond, CA) with M13 helper phage and was used as a template for site-directed mutagenesis (Kunkel, 1985). The amino acid sequences of B3(Fv), e23(Fv), and anti-Tac(Fv) have been previously described (Chaudhary et al., 1989; Batra et al., 1992; Brinkmann et al., 1991). The mutagenesis to change Arg-44 of B3(V_H) and Ser-105 of B3(V_L) [V_H44–V_L100 according to the Kabat numbering scheme (Kabat et al., 1991)] to cysteines and to introduce stop codons followed by an *Eco*RI site at the 3'-end of the B3(V_H) gene has previously been described (Brinkmann et al., 1993b). The oligonucleotide 5'-CAGATCCGCCAC-CACCGAAGCTTTTCCGCCTGAG GAGACAGTG-3' was used to introduce a *Hin*dIII site at the 3'-end of the B3(V_H) gene for deletion of B3(V_L). The oligonucleotide 5'-ACTG-GAGGAATTCATTATTTAATTTCCAGCTTTG-TCCC-3' was used to introduce translation stop codons followed by an *Eco*RI site at the end of B3(V_L).

For e23(Fv), the mutagenesis oligonucleotides were 5'-AGTCCAATCCACTCGAGGCACTTTCCATGGCT-CTGC-3' to change Asn-44 of e23(V_H) to cysteine, 5'-TATTTCCAGCTTGGACCCACATCCGAACGT-GGGTGG-3' to change Gly-99 of e23(V_L) (V_L100, Kabat numbering) to cysteine, and 5'-TGACTCCTGCAGCTG-CACTTCCATATGACCTTCAGAAGATTTACC-3' to introduce an *Nde*I site, an ATG translation–initiation codon, and a Glu at the N-terminus of e23(V_H) that was missing from the original sequence (Batra et al., 1992) but was conserved within FR1 according to Kabat et al. (1991). The oligonucleotides 5'-AGAAGATTTACCAGAACCAGGAAT-TCATTATTTTATTTCCAGCTTGGACC-3' and 5'-GAC-CACAACGGTTTCCCTCTAG-3' were used to introduce stop codons followed by an *Eco*RI site at the 3'-end of the e23(V_L) gene by PCR. The mutagenesis oligonucleotides for engineering anti-Tac(dsFv) were 5'-AATATATCCAATC-CATTCTAGACACTGTCCAGGCCTCTGTTT-3' to change Gly-44 in anti-Tac(V_H) to cysteine, 5'-CTTTGAGCTC-CAGCTTGGTACCACAACCGAACGTGAGTG-GGTAA-3' to change Ser-99 (V_L100, Kabat numbering) in anti-Tac(V_L), 5'-ACTGGGTGAGAACAATTTGCATATG-GCCGCCACCCGAGCCGCC-3' to introduce an *Nde*I site and an initiation codon in the anti-Tac(V_L) gene, and 5'-CGAGCCGCCACCGCCCGAGCGAATTCATTAT-GAGGAGACTGTGAGAGTGG-3' to introduce stop codons followed by an *Eco*RI site at the 3'-end of the anti-Tac(V_H) gene.

Restriction sites introduced into all of the above oligonucleotides to facilitate the identification of mutated clones or subcloning are underlined. All mutations were confirmed to be correct by DNA sequencing. Expression plasmids for the components of B3, e23, and anti-Tac dsFv's were made by subcloning of mutated clones, as described in Figure 2. The plasmids for the expression of e23(dsFv)–PE38KDEL encode e23(V_L) (pYR40) and e23(V_H)–PE38KDEL (pYR39). The plasmids for the expression of anti-Tac(dsFv)–PE38KDEL are anti-Tac(V_H) (pYR43) and anti-Tac(V_L)–PE38KDEL (pYR42). The plasmids pYR38–2, pULI39–1, pULI39–2, and pYR38–3 encode the components for the expression of B3(dsFv)–PE38KDEL or B3(dsFv)–PE38, in which the V_H or V_L of B3 is connected to the toxin [B3(V_H)–PE38 (pYR38–3) or B3(V_L)–PE38 (pULI39–1), respectively].

Expression of Recombinant Proteins. The components of the disulfide-stabilized immunotoxins V_L[Cys]–PE38KDEL, V_H[Cys], V_H[Cys]–PE38KDEL, and V_L[Cys] or single-chain immunotoxins were produced in separate *E. coli* BL21 (λDE3) (Studier & Moffatt, 1986) cultures containing the corresponding expression plasmid (see Figure 2). All recombinant proteins accumulated in cytoplasmic inclusion bodies (IBs).

```
          ----------FR1---------- -------CDR1------ ------FR2------
603 (VL)  DIVMTQSPSSLSVSAGERVTMSC KSSQSLLNSGNQKNFLA WYQQKPGQPPKLLIY
 B3 (VL)  DVLMTQSPLSLPVSLGDQASISC RSSQIIVHS.NGNTYLE WYLQKPGQSPKLLIY
e23 (VL)  DVQLTQSPAILSASPGEKVTMTC RATP.......SVSYMH WYQQKPGSSPKPWIY
aTac(VL)  QIVLTQSPAIMSASPGEKVTITC SASS.......SISYMH WFQQKPGTSPKLWIY

603 (VL)  GASTRES GVPDRFTGSGSGTDFTLTISSVQAEDLAVYYC QNDHSYPLT FGAGTK
 B3 (VL)  KVSNRFS GVPDRFSGSGSGTDFTLKISRVEAEDLGVYYC FQGSHVPFT FGSGTK
e23 (VL)  TTSNLAS GVPARFSGGGSGTSYSLTVSRVEAEDAATYYC QQWSRSPPT FGGGSK
aTac(VL)  TTSDLAS GVPARFSGSGSGTSYSLTISAMEAEDAATYYC HQASTYPLT FGSGTK
          --CDR2- --------------FR3-------------- ---CDR3-- --FR4-
                                                              Δ

          -------------FR1-------------- -CDR1 -----FR2------ -CDR2-
603 (VH)  EVKLVESGGGLVQPGGSLRLSCATSGFTFS DFYME WVRQPPGKRLEWIA ASRNKG
 B3 (VH)  DVKLVESGGGLVQPGGSLKLSCATSGFTFS DYYMY WVRQTPEKRLEWVA YISNDD
e23 (VH)  EVQLQESGPEVVKPGGSMKISCKTSGYSFT GHTMN WVKQSHGKNLEWIG LINPYN
aTac(VH)  QVHLQQSGAELAKPGASVKMSCKASGYTFT SYRMH FVKQRPGQGLEWIG YINPSP
                                                        Δ
603 (VH)  NKYTTEYSASVKG RFIVSRDTSQSILYLQMNALRAEDTAIYYCAR NYYGSTWYFDV
 B3 (VH)  SSAA..YSDTVKG RFTISRDNARNTLYLQMSRLKSEDTAIYYCAR GLAWGAW.FAY
e23 (VH)  GDTN..YNQKFKG KATFTVDKSSSTAYMELLSLTSEDSAVYYCAR RVTD..WYFDV
aTac(VH)  GYTE..YNQKFKD KATLTADKSSSTAYMQLSSLTFEDSAVYYCAR GGGV....FDY
          ------------ --------------FR3--------------- ----CDR3---

603 (VH)  WGAGTTVTVS
 B3 (VH)  WGQGTLVTVS
e23 (VH)  WGAGTTVTVS
aTac(VH)  WGQGTTLTVS
          ----FR4---
```

FIGURE 1: Identification of the positions for disulfide connections between V_H and V_L of e23(Fv) and anti-Tac(Fv). Comparison of the variable regions of the amino acid sequences of MAb e23 and humanized anti-Tac with MAb B3 and McPC603. Δ: Positions of cysteine replacement in the framework region 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. (1991).

Refolding and Purification of Disulfide-Stabilized Immunotoxins. Inclusion bodies of V_H, V_L–toxin, V_L, V_H–toxin, or single-chain immunotoxins were purified, solubilized, reduced, and diluted into redox-shuffling refolding buffer containing an aggregation-preventing additive, as described previously (Buchner et al., 1992). The V_H and V_L–toxin or V_L and V_H–toxin solubilized inclusion bodies were combined in a 2:1 molar ratio in the refolding solution and a final oxidation step, in which a 10-fold excess of oxidized glutathione was added after 24 h of refolding in the redox-shuffle buffer (Brinkmann et al., 1993b; Buchner et al., 1992). Properly folded disulfide-stabilized and single-chain immunotoxins were purified by ion-exchange (Q-Sepharose and Mono Q) and size-exclusion chromatography (Chaudhary et al., 1989).

Cytotoxicity Assays. The activity of disulfide-stabilized PE-derived immunotoxins was determined by the inhibition of protein synthesis, as described (Chaudhary et al., 1989). For competition experiments, MAb B3, HB21, e23, humanized anti-Tac, or human IgG was added 15 min before the addition of the disulfide-stabilized immunotoxins.

RESULTS

Engineering a Disulfide Connection between V_H and V_L in the Fv Framework Regions. The goal of this study was to determine whether recombinant Fv's, whose three-dimensional structures are not known, can be stabilized by inserting cysteine residues into the conserved framework residues. We previously found that when Arg-44 of B3(V_H) and Ser-105 of B3(V_L) (V_H44–V_L100, according to Kabat numbering) were changed to cysteine residues, a disulfide bond formed between the two domains, generating a stable Fv heterodimer that bound to the B3 antigen (Brinkmann et al., 1993b; Pastan et al., 1991b). To determine the locations in e23 and anti-Tac antibodies that would be used for disulfide bond formation, we simply aligned their sequences with those of B3 and McPC603, as shown in Figure 1. Because the locations for cysteine substitutions are in conserved framework regions, they can be readily identified. The positions identified for cysteine replacements in the framework regions of e23(Fv) are Asn-44 in V_H (FR2) and Gly-99 in V_L (FR4). The positions identified for cysteine replacements for disulfide stabilization of the anti-Tac(Fv) are Gly-44 in V_H (FR2) and Ser-99 in V_L (FR4). Neither molecular modeling nor knowledge of the structures of these Fv's was necessary to identify these positions.

Construction of Plasmids for the Expression of Disulfide-Stabilized Immunotoxins. The parent plasmids for the generation of plasmids expressing e23 and anti-Tac disulfide-stabilized immunotoxins encode the single-chain immunotoxins e23(Fv)–PE38KDEL and anti-Tac(Fv)–PE38KDEL, respectively. In these molecules, a gene encoding the V_H and V_L domains, held together by a $(Gly_4–Ser)_3$ peptide linker, is fused to the PE38KDEL gene encoding the translocation and ADP–ribosylation domains of *Pseudomonas* exotoxin (PE) with an altered carboxyl-terminal sequence, KDEL (Kreitman et al., 1993). The plasmids for expression of the components of e23(dsFv)– and anti-Tac(dsFv)–immunotoxins were made as described in the Experimental Procedures, by site-directed mutagenesis and subcloning from the mutagenized plasmids as outlined in Figure 2. Note that in the expression plasmids for anti-Tac(dsFv)–immunotoxin, the V_L domain is connected to the toxin and V_H is expressed separately, whereas in e23-(dsFv)–immunotoxin the V_H domain is fused to the toxin and the V_L domain is expressed separately. For B3(dsFv)–immunotoxins, both combinations, V_H+V_L–toxin and V_L+V_H–toxin, were made.

Expression, Refolding, and Purification of Disulfide-Stabilized Immunotoxins. Recombinant disulfide-stabilized immunotoxins were produced by separate *E. coli* BL21 (λDE3) cultures containing the corresponding plasmid encoding the components [Cys-44]e23(V_L) or [Cys-99]e23(V_H)–PE38-

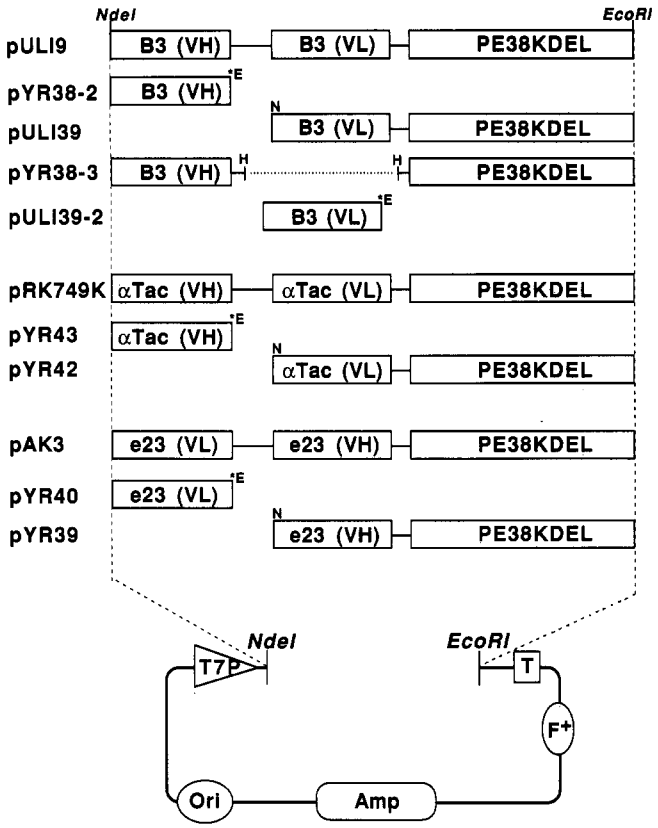

FIGURE 2: Plasmids for the expression of dsFv–immunotoxins. pULI7 codes for the scFv–immunotoxin B3(Fv)–PE38 (Brinkmann et al., 1991). pYR38–2 and pULI391 encoding [Cys-44]B3(V_H) and [Cys-105]B3(V_L)–PE38 were derived from pULI7 by site-directed mutagenesis and subcloning as described (Brinkmann et al., 1993b). pULI391 has the C-terminus of the toxin portion of [Cys-105]B3(V_L)–PE38KDEL encoded by pULI39 (Stemmer et al., 1993), changed from KDEL to the original C-terminus of PE, REDLK. pYR38–3 was made by simultaneously introducing the Cys-44 V_H mutation and a *Hin*dIII site at the C-terminus of B3(V_H) by Kunkel mutagenesis and subsequently deleting a *Hin*dIII fragment coding for B3(V_L) to obtain an in-frame [Cys-44]B3(V_H)-PE38 fusion protein. pULI39–2 was obtained from pULI39 by introducing stop codons followed by an *Eco*RI site at the C-terminus of [Cys-105]-B3(V_L) and subsequently deleting the toxin-coding *Eco*RI fragment. The plasmids for expression of the components of e23(dsFv)– and anti-Tac(dsFv)–immunotoxins were made by site-directed mutagenesis and subcloning essentially as described for B3(dsFv). pAK3 encodes the single-chain immunotoxin e23(Fv)–PE38KDEL (Batra et al., 1992). pYR40 is derived from pAK3 by the replacement of Gly-99 in e23(V_L) with cysteine, the introduction of stop codons followed by an *Eco*RI site at the C-terminus of e23(V_L), and the subsequent deletion of the e23(V_H)- and PE38KDEL-coding *Eco*RI fragment. To make pYR39, we introduced into pAK3 an *Nde*I site and an ATG translation initiation codon at the N-terminus of e23-(V_H), replaced Asn-44 in V_H with cysteine, and deleted an *Nde*I fragment coding for e23(V_L). pRK749K encodes the scFv–immunotoxin anti-Tac(Fv)–PE38KDEL (Chaudhary et al., 1989). pYR43 and pYR42 are derived from pRK749K similarly, as described for the e23(dsFv) components by site-directed mutagenesis, to replace Gly-44 in anti-Tac(V_H) and Ser-99 in anti-Tac(V_L) with cysteines and subcloning; i.e., deletion of the V_L–toxin portion as an *Eco*RI fragment to make pYR43 and deletion of the *Nde*I fragment coding for anti-Tac(V_H) to generate pYR42. *, stop codons; E, *Eco*RI site; N, *Nde*I site; H, *Hin*dIII site.

KDEL of e23(dsFv)–PE38KDEL, [Cys-44]anti-Tac(V_H) or [Cys-99]anti-Tac(V_L)–PE38KDEL of anti-Tac(dsFv)–PE-38KDEL, and [Cys-44]B3(V_H), [Cys-105]B3(V_L), [Cys-44]-B3(V_H)–toxin, or [Cys-105]B3(V_L)–toxin for the B3(dsFv)–immunotoxins. These cultures were induced to express the corresponding recombinant protein by isopropyl β-D-thiogalactoside. After cell disruption, the inclusion bodies were isolated separately, solubilized in 6 M guanidine hydrochloride, reduced, and refolded in a renaturation buffer, which contained redox-shuffling and aggregation-preventing additives. The IBs for each component of the dsFv–immunotoxins, such as e23(V_L) and e23(V_H)–PE38KDEL or anti-Tac(V_H) and anti-Tac(V_L)–PE38KDEL, were prepared separately, and the solubilized and reduced protein components V_H and V_L–toxin or V_L and V_H–toxin were mixed in a 2:1 molar ratio to a final total concentration of 100 μg/mL in the refolding solution. Refolding was performed for 24 h, and then a final oxidation step was performed in which an excess of oxidized glutathione was added to the refolding solution. This oxidation step increased the yield of properly folded functional immunotoxin, probably because the disulfide bond connecting the V_H and V_L domains is exposed on the surface of the Fv molecule and is accessible to the slightly reducing environment in the refolding solution.

Active dsFv–immunotoxins were recovered by a purification scheme previously established for single-chain immunotoxins (Chaudhary et al., 1989), which consists of ion-exchange chromatography (Q-Sepharose and Mono Q) followed by size-exclusion chromatography. Properly folded dsFv–immunotoxins must be separated from aggregates and also from "single-domain" toxins, which have chromatographic behavior similar to that of dsFv–immunotoxins and can severely contaminate column fractions containing properly folded dsFv–immunotoxins (Figure 3). Because the dsFv–immunotoxin elutes slightly earlier than the V_L–toxin, which has an extra charge because of the unbonded sulfhydryl group, the dsFv–immunotoxin could only be purified to near-homogeneity by consecutive cycles of chromatography on Mono Q, in which early fractions that did not contain contaminating single-domain molecules were saved (Figure 3). This purification method caused a significant loss of material. This problem is particularly pronounced when the immunotoxin is composed of V_H and V_L–toxin. It is much less evident with the V_L and V_H–toxin combination because single-domain V_L–toxin folding is very efficient and the product is quite soluble, whereas the V_H–toxin tends to aggregate and is less soluble (see the comparison of B3(V_H+V_L)–toxin to B3(V_L+V_H)–toxin below). Therefore, the yield of dsFv–immunotoxins is increased significantly when the V_H domain is fused to the toxin and the V_L domain is expressed separately (Table 1).

The yield of properly folded dsFv–immunotoxins is also higher than that of the corresponding single-chain immunotoxins. As shown in Table 1, 30–70 mg of dsFv–immunotoxin could be obtained from bacterial V_H and V_L–toxin or V_L and V_H–toxin cultures of 1 L, each induced at an OD_{600} of 9. The increased yield of active dsFv–immunotoxins probably results from the reduced aggregation of properly folded dsFv molecules after refolding compared to single-chain immunotoxins.

To test this idea, we compared the stabilities of purified scFv– and dsFv–immunotoxins under two conditions. One was in PBS (10 mM phosphate/150 mM NaCl); the other was in 20 mM Tris (pH 7.4) without added salt. As shown in Table 2, the dsFv-immunotoxin B3(dsFv)–PE38KDEL is much more stable in PBS than single-chain B3(Fv)–PE38KDEL. The scFv–immunotoxin was stable for 1–2 h and then began to lose activity. In marked contrast, the dsFv–immunotoxin retained almost full activity after 48 h. When incubated in Tris buffer without any added NaCl, the scFv–immunotoxin lost all of its activity after 1 h, whereas dsFv–immunotoxin was fully active after 1 h and less active after 2 h. We conclude that the improved stability and solubility

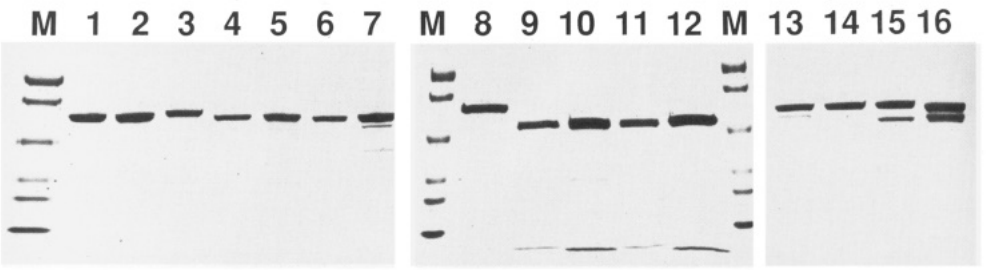

FIGURE 3: Purification of dsFv–immunotoxins. SDS–PAGE of purified dsFv–immunotoxins. Lanes: M, molecular weight standard; 1–3, purified nonreduced single-chain immunotoxins B3(Fv)–PE38KDEL, anti-Tac(Fv)–PE38KDEL, and e23(Fv)–PE38KDEL, respectively; 4–7, purified nonreduced disulfide-stabilized Fv immunotoxins B3(dsFv)–PE38KDEL[L-HT], B3(dsFv)–PE38KDEL[H-LT], anti-Tac(dsFv)–PE38KDEL, and e23(dsFv)–PE38KDEL, respectively; 8, purified reduced scB3(Fv)–PE38KDEL; 9–12, purified reduced disulfide-stabilized B3(dsFv)–PE38KDEL[H-LT], B3(dsFv)–PE38KDEL[L-HT], anti-Tac(dsFv)–PE38KDEL, and e23(dsFv)–PE38KDEL, respectively; 13–16, Mono Q chromatography peak fractions of e23(dsFv)–PE38KDEL, B3(dsFv)–PE38[L-HT], B3(dsFv)–PE38KDEL[H-LT], and anti-Tac(dsFv)–PE38KDEL, respectively. Molecular mass standards are (top to bottom) 106, 80, 49, 32, 27 and 18 kDa, respectively.

Table 1: Yield of Renatured Monomeric dsFv–Immunotoxins[a]

immunotoxin	renaturation solution (mg)	Q-Sepharose column (mg)	Mono Q column		
			sd–toxin[b] (mg)	Fv toxin (mg)	yield (%)
B3(scFV)(V_H–V_L–toxin)	100	8.5		2.7	2.7
B3(dsFv)(V_H+V_L–toxin)	100	13.8	2.4	9.8	9.8
B3(dsFv)(V_L+V_H–toxin)	100	28.4	0.1	15.4	15.4
anti-Tac(scFv)(V_H–V_L–toxin)	100	15.5		6.8	6.8
anti-Tac(dsFv)(V_H+V_L–toxin)	100	29	5	15	15
e23(scFv)(V_L–V_H–toxin)	100	12.5		10.5	10.5
e23(dsFv)(V_L+V_H–toxin)	100	35.6	0.6	20.5	20.5

[a] 1 L of a bacterial culture was induced at $OD_{600\ nm}$ = 9 with IPTG for 2 h and reached a final OD of about 18; approximately 4 g of inclusion bodies, which contained 350 mg of recombinant protein, were obtained from cells expressing the B3- and anti-Tac(dsFv)–immunotoxins. Expression of the e23(dsFv)–immunotoxin was slightly lower. Inclusion body protein (100 mg) was refolded in 1 L of renaturation solution for 24 h as described in the Experimental Procedures. Immunotoxins were purified as described (Brinkmann et al., 1993b). [b] sd–toxin are fractions which contain single-domain immunotoxins, either V_H–toxin or V_L–toxin. Protein concentrations were determined by the Bradford assay (Bradford, 1976).

Table 2: Stability of B3(dsFv)–Immunotoxins[a]

buffer	immunotoxin	% activity remaining after indicated time (h)								
		0	1	2	4	8	12	24	48	60
PBS	scFv	100	44	10	3	0.7	0.6	0		
	dsFv	100	100	100	100	100	80	80	80	67
Tris	scFv	100	5	0						
	dsFv	100	100	38	5	0				

[a] Each type of immunotoxin at 10 μg was incubated with Dulbecco's phosphate-buffered saline (PBS) or 20 mM Tris (pH 7.4) at 37 °C for the times shown and then assayed for cytotoxic activity on A431 cells.

of dsFv–immunotoxins contribute to the improved production yields of dsFv–immunotoxins.

Characterization of Disulfide-Stabilized Immunotoxins. Since PE-derived immunotoxins are highly cytotoxic agents whose cytotoxicity is mediated by the specific binding of their Fv components to target cells, measurements of the specific Fv-mediated toxicity of scFv– and dsFv–immunotoxins enable us to compare directly the binding and specificity of these Fv derivatives.

B3(dsFv)–Immunotoxins. As shown previously, a B3-(dsFv)–immunotoxin composed of V_H and a V_L–toxin fusion has the same activity as B3(scFv)–immunotoxin composed of V_H-linker–V_L–toxin (Brinkmann et al., 1993b). However, as shown in Figure 3, it is difficult to obtain pure B3(ds)–immunotoxin using our standard purification protocol, because the single-domain V_L–toxin molecules contaminate dsFv after Mono Q chromatography. Previous studies on single-domain immunotoxins made with MAb B3 showed that a V_H–toxin had a much higher tendency to aggregate than a V_L–toxin fusion and, when renatured from inclusion bodies, gave a lower yield of soluble monomers (Brinkmann et al., 1993a). Therefore, we designed a dsFv construct in which the V_H is connected to the toxin and the V_L is expressed separately. This should reduce the amount of contaminating single-domain immunotoxin in the dsFv–immunotoxin pool because the free V_H–toxin should aggregate and be readily removed. As shown in Figure 3 and Table 1, when compared to the V_H+V_L–toxin preparation, contamination of the dsFv with a single-domain immunotoxin was dramatically reduced. This contributed to the improved yields of the final purified material. To determine whether this switch of domains in B3(dsFv)–immunotoxins has any effect on activity, we compared the cytotoxicity of B3(dsFv)–immunotoxins composed of V_H– and V_L–toxin fusion, B3(dsFv)–PE38KDEL[H-LT], with that of a V_L and V_H–toxin fusion, B3(dsFv)–PE38KDEL[L-HT] (Figure 4). We found that the activity and specificity of both types of dsFv–immunotoxins were indistinguishable, indicating that except for the improved yields of the dsFv–immunotoxins their properties are the same.

anti-Tac(dsFv)–Immunotoxins. The anti-Tac(dsFv)–immunotoxin was produced in the same manner as the B3(dsFv)–immunotoxin, in which the V_L is connected to the toxin and V_H is expressed separately. As shown in Figure 3, single-domain toxin contamination was observed on monomeric Mono Q peaks, and this was overcome by consecutive purification on Mono Q, yielding a highly purified dsFv–immunotoxin

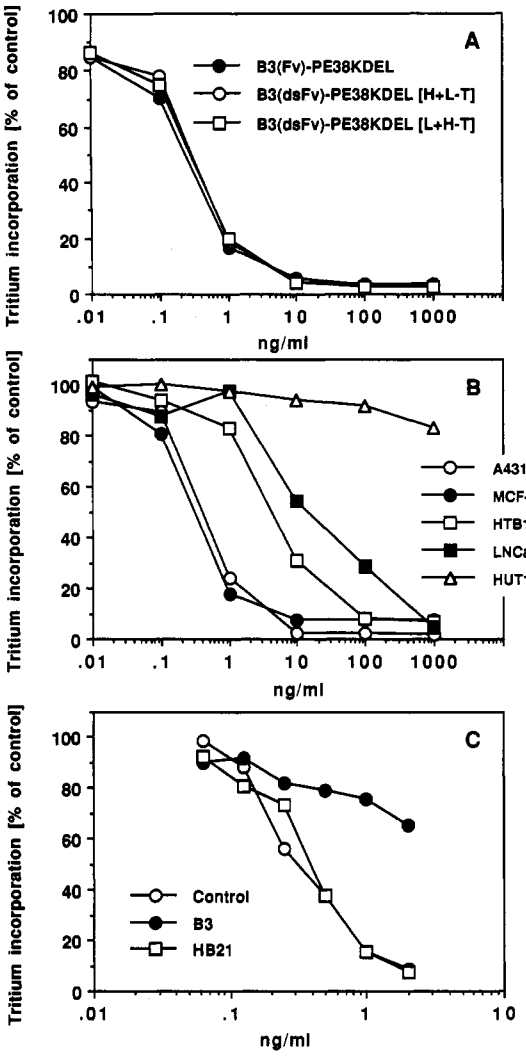

FIGURE 4: Specific cytotoxicity of B3dsFv-immunotoxins toward different cell lines: (A) comparison of the cytotoxicity of B3(Fv)–PE38KDEL and B3(dsFv)–PE38KDEL[H-LT] or B3(dsFv)–PE38KDEL[L-HT]; (B) cytotoxicity of B3(dsFv)–PE38KDEL[L-HT] toward various cell lines; (C) competition of cytotoxicity by the addition of excess MAb B3. Note that the addition of equal amounts of control MAb HB21, which binds to the cells but to a different antigen, does not compete.

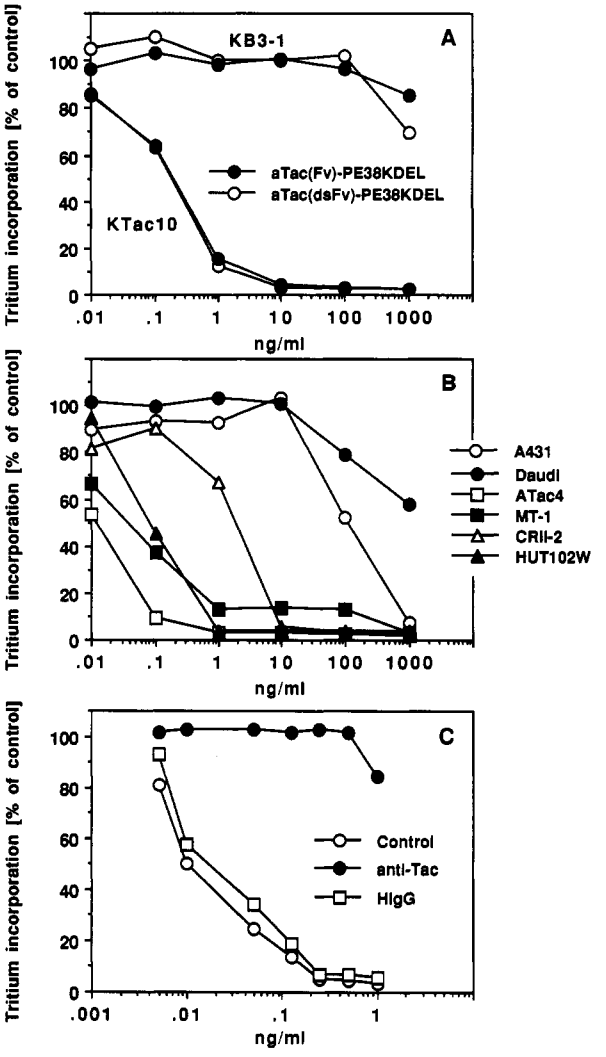

FIGURE 5: Specific cytotoxicity of anti-Tac(dsFv)–PE38KDEL toward different cell lines: (A) comparison of the cytotoxicity of anti-Tac(Fv)–PE38KDEL and anti-Tac(dsFv)–PE38KDEL; (B) cytotoxicity of anti-Tac(dsFv)–PE38KDEL toward various cell lines; (C) competition of cytotoxicity by the addition of excess humanized anti-Tac. Equal amounts of control human IgG do not compete.

(Figure 3). A comparison of the specific cytotoxicity mediated by the single-chain immunotoxin, anti-Tac(Fv)–PE38KDEL, and the corresponding disulfide-stabilized anti-Tac(dsFv)–PE38KDEL shows that both immunotoxins recognize the same spectrum of cells and are approximately equally active (Figure 5 and Table 3). The anti-Tac(dsFv)–PE38KDEL, like the single-chain immunotoxin, is toxic only to cells expressing the Tac antigen, the p55 subunit of the IL-2 receptor. HUT-102W, MT-1, and CRII-2 are adult T-cell leukemia cell lines. ATac4 and KTac10 cells are derived from IL-2 receptor negative A431 and KB cells by stably transfecting them with a cDNA encoding p55 (Kreitman et al., 1993). The cytotoxicity mediated by the disulfide-stabilized anti-Tac immunotoxin was competed for by the addition of an excess of humanized anti-Tac, but not by an excess of a humanized nonrelevant IgG, showing that the cytotoxic activity of anti-Tac(dsFv)–PE38KDEL is due to specific binding to the Tac antigen.

e23(dsFv)–Immunotoxins. The third example of a disulfide-stabilized Fv immunotoxin is e23(dsFv)–PE38KDEL, in which the V_H domain is connected to the toxin and the V_L is produced separately (Figure 2). Recovery of e23(dsFv)–PE38KDEL from the refolding solution by purification on Q-sepharose and Mono Q revealed a single monomeric species that corresponds to dsFv–immunotoxin without significant single-domain toxin contamination. This observation is consistent with the results from the B3(dsFv) (V_H+V_L–toxin and V_L+V_H–toxin) and the anti-Tac(dsFv) (V_H+V_L–toxin), which demonstrate that marked single-domain immunotoxin contamination is found only when the dsFv–immunotoxin is composed of V_H+V_L–toxin and not with the combination V_L+V_H–toxin.

Figure 6 shows some typical cytotoxicity assays with e23(dsFv)–PE38KDEL and e23(Fv)–PE38KDEL. In Figure 6A, the cytotoxic activity of the two molecules is compared on sensitive MCF7 cells and resistant KB-3-1 cells. Panel B shows typical data from a single experiment with cell lines expressing different levels of erbB2, and Table 3 shows the mean ID_{50}'s from eight different experiments. In general, those cell lines expressing higher levels of antigen are more susceptible to the immunotoxin, although some variations are observed. What is most important is that cell line KB-3-1, which does not express erbB2, is resistant to the action of the recombinant toxin. The disulfide-linked toxin is 2–10-fold more cytotoxic than the scFv–immunotoxin, e23(Fv)–PE38KDEL, depending upon which cell line was tested (Table

Table 3: Activities of dsFv–Immunotoxins on Various Cell Lines[a]

PE38KDEL immunotoxin	cell line	type	antigen expression	cytotoxicity ID_{50} (ng/mL)	
				scFv	dsFv
B3	A431	epidermoid	+++	0.45	0.5/0.4
	MCF-7	breast	+++	0.5	0.5/0.3
	LNCaP	prostate	+	8.0	7.0/9.0
	HTB103	gastric	+	4.0	3.0/4.0
	HUT102W	leukemia	–	>1000	>1000/>1000
anti-Tac	HUT102W	leukemia (ATL)	+++	0.4	0.2
	MT-1	leukemia (ATL)	+++	0.1	0.08
	CRII-2	leukemia (ATL)	+++	4.0	2.0
	KB3-1	epidermoid	–	>1000	>1000
	KTac10	KB3-1/Tac	+++	0.075	0.075
	A431	epidermoid	–	300	100
	ATac4	A431/Tac	+++	0.03	0.012
	Daudi	B-cell lymphoma	–	>1000	>1000
e23	HTB20	breast	+++	0.6	0.1
	HTB30	breast	+	2.0	0.5
	MCF-7	breast	+++	0.4	0.15
	N87	gastric	+++	0.06	0.015
	HTB103	gastric	+	6.0	1.0
	HEPG-2	hepatic	+	2.0	0.4
	A431	epidermoid	+	10	1.0
	KB3-1	epidermoid	–	>1000	>1000

[a] Cytotoxicity assays were performed by measuring the incorporation of [^{3}H]leucine into cell protein as described (Chaudhary et al., 1989). Data are given as ID^{50} values, the concentration of immunotoxin that causes a 50% inhibition in protein synthesis after a 24-h incubation with immunotoxin. Immunotoxins tested were single-chain Fv–immunotoxins (scFv) B3(Fv)–PE38KDEL, anti-Tac(Fv)–PE38KDEL, and e23(Fv)–PE38KDEL; disulfide-stabilized Fv–immunotoxins (dsFv) B3(dsFv)–PE38KDEL[H-LT]/[L-HT], anti-Tac(dsFv)–PE38KDEL, and e23(dsFv)–PE38KDEL. ATac4 and KTac10 cells are derived from IL-2R negative A431 and KB3-1 epidermoid cells stably transfected with and expressing a p55 (Tac) encoding gene. The level of antigen expression as assessed by Western blotting of cell extracts is marked +++, +, and – for high, low, and no detectable expression, respectively.

3). This increased activity could be due to the enhanced stability of the disulfide-stabilized immunotoxin, which enables longer exposure time to the cells or better binding of the dsFv–immunotoxin. The cytotoxicity of e23(dsFv)–PE38KDEL is specific because it can be competed for by the addition of excess MAb e23 but not an excess of HB21, an antibody to the human transferrin receptor (Figure 6C). The fact that the activity and specificity of the dsFv–immunotoxins are at least as good and often better than those of the single-chain immunotoxin indicates that the antigen binding region is well-conserved in the dsFv toxins and that the disulfide stabilization of Fv's, as demonstrated here for these different antibodies, should be generally applicable for other Fv's.

DISCUSSION

In this study, we have demonstrated that stable recombinant Fv molecules can be formed by inserting cysteine residues into specific sites in conserved framework residues and that these positions can be identified simply by aligning the sequence of the Fv with those of MAb B3 and McPC603. The first disulfide-bond-stabilized molecule produced was a recombinant immunotoxin, B3(dsFv)–PE38KDEL, in which the unstable Fv heterodimer V_H and V_L was stabilized by a genetically engineered disulfide bond (Brinkmann et al., 1993b). We have now made and analyzed an alternate form of the B3(dsFv)–immunotoxin, as well as two other dsFv-containing immunotoxins. These contain either the e23(dsFv), which binds to the erbB2 carcinoma-related antigen (Batra et al., 1992), or the anti-Tac(dsFv), which binds to the Tac antigen that is the p55 subunit of the IL-2 receptor (Chaudhary et al., 1989). The toxic module of these immunotoxins is a truncated form of *Pseudomonas* exotoxin (PE38KDEL), which contains the translocation and ADP–ribosylation domains of PE and an altered carboxyl-terminal sequence, KDEL, which is the consensus endoplasmic reticulum retention sequence and acts to increase the activity of recombinant immunotoxins.

Location of Disulfide Bonds. The positions for disulfide stabilization were identified initially by using a computer-modeled structure of the B3(Fv) derived from the known crystal structure of MAb McPC603 (Jung et al., 1993). In the current study, the positions for locating cysteine residues in the framework regions of e23(Fv) and anti-Tac(Fv) were determined by simply aligning the amino acid sequences of these proteins with those of MAb B3 and McPC603, as shown in Figure 1. The precise sequence assignments of the framework regions 1–4 (FR1–4) in V_H and V_L of e23(Fv), anti-Tac(Fv) with FR1–4 of B3(Fv), and McPC603 were made according to Kabat et al. (1991). We also used the intrachain cysteines whose positions are highly conserved within antibodies to aid in the sequence alignment. It should now be possible to align any other Fv with the Fv sequences described in this publication for identification of the correct positions for cysteine placements to produce a disulfide-stabilized Fv (as shown in Figure 1). Molecular modeling and structural information are not needed for this purpose.

Properties of Single-Chain Immunotoxins. Comparison of the specificity and activity of the disulfide-stabilized Fv–immunotoxins presented in this study with those of their single-chain counterparts shows that the disulfide-linked molecules have at least equal, and in some cases better, activity, indicating that the positions used for disulfide stabilization do not interfere with antigen binding. Some scFv's have been found to have a reduced affinity for antigen when compared to the corresponding IgGs or Fab fragments; this reduction could be caused by the peptide linker somehow interfering with antigen binding (Pantoliano et al., 1991; Stemmer et al., 1993). The e23(dsFv)–immunotoxin is probably such an example: its binding to the erbB2 antigen was found to be reduced when compared to the Fab fragment or the whole antibody (Batra et al., 1992). We found that the e23(dsFv)–PE38KDEL immunotoxin was 2–10-fold more active on various cell lines compared to its single-chain counterpart, e23(Fv)–PE38KDEL. This increased activity could be due to either the increased

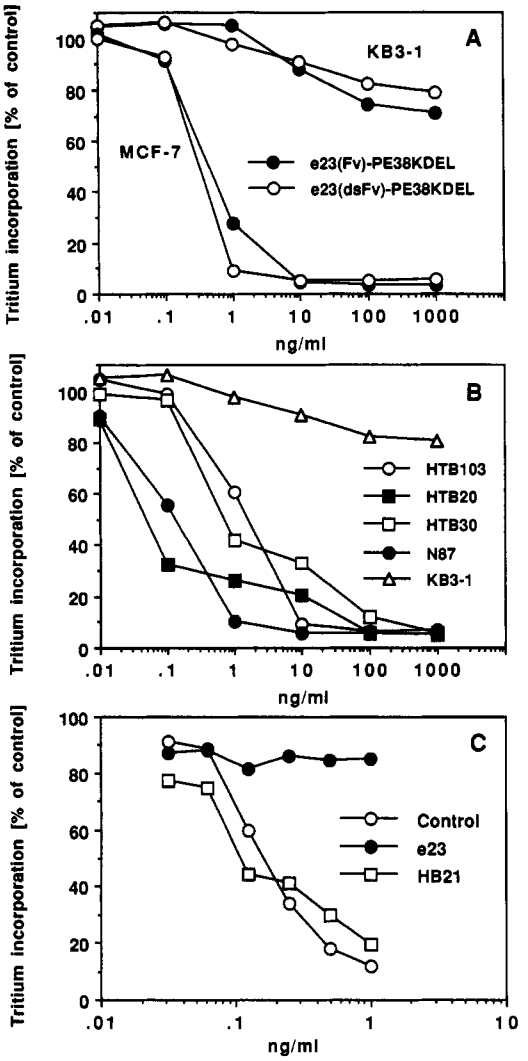

FIGURE 6: Specific cytotoxicity of e23(dsFv)–PE38KDEL toward different cell lines: (A) comparison of the cytotoxicity of e23(Fv)–PE38KDEL and e23(dsFv)–PE38KDEL; (B) cytotoxicity of e23-(dsFv)–PE38KDEL toward various cell lines; (C) competition of cytotoxicity by the addition of excess MAb e23. Equal amounts of MAb HB21 do not compete.

stability of the dsFv–immunotoxin, which enables the cytotoxic agent to interact with target cells for a longer period of time, or the better binding of dsFv than scFv. Because both the B3(dsFv)- and anti-Tac(dsFv)–immunotoxins are more stable than their corresponding scFv–immunotoxins but indistinguishable from the scFv's in activity, we believe the enhanced activity of e23(dsFv)- over e23(scFv)–immunotoxin is due to better binding of the dsFv. Experiments to evaluate this hypothesis are currently in progress.

Purification and Production of Disulfide-Linked Immunotoxins. The protocol for the production and purification of dsFv immunotoxins is only slightly different from that for single-chain immunotoxins. We observed that dsFv–immunotoxins in which toxin was fused to V_H instead of V_L could be purified more easily and resulted in higher yields than dsFv–immunotoxins composed of V_H and V_L fused to the toxin. This is because V_H–toxin molecules associated with V_L are less soluble than V_L–toxin molecules and tend to aggregate (Brinkmann et al., 1993a). As a consequence, single-domain immunotoxin contamination of dsFv–immunotoxin preparations is reduced dramatically when the combination V_L+V_H–toxin is used, because the V_H–toxin precipitates or aggregates and is easily separated from correctly folded dsFv–immunotoxins. With optimized refolding and purification procedures, the yield of dsFv–immunotoxins was up to 70 mg of highly purified active dsFv–immunotoxin from 1 L of bacterial culture induced at an OD_{600} of 9. This represents a yield of 20% of total protein added to the renaturation system. This yield is better than that observed for scFv–immunotoxins. Important contributing factors are the enhanced stability of the disulfide-linked molecules even at low ionic strength (Table 2), a decreased tendency of properly folded molecules to aggregate, and perhaps improved refolding.

Other Types of Molecules. In this and a preceding publication (Brinkmann et al., 1993b), we have demonstrated the specific binding of disulfide-stabilized Fv's by fusing them to a toxin and using the resulting immunotoxins as a convenient "assay system". This is possible, since immunotoxin cytotoxicity is mediated by specific binding of the Fv components to target cells. Because of this, measurements of the specific Fv-mediated cytotoxicity of scFv- and dsFv–immunotoxins enable us to compare directly the binding and specificity of these Fv derivatives. We have also produced single-chain disulfide-stabilized Fv molecules by themselves and found these to bind to antigen (K. O. Webber and I. Pastan, unpublished results). Therefore, disulfide-stabilized Fv's could be used not only to generate immunotoxins but also for all of the diagnostic and therapeutic uses proposed for single-chain antibodies or antigen binding proteins (Huston et al., 1988; Bird et al., 1998; Pastan & FitzGerald, 1991).

Conclusion. Because immunotoxins composed of disulfide-linked Fv's have cytotoxic activity equal to or better than scFv's containing a peptide linker, they can be produced more easily than scFv–immunotoxins, and they are more stable, dsFv–immunotoxins may be more useful and versatile than scFv–immunotoxins in applications that require large amounts of protein with good binding affinity, such as for clinical use.

ACKNOWLEDGMENT

We thank E. Lovelace and A. Harris for cell culture assistance, A. Kihara for the plasmid pAK3, and J. Evans and A. Jackson for editorial assistance.

REFERENCES

Batra, J. K., Kasprzyk, P. G., Bird, R. E., Pastan, I., & King, R. C. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 5867–5871.

Bird, R. E., Hardman, K. D., Jacobson, J. W., Johnson, S., Kaufman, B. M., Lee, S.-M., Lee, T., Pope, S. H., Riordan, G. S., & Withlow, M. (1988) *Science 242*, 423–426.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 848–854.

Brinkmann, U., Pai, L. H., FitzGerald, D. J., Willingham, M., & Pastan, I. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 8616–8620.

Brinkmann, U., Lee, B. K., & Pastan, I. (1993a) *J. Immunol. 150*, 2774–2782.

Brinkmann, U., Reiter, Y., Jung, S.-H., Lee, B., & Pastan, I. (1993b) *Proc. Natl. Acad. Sci. U.S.A. 90*, 7538–7542.

Buchner, J., Pastan, I., & Brinkmann, U. (1992) *Anal. Biochem. 205*, 263–270.

Chaudhary, V. K., Queen, C., Junghans, R. P., Waldmann, T. A., FitzGerald, D. J., & Pastan, I. (1989) *Nature 339*, 394–397.

Glockshuber, R., Malia, M., Pfitzinger, I., & Pluckthun, A. (1990) *Biochemistry 29*, 1362–1367.

Huston, J. S., Levinson, D., Mudgett-Hunter, M., Tai, M. S., Novotny, J., Margolies, M. N., Ridge, R. J., Bruccoleri, E. H., Crea, R., & Oppermann, H. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 5879–5883.

Jung, S.-H., Lee, B. K., & Pastan, I. (1993) *Proteins: Struct., Funct., Genet.* (in press).
Kabat, E. A., Wu, T. T., Perry, H. M., Gottesman, K. S., & Foeller, C. (1991) *Sequences of Proteins of Immunological Interest*, 5th ed., U.S. Dept. of Health and Human Services, Public Health Service, NIH Publication No. 91-3242, National Institutes of Health, Bethesda, MD.
Kreitman, R. J., Bailon, P., Chaudhary, V. K., FitzGerald, D. J. P., & Pastan, I. (1993) *Blood* (in press).
Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488–492.
Milenic, D. E., Yokota, T., Filpula, D. R., Finkelman, M. A. J., Dodd, S. W., Wood, J. F., Withlow, M., Snoy, P., & Schlom, J. (1991) *Cancer Res. 51*, 6363–6371.
Pantoliano, M. E., Bird, R. E., Johnson, S., Asel, E. D., Dodd, S. W., Wood, J. F., & Hardman, K. D. (1991) *Biochemistry 30*, 10117–10125.
Pastan, I., & FitzGerald, D. (1991) *Science 254*, 1173–1177.
Pastan, I., Lovelace, E. T., Gallo, M. G., Rutherford, A. V., Magnani, J. I., & Willingham, M. C. (1991) *Cancer Res. 51*, 3781–3787.
Seetharam, S., Chaudhary, V. K., FitzGerald, D., & Pastan, I. (1991) *J. Biol. Chem. 266*, 17376–17381.
Stemmer, W. P. C., Morris, B. S., & Wilson, B. S. (1993) *Biotechniques 14*, 256–262.
Studier, F. W., & Moffatt, B. A. (1986) *J. Mol. Biol. 189*, 113–130.
Yokota, T., Milenic, D. E., Withlow, M., & Schlom, J. (1992) *Cancer Res. 52*, 3402–3408.