



## A Bivalent Disulfide-stabilized Fv with Improved Antigen Binding to erbB2

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We have used protein engineering to generate a stable bivalent Fv molecule of the anti-erbB2 monoclonal antibody e23. The  $V_H$  and  $V_L$  domains of the Fv are linked to each other by a disulfide bond and the two Fvs are connected by a flexible 15 amino acid residue (Gly<sub>4</sub>-Ser)<sub>3</sub> linker. The e23 (dsFv)<sub>2</sub> molecule is fused to a truncated form of *Pseudomonas* exotoxin to generate a bivalent disulfide-stabilized, (dsFv)<sub>2</sub>, immunotoxin. The immunotoxin was expressed in *Escherichia coli*, refolded *in vitro* and purified to about 95% purity. Binding studies demonstrated that the (dsFv)<sub>2</sub> molecule has a much higher affinity for erbB2 than a monovalent dsFv molecule and a similar binding affinity as the parental antibody e23. The (dsFv)<sub>2</sub> immunotoxin was 5 to 20-fold more cytotoxic to two e23 antigen-positive cell lines than the monovalent dsFv immunotoxin. The bivalent dsFv molecule is very stable, retaining 94% of its activity after a 24 hours incubation in human serum at 37°C. Two other molecules with shorter linkers five and ten amino acid residues in length were produced and showed similar activities as the molecule containing a 15 amino acid residue linker. The bivalency, stability and the relative ease of purification makes these e23 (dsFv)<sub>2</sub> molecules valuable reagents for cancer immunotherapy and diagnosis.

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### Introduction

Fv fragments of antibodies are heterodimers made up of antibody  $V_H$  and  $V_L$  domains and are the smallest antibody fragments that contain all the structural information necessary for specific antigen binding. Because small molecules penetrate tumors much faster than large molecules, Fv fragments are particularly useful in applications that require rapid tissue or tumor penetration (Yokota *et al.*, 1992; Kreitman & Pastan, 1998). Fv fragments are also valuable reagents for diagnostic and therapeutic applications, such as imaging of tumors or other forms of targeted cancer therapy. Although native unstabilized Fv heterodimers have been made from unusual antibodies (Skerra & Pluckthun, 1988; Webber *et al.*, 1995), generally Fv fragments by themselves are unstable because the

$V_H$  and  $V_L$  domains of the heterodimer can rapidly dissociate (Glockshuber *et al.*, 1990). This results in drastically reduced binding affinity and is often accompanied by aggregation.

A solution to the stabilization problem resulted from a combination of genetic engineering and recombinant protein expression techniques. The first general method of stabilizing Fvs is the covalent connection of  $V_H$  and  $V_L$  by a flexible peptide linker which results in single-chain (sc)Fv molecules (Bird *et al.*, 1988; Huston *et al.*, 1988). scFvs are more stable than Fvs alone, and several scFvs and scFv-fusion proteins (e.g. recombinant immunotoxins) have been made with *Pseudomonas* exotoxin A (PE), that may be useful for the imaging or therapy of tumors (Yokota *et al.*, 1992; Webber *et al.*, 1995; Chaudhary *et al.*, 1989; Brinkmann & Pastan, 1994; Pastan *et al.*, 1995; Brinkmann *et al.*, 1991). Another way to generate stable recombinant Fvs is to connect the  $V_H$  and  $V_L$  by an interdomain disulfide bond instead of a linker peptide; this results in disulfide-stabilized (ds)Fvs. dsFvs, when they can be successfully produced, solve most problems that are associated with scFvs; they are very stable, often show full antigen binding activity, and sometimes have

Abbreviations used: IPTG, isopropyl- $\beta$ -D-thiogalactopyranoside; scFv, single-chain Fv; dsFv, disulfide-stabilized Fv;  $V_H$  and  $V_L$  variable region of heavy or light-chain; (dsFv)<sub>2</sub>, bivalent disulfide-stabilized Fv fragment; PCR, polymerase chain reaction; GuCl, guanidine chloride; DTE, dithioerythritol; IB, inclusion body; IG, immunoglobulin.

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better affinity than scFvs (Webber *et al.*, 1995; Brinkmann *et al.*, 1993; Reiter *et al.*, 1994a,b,c).

Although the Fvs are the smallest functional modules that confer specific antigen binding, they lack one vital property of the whole antibody. The Fvs are monovalent as compared to a bivalent IgG or even a decavalent IgM. As a result, the binding of multivalent antibodies is higher than that of corresponding Fvs. Sometimes the bivalent interaction between the antigen and the antibody contributes to the internalization of the antigen-antibody complex.

Protein engineering has been used to design different types of recombinant antibodies such as monospecific or bispecific bivalent Fvs (Holliger & Winter, 1993; Holliger *et al.*, 1993; Pluckthun, 1992). In a bispecific scFv, two scFvs from two antibodies are linked together by a flexible polypeptide linker (Mallender *et al.*, 1994). These molecules are capable of binding to two adjacent epitopes on the same antigen (Neri *et al.*, 1995) or cross-linking antigens on two different cells (Kurucz *et al.*, 1995). In the case of diabodies, instead of a single-chain polypeptide of four domains, the scFvs from each antibody form a dimer (Holliger *et al.*, 1993). In this case, the polypeptide linkers between the Fvs are too short to allow pairing between domains on the same chain and as a result facilitates mispairing between complementary domains of another chain of a similar or different antibody. Diabodies are rigid and less flexible than bispecific scFv molecules because of their short linker.

The erbB2 proto-oncogene belongs to the epidermal growth factor receptor family and has been implicated in malignant transformation. Amplification and overexpression of the erbB2 gene has been shown to occur in many human cancers including lung, breast, ovary, and stomach (King *et al.*, 1985; Slamon *et al.*, 1989; Yokota *et al.*, 1986). The overexpression of erbB2 has been directly linked to the malignant conversion of cancer cells and thus makes it an excellent target for immunotoxin therapy because cancer cells are unlikely to be able to escape treatment by loss of the antigen (Di Fiore *et al.*, 1989; Hudziak *et al.*, 1987).

Here, we describe a method to generate stable bivalent Fv fragments in which the  $V_H$  and  $V_L$  domains of the anti-erbB2 antibody e23 are covalently linked to each other by a disulfide bond and two Fvs are associated by a 15 amino acid residue linker. Using this approach the bivalent Fv can be connected to a single toxin domain to make an immunotoxin. We have used the Fv fragments of the anti-erbB2 monoclonal antibody e23 to generate bivalent dsFv immunotoxins with increased affinity and activity.

## Results

The goal of the present study is to make a recombinant bivalent Fv molecule using the anti-erbB2 antibody e23. Previous studies have demon-

strated that the bivalent e23 antibody binds to its antigen with a higher affinity than the monovalent e23 Fab or e23 Fv (Reiter *et al.*, 1994a). Since our laboratory is interested in developing immunotoxins using cancer-specific antibodies, we believed that better binding of the antibody to the antigen would translate into better activity of the immunotoxin.

## Design and construction of plasmids for expression of bivalent e23 dsFv

Because disulfide-stabilized Fvs are much more stable than single-chain Fvs, we used the design for the  $(dsFv)_2$  that is illustrated in Figure 1, in which the heavy and the light chains are connected to each other by a disulfide bond and the heavy chains of the two dsFv domains are connected by a 15 amino acid residue linker. A structural model of such a  $(dsFv)_2$  derived from e23, a murine antibody that recognizes the erbB2 antigen, is also shown in Figure 1. The linear composition of the composite gene that encodes the  $(dsFv)_2$  protein is shown in Figure 2. We fused one Cys44  $V_H$  domain of e23 Fv using a  $(Gly_4-Ser)_3$  linker peptide into a plasmid expressing another Cys44  $V_H$  domain. To produce an immunotoxin, the  $V_H$  linker  $V_H$  molecule was fused to PE38, a truncated form of PE. This fusion also resulted in a protein that was easy to produce and purify and could be used for analyses of specific binding and cytotoxic activity.

To generate plasmids for expression of bivalent e23(dsFv), a gene that codes for two heavy chain variable domains of the e23 antibody connected by a 15 amino acid residue linker was constructed by PCR using the Cys44 mutated  $V_H$  domain of the e23 antibody gene (pYR39-1) as the PCR template. The resulting PCR product was then introduced into a plasmid containing the Cys44 mutated  $V_H$  domain of e23 antibody gene (pYR39-1). The gene in pTKB22.19 was fused in-frame to the gene encoding PE38 for production of a recombinant immunotoxin in *Escherichia coli* (Brinkmann & Pastan, 1995). The  $V_L$  domain of e23 antibody containing a Cys99 mutation was constructed as described (Reiter *et al.*, 1994a) and was expressed separately in *E. coli* as a recombinant protein.

## Expression in inclusion bodies, refolding and purification of a recombinant e23 (dsFv) $_2$ immunotoxin

*E. coli* BL21 ( $\lambda$ DE3) cells containing the plasmids pTKB22.19 and pYR40 for expression of the components of e23 (dsFv) $_2$  immunotoxin were grown and induced with IPTG separately. The fusion proteins accumulated in insoluble intracellular inclusion bodies (IBs). These IBs contain almost pure recombinant protein, but in an insoluble and aggregated conformation. To generate protein with a native conformation, we solubilized and reduced the IBs in GuCl and DTE, and refolded the protein

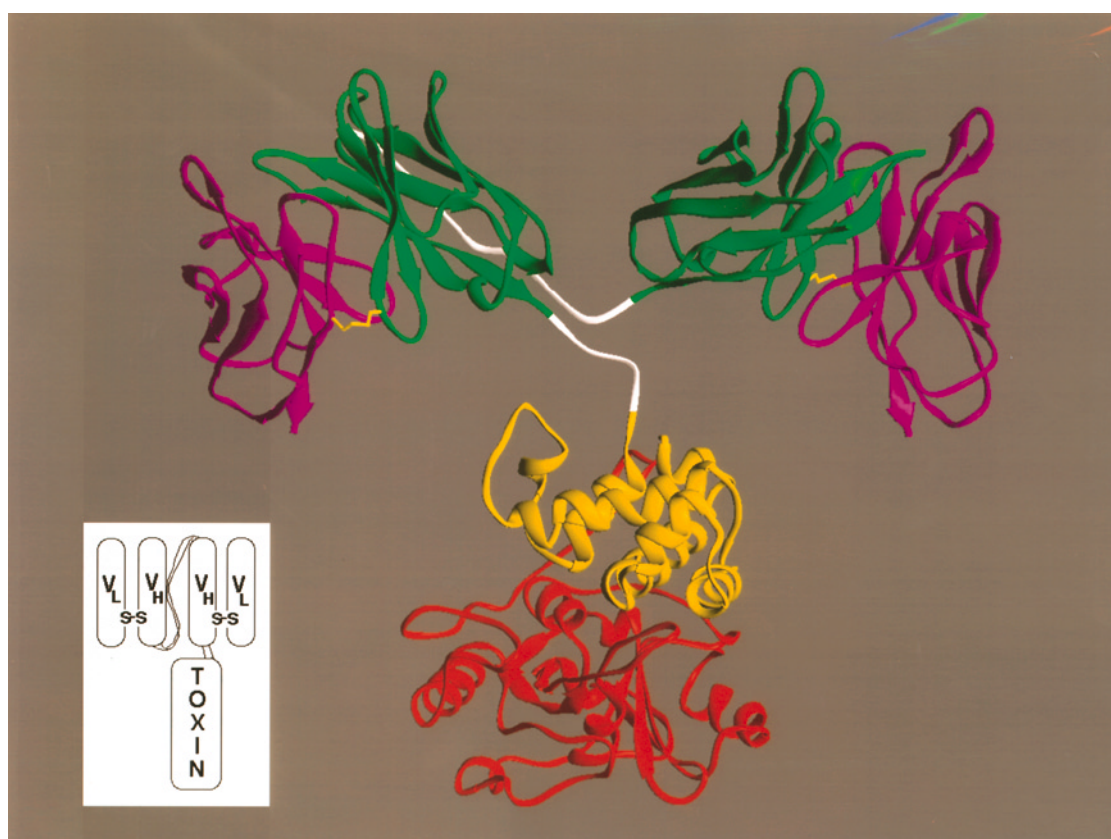


**Figure 1.** Design and structural model of  $(dsFv)_2$ . The structure was generated by “homology modeling” from known Fv sequences into building blocks of known antibody crystal structures and from the crystal structure of PE (Vasmatazis *et al.*, 1995; Carson, 1987; Freund *et al.*, 1994).  $V_H$  is shown in green,  $V_L$  in magenta and the interchain disulfide bond that stabilizes the Fv heterodimers is indicated in yellow. The translocation domain of PE is yellow and the ADP ribosylation domain is red. The flexible peptide linkers that connect the two  $V_H$  domains to each other and to the N terminus of the toxin moiety are shown in white. The (Ser-Gly-Gly-Pro-Glu-Gly-Gly) connector after the C terminus of the second  $V_H$  keeps the toxin flexible and at an adequate distance from the binding moiety, in order to prevent interference with antigen binding. Inset: Schematic of a bivalent disulfide-stabilized Fv immunotoxin. Positions of cysteine replacement in the framework region of e23 (Fv) are Asn44 to Cys in  $V_H$  and Gly99 to Cys in  $V_L$ . The linker which connects the two  $V_H$  is a 15 amino acid residue  $(Gly_4-Ser)_3$  polypeptide.

by dilution in a buffer containing arginine as described in Materials and Methods (Brinkmann & Pastan, 1995; Buchner *et al.*, 1992). Refolded, soluble monomeric protein was then purified from other bacterial and improperly folded proteins by ion exchange (Q-sepharose, Mono Q) and size exclusion chromatography to near homogeneity. Figure 3(a) shows the highly purified recombinant  $(dsFv)_2$  immunotoxin recovered after the three-column purification steps. A comparison of the purification profile of the  $(dsFv)_2$  immunotoxin with the dsFv immunotoxin shows that both proteins behave in a very similar manner. The  $(dsFv)_2$  immunotoxin appears as characteristic chromatography peaks in approximately the same position as the dsFv immunotoxin (data not shown). Although the amount of recombinant protein in induced cells and in inclusion bodies was the same as with the dsFv components, there was a lower yield of properly folded active  $(dsFv)_2$  immunotoxin compared to the dsFv immunotoxin. Using a standard refolding protocol (for antibodies and immunotoxins) about 1% of the input protein was obtained as the

monomeric  $(dsFv)_2$  immunotoxin (1 mg of purified immunotoxin from 100 mg of inclusion bodies), compared to a yield of 4 to 6% with the e23 dsFv immunotoxin. We observed that this low yield correlates with the formation of increased amounts of aggregates during refolding of the  $(dsFv)_2$  compared to the dsFv. The reason for the lower yield of  $(dsFv)_2$  might be because of its higher tendency for aggregation during refolding and thus may be related to the same phenomenon reported by Bjork & Tanford (1971) for H-chain refolding. The refolding conditions used were optimized for making the dsFv immunotoxin molecules and are probably not the optimal conditions for refolding of the  $(dsFv)_2$  immunotoxin.

#### Improved binding of the bivalent e23 dsFv

The binding affinities of e23 dsFv and  $(dsFv)_2$  immunotoxin were determined by surface plasmon resonance (BiaCore) analyses and by a competition binding assay on an erbB2-expressing cell line. For BiaCore assay, the recombinant dsFv or  $(dsFv)_2$

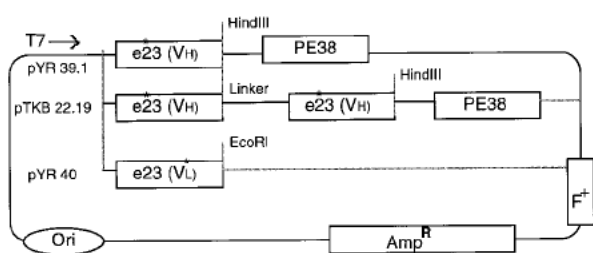


**Figure 2.** Plasmids for expression of e23 (dsFv)<sub>2</sub> immunotoxin. The parental plasmid pYR 39.1 encodes the e23 V<sub>H</sub>-PE38 fusion protein in which Asn44 of the e23 V<sub>H</sub> is mutated to Cys in the framework region. The expression plasmid pTKB22.19 encodes two e23 V<sub>H</sub> domains which are fused in-frame to the PE38 toxin. Both V<sub>H</sub> domains have a Asn44 to Cys mutation and are held together by a 15 amino acid residue (Gly<sub>4</sub>-Ser)<sub>3</sub> linker. The expression plasmid pYR40 encodes for the e23 V<sub>L</sub> domain which has a Gly99 to Cys mutation.

toxin fusions were passed over erbB2-coated sensor chips. A determination of association and dissociation rates reveals a  $k_{\text{ass}}$  value of  $4.77(\pm 0.55) \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$  for the dsFv and  $1.61(\pm 0.10) \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$  for the (dsFv)<sub>2</sub>. The dissociation rates of the dsFv is about four-fold higher than the (dsFv)<sub>2</sub> toxin with a  $k_{\text{diss}}$  values of  $1.28(\pm 0.02) \times 10^{-3} \text{ s}^{-1}$  for the dsFv and  $2.84(\pm 0.13) \times 10^{-4} \text{ s}^{-1}$  for the (dsFv)<sub>2</sub>. The  $K_D$  value at binding equilibrium, calculated as  $K_D = k_a/k_d$  is 27 nM for the dsFv and 1.8 nM for the (dsFv)<sub>2</sub> (Table 1). These data indicate that the bivalent dsFv of e23 antibody possesses about 10 to 15-fold higher affinity than its monovalent counterpart and about similar affinity as e23 monoclonal IgG.

For competition binding assays, the binding affinities of e23 dsFv and of the newly constructed bivalent dsFv were measured on erbB2 expressing A431 cells. Various amounts of recombinant dsFv or (dsFv)<sub>2</sub> toxin fusion protein were used to compete for binding of radiolabeled bivalent e23 IgG to erbB2-expressing A431 cells. Table 1 shows that bivalent dsFv competes 3.5-fold better for binding than the monovalent dsFv on A431 cells. The concentration of competitor which caused 50% inhibition of the binding of [<sup>125</sup>I]e23 IgG was 35 nM for (dsFv)<sub>2</sub>-PE38 and 100 nM for dsFv-PE38 immunotoxin. Thus, the bivalency improves the binding of the recombinant e23 (dsFv)<sub>2</sub> molecules.

### Improved cytotoxicity of bivalent e23 dsFv immunotoxins

PE38 is a truncated but enzymatically active form of *Pseudomonas* exotoxin (Brinkmann & Pastan, 1994; Pastan *et al.*, 1995). Fusion proteins of antibody fragments with PE38 are cytotoxic to cells that bind and internalize the fusion protein but are not cytotoxic to antigen-negative cells. Thus, cytotoxicity reflects specific antigen binding. Because the degree of cytotoxicity to a given antigen (with

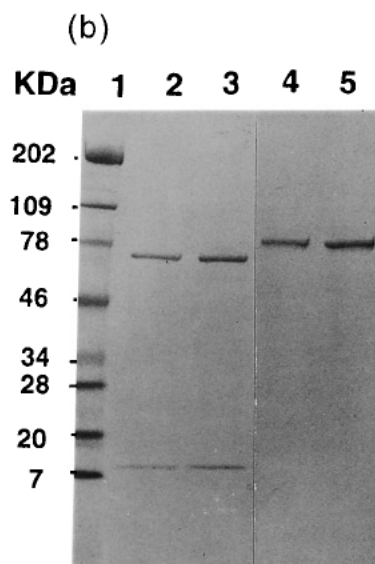
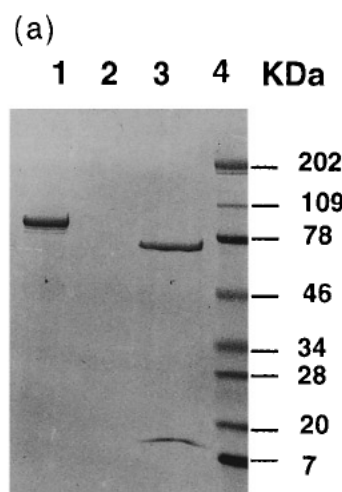


**Figure 3.** Gel analysis of recombinant e23 (dsFv)<sub>2</sub> immunotoxin. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of purified e23 (dsFv)<sub>2</sub>-PE38 immunotoxins. (a) Lane 1, sample in non-reducing loading buffer; lane 2, blank; lane 3, sample in reducing loading buffer; lane 4, prestain protein molecular mass standard. (b) Lane 1, prestain protein molecular mass standard; lanes 2 and 4, five amino acid linker-containing e23 (dsFv)<sub>2</sub> immunotoxin in reducing and non-reducing loading buffer, respectively; lanes 3 and 5, ten amino acid linker containing e23 (dsFv)<sub>2</sub> immunotoxin in reducing and non-reducing loading buffer, respectively.

defined antigen density) depends primarily on binding affinity, cytotoxicity assays of Fv-immunotoxin fusion proteins can serve as an accurate test system for specificity as well as for the relative affinity of engineered antibody fragments (Brinkmann & Pastan, 1995). To test whether the (dsFv)<sub>2</sub> of e23 retains not only the affinity but also specificity, we analyzed and compared the activity of e23 dsFv and (dsFv)<sub>2</sub> immunotoxins towards different antigen-positive and antigen-negative cell lines. The

**Table 1.** Affinity of various forms of the anti-erbB2 antibody e23

| Molecules                        | Antigen           | Assay           | $K_D$ (M)            | Reference                    |
|----------------------------------|-------------------|-----------------|----------------------|------------------------------|
| <i>A. Tracer, monovalent Fab</i> |                   |                 |                      |                              |
| MAb                              | N87 cells         | Fab/competition | $3 \times 10^{-9}$   | Reiter <i>et al.</i> (1994a) |
| Fab                              | N87 cells         | Fab/competition | $8 \times 10^{-9}$   | Reiter <i>et al.</i> (1994a) |
| scFv                             | N87 cells         | Fab/competition | $4 \times 10^{-8}$   | Reiter <i>et al.</i> (1994a) |
| dsFv                             | N87 cells         | Fab/competition | $1 \times 10^{-8}$   | Reiter <i>et al.</i> (1994a) |
| <i>B. Tracer, bivalent MAb</i>   |                   |                 |                      |                              |
| dsFv                             | A431 cells        | MAb/competition | $10 \times 10^{-8}$  | This work                    |
| (dsFv) <sub>2</sub>              | A431 cells        | Mab/competition | $3.5 \times 10^{-8}$ | This work                    |
| dsFv                             | Recombinant erbB2 | BiaCore         | $2.7 \times 10^{-8}$ | This work                    |
| (dsFv) <sub>2</sub>              | Recombinant erbB2 | BiaCore         | $1.8 \times 10^{-9}$ | This work                    |

The affinity of dsFv and (dsFv)<sub>2</sub> immunotoxins were determined by competition binding analysis to cells expressing e23 antigen or by BiaCore assay. The antigen positive cells used were N87 or A431. The relative binding affinity was calculated from the concentration of competitor which caused 50% inhibition of the binding of [<sup>125</sup>I]e23 IgG to the e23 expressing cell lines. Note also that labeled tracer used in A was monovalent Fab while in B it was bivalent MAb. This explains that the amount of dsFv needed to compete in B is higher than in A. For BiaCore assay  $K_{ass}$ ,  $k_{diss}$  and  $K_D$  ( $K_D = k_{diss}/k_{ass}$ ; binding at equilibrium) were calculated from the sensorgrams using the BIAevaluation software package.

antigen (erbB2)-positive cell lines tested were A431 and MCF7 cells. Antigen negative controls were HUT102 (human) and SP2/Tac (mouse) cells. Table 2 and Figure 4 show that the e23 (dsFv)<sub>2</sub> immunotoxin is cytotoxic to antigen-positive cells but not to antigen-negative cells. In addition, it is about 10 to 15-fold more cytotoxic than its dsFv counterpart. The IC<sub>50</sub> values of the dsFv immunotoxin to the antigen-positive A431, and MCF7 cells are 2.4 ng/ml and 3.0 ng/ml, respectively. The IC<sub>50</sub> values of the corresponding (dsFv)<sub>2</sub> immunotoxin to A431 and MCF7 cells were 0.3 ng/ml, and 0.4 ng/ml, respectively. Neither type of e23 dsFv immunotoxin affected antigen-negative cells (HUT102 and SP2/Tac) even at 1000 ng/ml (Table 2). These results demonstrate that the e23 (dsFv)<sub>2</sub> immunotoxin has a dramatically improved cytotoxic activity to two erbB2-expressing cell lines compared to the monovalent dsFv and with the same specificity.

### Stability of e23 (dsFv)<sub>2</sub>

One important application of Fvs is the diagnosis and therapy of cancer, e.g. in the form of radio-labeled Fvs for tumor imaging or Fv-toxin fusion proteins for therapy (immunotoxins). For such applications, like other factors such as plasma half-

life, tumor penetration, it is also important that Fvs are stable at 37°C in human serum so they will retain activity for a long time after injection into patients. The dsFv immunotoxins are very stable (Reiter *et al.*, 1994a,b,c). To analyze if the (dsFv)<sub>2</sub> immunotoxin is stable, we assayed the stability of the e23 (dsFv)<sub>2</sub> immunotoxin in human serum albumin at 37°C. Immunotoxins were incubated at 37°C for different times in phosphate-buffered saline containing 0.2% (w/v) human serum albumin at a concentration of 10 µg/ml. The remaining activity of the incubated immunotoxin was determined by a protein synthesis inhibition assay. Since the toxin moiety of PE-derived immunotoxin is very stable, any instability can be attributed to the binding moiety of the immunotoxin (Brinkmann *et al.*, 1993; Reiter *et al.*, 1994b,c). We found that the (dsFv)<sub>2</sub> immunotoxin retains 94% of its activity even after 24 hours incubation at 37°C in human serum, and thus it is as stable as the corresponding monovalent dsFv (Y. Reiter *et al.*, personal communication).

### Effects of linker length between dsFvs on overall properties of the (dsFv)<sub>2</sub> molecule

To generate e23(dsFv)<sub>2</sub> molecules we initially used the conventional 15 amino acid residue

**Table 2.** Specific cytotoxicity of e23 dsFv and (dsFv)<sub>2</sub> immunotoxins

| Cell Line | e23 antigen | IC <sub>50</sub> (ng/ml) |                     |            |             |
|-----------|-------------|--------------------------|---------------------|------------|-------------|
|           |             | dsFv                     | (dsFv) <sub>2</sub> |            |             |
|           |             |                          | L3                  | L2         | L1          |
| A431      | +           | 2.4 ± 1.5                | 0.3 ± 0.1           | 0.26 ± 0.1 | 0.36 ± 0.15 |
| MCF7      | +           | 3.0 ± 1.0                | 0.4 ± 0.15          | 0.36 ± 0.1 | 0.45 ± 0.15 |
| HUT-102   |             | >1000                    | >1000               | >1000      | >1000       |
| SP2/Tac   |             | >1000                    | >1000               | >1000      | >1000       |

Cytotoxicity assays were performed by measuring incorporation of [<sup>3</sup>H]Leu into cellular proteins as described in Materials and Methods. IC<sub>50</sub> is the concentration that causes 50% inhibition of protein synthesis after 20 hours incubation with immunotoxin. L1, Gly-Gly-Gly-Gly-Ser; L2, (Gly-Gly-Gly-Gly-Ser)<sub>2</sub>; L3, (Gly-Gly-Gly-Gly-Ser)<sub>3</sub>. +, Antigen present; -, antigen absent.

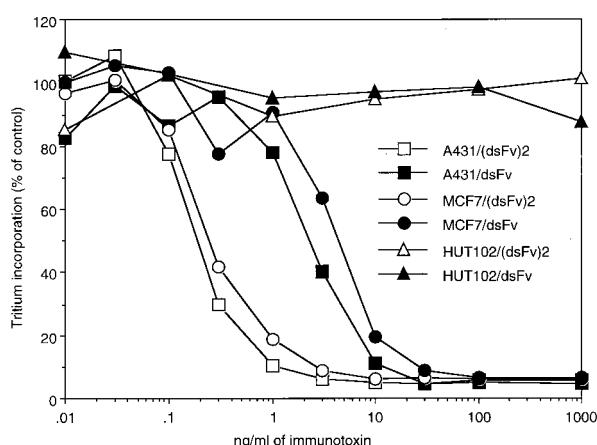


**Figure 4.** Specific cytotoxicity of e23 (dsFv)<sub>2</sub>-PE38 and dsFv-PE38 immunotoxins towards different cell lines. Squares, A431; circles, MCF7 antigen-positive cell lines; triangles, HUT102 antigen-negative cell line.

(Gly<sub>4</sub>Ser)<sub>3</sub> linker (Huston *et al.*, 1991) to connect two V<sub>H</sub> domains of the dsFvs. To determine whether the linker length between two dsFv fragments has any effect on the overall properties of the molecule, we have also made a bivalent dsFv molecule containing a (Gly<sub>4</sub>Ser)<sub>1</sub> or a (Gly<sub>4</sub>Ser)<sub>2</sub> linker. To generate plasmids for expression of e23(dsFv)<sub>2</sub> containing a five or ten amino acid residue linker, a gene that codes two heavy chain variable domain of e23 antibody connected by one of those two linkers was constructed by PCR using the Cys44 mutated V<sub>H</sub> domain of e23 antibody gene (pYR39-1) as PCR template as used for the 15 amino acid residues linker-containing (dsFv)<sub>2</sub> construct. The resulting PCR product was then introduced into the plasmid expressing the Cys44 mutated V<sub>H</sub> domain of e23 antibody gene (pYR39-1). The V<sub>L</sub> domain of e23 antibody containing a Cys99 mutation was constructed as described (Reiter *et al.*, 1994a) and expressed in *E. coli* as a recombinant protein.

Both molecules could be produced as monomers with a final yield of about 0.5%. The purity of those molecules, after refolding and subsequent purification, is shown in Figure 3B and is comparable to the bivalent immunotoxin with the 15 amino acid residue linker. To test whether these (dsFv)<sub>2</sub> molecules retained specific cytotoxicity, we compared the activity of the e23 (dsFv)<sub>2</sub> immunotoxin molecules containing a five, ten or 15 amino acid residue linker, towards the e23 antigen-positive and negative cancer cell lines. As shown in Table 2, both five and ten amino acid residue linker-containing (dsFv)<sub>2</sub> immunotoxin molecules have the same activity as the 15 amino acid residue linker-containing immunotoxin and are about five- to tenfold more cytotoxic than the monovalent immunotoxin on antigen-positive A431 and MCF7 cells. In addition, both molecules showed no killing on antigen-negative HUT102 cells.

## Discussion

We have constructed a new type of recombinant bivalent Fv fragment, in which the V<sub>H</sub>/V<sub>L</sub> heterodimer is stabilized by an intrachain disulfide bond and the two Fvs are covalently connected by a flexible peptide linker. Generally we find that disulfide-linked Fvs are extremely stable and are easy to prepare. We have used the principle of disulfide stabilization to make a bivalent Fv of an antibody that specifically targets the erbB2 oncogene product. The molecule shows the same specificity and stability of a monovalent dsFv but a higher apparent affinity.

## Comparison with other approaches to generate bivalent recombinant Fvs

Several approaches have been used previously to generate recombinant bivalent Fvs by different laboratories. Also a variety of protein designs have been investigated which includes miniantibodies, diabodies and disulfide-linked fragments (DeJonge *et al.*, 1995; Holliger *et al.*, 1996; Gruber *et al.*, 1994; Hayden *et al.*, 1994; Mallender *et al.*, 1994; Haunschild *et al.*, 1995; Cumber *et al.*, 1992). To generate bivalent and even tetravalent Fvs, a variety of self-associating secondary structures such as helix bundles or coiled-coils were investigated (Pluckthun, 1992; Pluckthun & Pack, 1997). To make the bivalent miniantibody the carboxyl-terminal end of the scFv fragment was extended by attaching the upper hinge region from mouse IgG3 and two identical helices from the antiparallel helix-turn-helix dimerization motif. In this case, the putative four-helix bundle can be formed from two scFv molecules, each contributing two helices. The final yield in this approach is approximately 200 mg of correctly folded bivalent miniantibodies per litre culture in high cell density fermentation of *E. coli* (Pack *et al.*, 1993). Tetravalent miniantibodies were made by attaching the modified leucine zipper sequences at the carboxyl end of each scFv fragment *via* a hinge region. The yield of the resulting tetravalent molecules is 0.8 mg/l per A550 unit in a shake-flask, which is lower than the bivalent miniantibodies (Pack *et al.*, 1995). The stability of these bivalent and tetravalent miniantibodies under physiological conditions has not been reported. Another approach to make bivalent scFvs with enhanced functional affinity is the *in vitro* formation of an interdomain disulfide bridge by the oxidation of additional cysteine residues introduced at the carboxyl terminus. This strategy was shown to work with either a scFv-cys (Adams *et al.*, 1993, 1995; McCartney *et al.*, 1995) or a Fab fragment (Rodrigues *et al.*, 1993) and resulted in functional bivalent molecules. In this approach since the scFvs are linked together by a disulfide linkage, it forms a stable dimer but the overall stability of the molecule under physiological conditions has not been reported. Our approach to generate a bivalent Fv is based on the fact that

dsFv is extremely stable and therefore the resulting molecule will be very stable under physiological conditions, which is supported by our experimental results.

Compared to these other methods, our approach to generate recombinant bivalent Fvs is simple and results in homogenous and properly folded bivalent Fvs with a reasonable yield.

### Applications of bivalent Fvs in cancer diagnosis and therapy

The (dsFv)<sub>2</sub> of the anti-erbB2 antibody showed better binding affinity than the monovalent dsFv, because of its bivalent binding. While the monovalent dsFv has the same affinity as the monovalent e23 Fab (derived from e23 IgG by proteolysis), the (dsFv)<sub>2</sub> has very similar antigen binding affinity as the bivalent e23 IgG. We expect that the better binding property of the (dsFv)<sub>2</sub> will translate into improved diagnostic and therapeutic applications such as tumor imaging or targeted cancer therapy. A stable bivalent dsFv without a toxin attached to it should be suitable for numerous *in vivo* applications. For example, (dsFv)<sub>2</sub> can be used for tumor imaging after linkage to a radioactive molecule, and because of its bivalency tumor imaging will be more effective than with the corresponding dsFv. The bivalent dsFv can be fused to different toxin molecules and also to prodrug activating enzymes for therapeutic applications (Jungheim & Shepherd, 1994; Bagshawe *et al.*, 1994). Finally, because the (dsFv)<sub>2</sub>-containing immunotoxin, e23 (dsFv)<sub>2</sub>-PE38, has the same cytotoxic specificity but improved cytotoxicity towards antigen-expressing cancer cells, compared to the corresponding monovalent dsFv immunotoxin this should translate into increased antitumor activity in animals. Analyses of the *in vivo* behavior and antitumor activity of the bivalent immunotoxins are underway.

## Materials and Methods

### Plasmid constructions

The plasmid pYR39-1 encodes the Cys44 V<sub>H</sub> toxin, e23 V<sub>H</sub>-PE38 (Reiter *et al.*, personal communication). e23 Fv is derived from the murine MAb anti-erbB2. PE38 is a truncated but enzymatically active form of *Pseudomonas* exotoxin (Brinkmann & Pastan, 1994; Pastan *et al.*, 1995; Hwang *et al.*, 1987). Proteins in which the antibody fragments are fused to PE38 are cytotoxic to cells that bind the antibody, but not to antigen-negative cells (Brinkmann & Pastan, 1994; Pastan *et al.*, 1995). The plasmid pTKB22.19 that encodes two Cys44 V<sub>H</sub> domains separated by a 15 amino acid residue linker, fused to PE38 was generated by PCR using the pYR39-1 plasmid as the PCR template as described in detail in Figure 3. The primer T136, 5'ATC TCT TAA GCT TCA GGC GGA GGA GGC GGA TCA GGT GGT GGC GGA TCT GGA GGT GGC GGA AGC GTG CAG CTG CAG GAG TCA GGA CCT3' was used to provide the 15 amino acid residue linker (Gly<sub>4</sub>Ser)<sub>3</sub> in between two V<sub>H</sub> and the cloning site *Hind*III (*Hind*III site underlined). The 3' primer T137,

5'GGT CCA AGC TTT GGA GAC GGT GAC CGT GGT CCC TGC GCC CCA GAC ATC3' was used to provide cloning site *Hind*III to the C-terminal half of V<sub>H</sub>. Then primers T136 and T137 were added to the 100 µl final reaction mix, and amplified for 25 cycles to obtain the Cys44 V<sub>H</sub> domain containing 15 amino acid residue linker at the N terminus. High fidelity polymerase mix (Boehringer Mannheim) was used to avoid PCR errors. The resulting fragment was cloned into the *Hind*III site of pYR39-1 (Reiter *et al.*, personal communication), which codes for e23 Cys44 V<sub>H</sub> fused to PE38, a truncated form of *Pseudomonas* exotoxin. The *Hind*III site fuses the inserted fragment in frame to the Cys44 V<sub>H</sub> fragment of e23 and the truncated toxin (Hwang *et al.*, 1987) in one of two possible orientation. The vector contains the phage T7 promoter for expression in Studier's *E. coli* BL21(λDE3) expression system (Studier & Moffatt, 1986). The plasmid pYR40 encodes the V<sub>L</sub> domain of e23 antibody and contains a Cys99 mutation (Reiter *et al.*, 1994a). All expression plasmids were confirmed to be correct by DNA sequencing on an ABI 373A sequencer using the Dyedeoxy chain terminator sequencing kit.

### Production of recombinant protein

The components of e23 (dsFv)<sub>2</sub> and dsFv immunotoxins were expressed in *E. coli* BL21(λDE3), and accumulated in inclusion bodies (IBs) as described for other recombinant immunotoxins (Brinkmann & Pastan, 1995). IBs were solubilized in GuCl, reduced with DTE and refolded by dilution in a refolding buffer containing arginine, to prevent aggregation, and oxidized and reduced glutathione to facilitate redox shuffling (Buchner *et al.*, 1992; Reiter *et al.*, 1994a). Active monomeric protein was purified from the refolding solution by ion exchange and size exclusion chromatography to near homogeneity as described (Buchner *et al.*, 1992). Protein concentrations were determined by Bradford assay (BioRad Coomassie Plus).

### Binding and cytotoxicity assays

The affinities of e23(dsFv) and (dsFv)<sub>2</sub> were assayed and compared by surface plasmon resonance (BIAcore, Pharmacia Biosensor). Recombinant erbB2 was coupled to BIAcore sensor-chips (CM5, research grade, Pharmacia Biosensor) according to the manufacturer's specifications. The dsFv and (dsFv)<sub>2</sub> immunotoxin was applied to the chips and binding and dissociation ( $k_{\text{ass}}$  and  $k_{\text{diss}}$ ) was determined from association and dissociation curves of the sensorgrams with the BIAevaluation software package (Pharmacia Biosensor).  $K_D$  at equilibrium was calculated as  $K_D = k_{\text{diss}}/k_{\text{ass}}$ .

The affinity of the monovalent and bivalent e23 dsFv toxin molecules was also determined by competition binding assay on erbB2 expressing A431 cells as described (Reiter *et al.*, 1994a). The antibody e23 IgG, radioiodinated by Bolton-Hunter reagent (New England Nuclear, Boston, MA) was used as tracer in this assay.

The specific cytotoxicity of the e23 (dsFv)<sub>2</sub> and dsFv immunotoxins was assessed by protein synthesis inhibition assays (inhibition of incorporation of tritium-labeled leucine into cellular protein) in 96 well plates as described (Brinkmann *et al.*, 1991). The activity of the molecule is defined by the IC<sub>50</sub>, the toxin concentration that reduces incorporation of radioactivity by 50% compared to cells that were not treated with toxin. The specificity is obtained by comparing the activity towards

antigen-positive cells *versus* toxicity against antigen-negative cells.

### Stability assays

The stability of the e23 (dsFv)<sub>2</sub> and dsFv immunotoxins were determined by incubating them at 10 µg/ml human serum albumin at 37°C (Reiter *et al.*, 1994c). Active immunotoxin remaining after incubation was determined by cytotoxicity assays on A431 cells.

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