THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 269, No. 28, Issue of July 15, pp. 18327–18331, 1994
*Printed in U.S.A.*

# Improved Binding and Antitumor Activity of a Recombinant Anti-erbB2 Immunotoxin by Disulfide Stabilization of the Fv Fragment*

(Received for publication, January 11, 1994, and in revised form, April 11, 1994)

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**e23(dsFv)-PE38KDEL is a recombinant immunotoxin composed of the Fv region of anti-erbB2 monoclonal antibody e23 connected to a truncated form of *Pseudomonas* exotoxin (PE38KDEL), in which the inherently unstable Fv heterodimer (composed of $V_H$ and $V_L$) is stabilized by a disulfide bond engineered between structurally conserved framework positions of $V_H$ and $V_L$.**

**We have now found that e23(dsFv)-PE38KDEL is considerably more cytotoxic to antigen-positive cell lines than the corresponding single-chain immunotoxin. The basis for the enhanced cytotoxic activity is that the e23 dsFv-immunotoxin binds to erbB2 with greater affinity than the single-chain counterpart. The dsFv-immunotoxin had 4-fold increased binding compared to the scFv and almost identical to the binding affinity of e23 Fab. e23(dsFv)-PE38KDEL was also considerably more stable at 37 °C than the single-chain immunotoxin.**

**The therapeutic potential of the disulfide-stabilized immunotoxin was compared with its single-chain counterpart using two animal models of immunodeficient mice bearing subcutaneous tumor xenografts of human gastric tumor N87 cells or human A431 epidermoid carcinoma cells.**

**The antitumor effect of e23(dsFv)-PE38KDEL was significantly better than that of the single-chain immunotoxin. e23(dsFv)-PE38KDEL caused complete regression of tumors at doses which caused no toxic effects in mice, whereas the single-chain immunotoxin did not cause complete regressions at the same doses.**

Fv fragments of antibodies are heterodimers of the heavy chain variable domain ($V_H$)[1] and the light chain variable domain ($V_L$). They are the smallest functional modules of antibodies required for high affinity antigen binding. The polypeptide chains of whole IgG or Fab fragments are joined by a disulfide bond. Fv fragments have no such interchain disulfide bridge and are therefore unstable (1–9). Stable Fv fragments can be produced by making recombinant molecules in which the $V_H$ and $V_L$ domains are connected by a peptide linker so that the antigen binding site is regenerated in a single protein (2, 3). Until recently, this was the only available method to generally stabilize Fv fragments. In many cases such single-chain Fv fragments (scFv) retain the specificity and affinity of the antibody. Single-chain Fv fragments have been successfully used for tumor imaging and as the basis for construction of multifunctional fusion proteins (4–8). Fv domains have been fused to toxins to make immunotoxins in which the Fv domain serves as a cell-targeting moiety for a potent toxin (5–8). Recombinant immunotoxins selectively bind to and kill cells that are recognized by the antigen binding domain, *i.e.* the Fv moiety. Several potent single-chain immunotoxins have been made that are specifically cytotoxic to antigen bearing tumor cells and cause complete or partial regression of human tumor xenografts in nude mice (7, 8). One example is e23(Fv)-PE38KDEL directed against the erbB2 proto-oncogene product (7). In these immunotoxins, the scFv is connected to a truncated form of *Pseudomonas* exotoxin (PE38KDEL) which contains the PE protein domains responsible for intracellular translocation, ADP-ribosylation activity, and a mutant carboxyl-terminal sequence, KDEL (10). These proteins lack the PE protein domain responsible for cell binding. Some scFv fragments, such as e23(Fv), have a lower affinity for antigen than the Fab counterpart. This lower affinity could result from the peptide linker somehow interfering with antigen binding or the linker may be unable to stabilize the Fv sufficiently.

Recently, we identified an alternative method of stabilizing the Fv moiety in recombinant immunotoxins. In this approach, the Fv fragment is stabilized by a disulfide bond that is engineered between the framework regions of the two Fv domains and the toxin is fused to either of the Fv domains (11, 13). The appropriate disulfide positions were identified by molecular modeling techniques (12). These positions are in the conserved framework regions, distant from CDRs, and therefore are generally applicable to many Fv fragments without affecting antigen binding. Several disulfide-stabilized Fv (dsFv) immunotoxins have been made and tested, and they have about the same cytotoxicity as their single-chain counterparts and also similar antitumor activities (14, 15). However, the initial *in vitro* characterization of e23(dsFv)-PE38KDEL showed that it is more active than its single-chain counterpart, but the basis of this increased activity was not examined. We report here that the dsFv-immunotoxin has significantly improved binding and antitumor activity compared with its single-chain counterpart. The dsFv-immunotoxin is also more stable *in vitro*. Our results suggest that in some cases disulfide stabilization of Fv fragments can improve binding and activity of Fv fragments.

## MATERIALS AND METHODS

*Construction of Plasmids for Expression of the dsFv-immunotoxin and Production of Recombinant Proteins: e23(dsFv)-PE38KDEL*—The introduction of cysteines by site-directed mutagenesis in between e23

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‡ Supported by a grant from the Rothschild Foundation.

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[1] The abbreviations used are: V, variable; H, heavy; L, light; scFv and dsFv, single-chain and disulfide-stabilized Fv, respectively; CDR, complementary-determining region; PE, *Pseudomonas* exotoxin; MES, 4-morpholineethanesulfonic acid; PBS, phosphate-buffered saline.

$V_H$ and $V_L$ for stabilization of e23(Fv) and the amino acid sequence of e23(Fv) has been described previously (13). In the dsFv $Asn^{44}$ of e23($V_H$) and $Gly^{99}$ of e23($V_L$) are changed to cysteines. The plasmids for the components of e23(dsFv)-PE38KDEL were made by subcloning as described (13).

Recombinant proteins were produced from inclusion bodies as described previously (11, 13, 16). Properly folded disulfide-stabilized and single-chain immunotoxins were purified as described previously (8, 11).

*Cytotoxicity Assays*—Specific cytotoxic activity of immunotoxins was determined by inhibition of protein synthesis as described (8).

*Binding Assays*—In binding assays $^{125}$I-labeled e23 Fab was added to $10^5$ N87 cells as a tracer with various concentrations of the competitor. The binding assays were performed at 4 °C for 2 h in RPMI containing 1% bovine serum albumin and 50 mM MES (Sigma) as described (7).

*Stability Assays*—The stability of e23(dsFv)-PE38KDEL and e23(Fv)-PE38KDEL immunotoxins was determined by incubating them at 10 μg/ml at 37 °C in PBS. Active immunotoxin remaining after 5 h of incubation was determined by cytotoxicity assays on N87 cells.

*Antitumor Activity of dsFv-immunotoxin in Nude Mice*—Antitumor activity of e23(dsFv)-PE38KDEL was determined in nude mice bearing human gastric (N87) or epidermoid carcinoma (A431) tumors. N87 tumor cells ($5 \times 10^6$) were injected subcutaneously on day 0 into immunodeficient "nude" mice. Tumors about 5 × 5 mm in size developed in all animals by day 7. Starting on day 7 after tumor implantation, animals were treated with intravenous injections of e23(dsFv)-PE38KDEL or e23(Fv)-PE38KDEL. Therapy was given every other day. For A431 tumors cells ($3 \times 10^6$) were injected and tumors (5 × 5 mm) were developed by day 4. Starting on day 4 animals were treated with immunotoxins. Each treatment group consisted of five animals. Tumors were measured with a caliper every 2 days and the volume of the tumor was calculated by using the formula: tumor volume (in $mm^3$) = length × $(width)^2 \times 0.4$.

## RESULTS

*Improved Cytotoxic Activity of the Disulfide-stabilized Immunotoxin*—The goal of this study is to characterize the binding and *in vivo* activity of a recombinant anti-erbB2 immunotoxin, e23(dsFv)-PE38KDEL, in which the Fv fragment is stabilized by a designed disulfide bond (Ref. 13; Fig. 1*A*). The e23(Fv)-immunotoxins are PE-derived immunotoxin, highly cytotoxic agents whose specificity is mediated by specific binding of the Fv component to target cells and toxicity by PE38KDEL which is the translocation and ADP-ribosylation domains of PE. Therefore, the specific Fv-mediated cytotoxicity of dsFv and scFv could be used to compare directly the binding of these Fv derivatives. Analysis of the cytotoxic activity of the e23dsFv and scFv immunotoxins shows that they bind to and kill the same spectrum of antigen-positive cells, but do not bind and kill antigen-negative cells. We found the dsFv-immunotoxin to be 3–10-fold more active than the scFv-immunotoxin, depending on the cell line used using immunotoxin preparations with the same purity (13). Repeated determinations of the activity of the dsFv-immunotoxin on N87 and A431 cells indicate a statistically different activity ($p < 0.0001$) (Table I).

*Improved Binding of the dsFv-immunotoxin*—To determine whether the improved cytotoxic activity of the dsFv-immunotoxin is due to improved binding, a competitive binding analysis was performed using $^{125}$I-labeled e23 Fab as a tracer and increasing concentrations of the e23dsFv- or scFv-immunotoxin. Fig. 2 shows that e23(dsFv)-PE38KDEL binds to N87 cells with 4-fold greater affinity compared with the single-chain immunotoxin. The single-chain immunotoxin binds with a 4-fold lower affinity than the Fab (40 nM for e23(Fv)-PE38KDEL compared with 8 nM for e23 Fab) (Fig. 2). In contrast, the binding of the dsFv-immunotoxin, e23(dsFv)-PE38KDEL, is almost identical to that of e23 Fab (10 nM). These results indicate that disulfide stabilization of e23 Fv significantly improves its binding and suggest that the peptide linker in the scFv somehow interferes with binding of the single-chain immunotoxin or the linker does not sufficiently stabilize the Fv heterodimer structure.

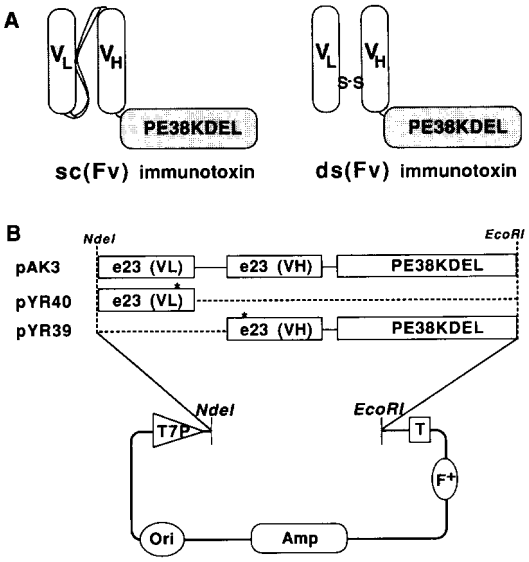


FIG. 1. **Disulfide connection between $V_H$ and $V_L$ of recombinant immunotoxin and plasmids for expression of dsFv-immunotoxin.** *A*, design of a disulfide-stabilized Fv-immunotoxin. Positions of cysteine replacement in framework region of e23(Fv) are $Asn^{44} \rightarrow$ Cys in $V_H$ and $Gly^{99} \rightarrow$ Cys in $V_L$ as described previously (13). *B*, pAK3 codes for the scFv immunotoxin e23(Fv)-PE38KDEL and is the parent plasmid for the generation of plasmids encoding the components of the disulfide-stabilized immunotoxin (7). In this molecule, the $V_H$ and $V_L$ domains of monoclonal antibody e23 are held together by a peptide linker ($Gly^4 \rightarrow Ser$)$^3$ and then fused to the PE38KDEL gene encoding the translocation and ADP-ribosylation domains of PE. pYR39 and pYR40 encoding e23($V_H$ $Cys^{44}$)-PE38KDEL and e23($V_L$ $Cys^{99}$) are the expression plasmids for the components of the dsFv-immunotoxin e23(dsFv)-PE38KDEL and are derived from pAK3 by site-directed mutagenesis and subcloning as described (13).

TABLE I

*Activity of e23(Fv)-PE38KDEL immunotoxins on various cell lines*

Cytotoxicity assays were performed by measuring incorporation of [$^3$H]leucine into cell proteins as described (8). Data are given as $ID_{50}$ values, the concentration of immunotoxin that causes a 50% inhibition of protein synthesis after a 24-h incubation with immunotoxin. Immunotoxins tested were: single-chain e23(Fv)-PE38KDEL (scFv) and disulfide-stabilized e23(dsFv)-PE38KDEL (dsFv). ND, not determined.

| Cell line | Type | Cytotoxicity | | | |
|---|---|---|---|---|---|
| | | $ID_{50}$ | | -Fold increase | Statistical significance |
| | | scFv | dsFv | | |
| | | *ng/ml* | | | |
| HTB20 | Breast | 0.4 | 0.07 | 5.7 | $p < 0.0001$ |
| N87 | Gastric | 0.3 | 0.06 | 5.0 | $p < 0.0001$ |
| HEPG-2 | Hepatic | 1.2 | 0.3 | 4.0 | ND |
| A431 | Epidermoid | 10 | 1.0 | 10.0 | $p < 0.0001$ |
| KB3-1 | Epidermoid | >1000 | >1000 | | ND |

*Improved Stability of the dsFv-immunotoxin*—We have shown previously for other dsFv-immunotoxins that disulfide stabilization of the Fv improves its stability in human serum and in buffers (11, 13). Fig. 3 shows a stability analysis of e23(Fv)-immunotoxins in which their binding and cytotoxic activity were tested on N87 cells after incubation in PBS for 5 h at 37 °C. It is evident that the single-chain immunotoxin is unstable at 37 °C, and almost all its cytotoxic activity is lost after 5 h in PBS. The loss of cytotoxicity is paralleled by a loss of binding activity (Fig. 3). In marked contrast, the dsFv-immunotoxin, e23(dsFv)-PE38KDEL, is stable. Its cytotoxic activity is reduced only slightly and its binding to N87 cells is almost unchanged after 5 h incubation in PBS.

*Improved Antitumor Activity of e23(dsFv)-PE38KDEL*—To determine whether the improved binding and cytotoxic activity *in vitro* is accompanied by an increase in *in vivo* activity, we

assayed the antitumor activity of the dsFv- and the scFv-immunotoxin against two different models of tumor xenografts in nude mice. The first model is one in which N87 human gastric tumors are grown in nude mice. To obtain tumors, N87 cells ($5 \times 10^6$) were injected subcutaneously into 4–6-week-old 20-g mice (day 0). Treatment was started on day 7 after tumor implantation when tumors measured about 50–70 $mm^3$. Animals were treated intravenously on days 7, 9, and 11. Shown in Fig. 4 is the antitumor activity of the disulfide-stabilized immunotoxin (e23(dsFv)-PE38KDEL) and its single-chain counterpart (e23(Fv)-PE38KDEL). Compared with untreated mice, the treated mice showed significant dose-related tumor regressions. The antitumor activity of the dsFv-immunotoxin was significantly better than that of the scFv-immunotoxin when equivalent doses of immunotoxin were compared. The mean tumor volume in mice treated with the dsFv-immunotoxin was 2–3-fold smaller than tumor size of the mice treated with the scFv-immunotoxin, depending on the dose used (Table II). In this model, the tumors do not usually completely regress, but still it is clear that the dsFv-immunotoxin is more active.

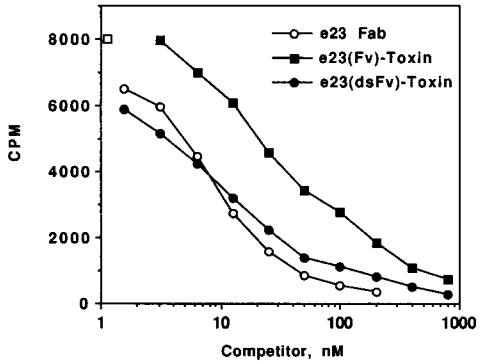


FIG. 2. **Binding of e23(Fv)-PE38KDEL and e23(dsFv)-PE38KDEL to N87 cells.** Competitive binding analysis of the ability of purified e23(Fv)-PE38KDEL and e23(dsFv)-PE38KDEL to inhibit the binding of $^{125}$I-labeled e23 Fab to cells overexpressing erbB2. The concentration of competitor which caused 50% inhibition of the binding of $^{125}$I-e23Fab was 3 nM for e23 IgG, 8 nM for e23 Fab, 40 nM for e23 single-chain Fv-immunotoxin, and 10 nM for disulfide-stabilized e23Fv-immunotoxin.

The other tumor model that we used was human epidermoid cancer cell line A431, which rapidly forms large tumors in nude mice (18) and toward which the dsFv-immunotoxin is 10-fold more active *in vitro* than the scFv-immunotoxin ($ID_{50}$ of 1 ng/ml for dsFv-immunotoxin and 10 ng/ml for scFv-immunotoxin, Table I). Accordingly, $3 \times 10^6$ A431 cells were injected subcutaneously in nude mice on day 0. Animals were treated intravenously on days 4 (tumor size 50–70 $mm^3$), 6, and 8. As shown in Fig. 5, both immunotoxins have significant dose-related antitumor activity. However, whereas the scFv-immunotoxin, e23(Fv)-PE38KDEL, caused regression of A431 tumors at 1.5- or 2.5-μg dose levels, the dsFv-immunotoxin at the same dose levels caused complete remission of the tumors after treatment with both dose levels of e23(dsFv)-PE38KDEL. Furthermore, the tumors treated with the scFv-immunotoxin regrew after treatment while cures lasting at least 2 months were observed in 9 out of 10 animals treated with the dsFv-immunotoxin. These results indicate that the dsFv-immunotoxin has significantly better antitumor activity compared with the single-chain immunotoxin. Thus, the improved binding and cytotoxic activity *in vitro* translates into improved *in vivo* antitumor activity.

## DISCUSSION

In this study, we have shown that a recombinant immunotoxin composed of a disulfide-stabilized Fv fragment of monoclonal antibody e23 and a truncated form of *Pseudomonas* exotoxin is a very potent antitumor agent with significant improved characteristics *in vitro* and *in vivo* compared with its single-chain linker-stabilized counterpart. This study demonstrates for the first time that disulfide stabilization of an Fv can improve its activity compared with the linker-stabilized single-chain Fv. The disulfide stabilization of e23(Fv) in this recombinant immunotoxin improves its binding, cytotoxic activity *in vitro*, stability *in vitro*, and most important its antitumor activity. In contrast to the corresponding single-chain immunotoxin, the disulfide-stabilized immunotoxin can cause complete regression of human tumor xenografts in nude mice.

*Improved Binding of e23(dsFv)*—The disulfide-stabilized e23(Fv)-immunotoxin, e23(dsFv)-PE38KDEL, is directed to the proto-oncogene product erbB2. In this immunotoxin the Fv

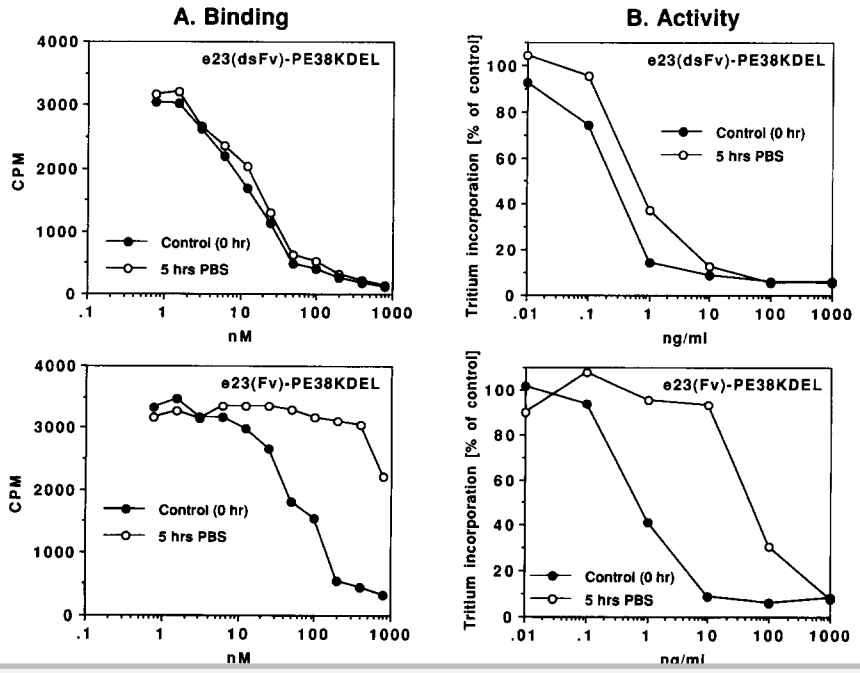


FIG. 3. **Stability of e23(dsFv) and e23(Fv)-immunotoxins in buffered saline.** The stability of e23(dsFv)-PE38KDEL and e23(Fv)-PE38KDEL was determined by incubating them for 5 h at 37 °C in PBS. Active immunotoxin remaining after incubation was determined by cytotoxicity assays on N87 cells. The binding of immunotoxins after incubation in PBS was determined by competitive binding analysis as described in the legend to Fig. 2.

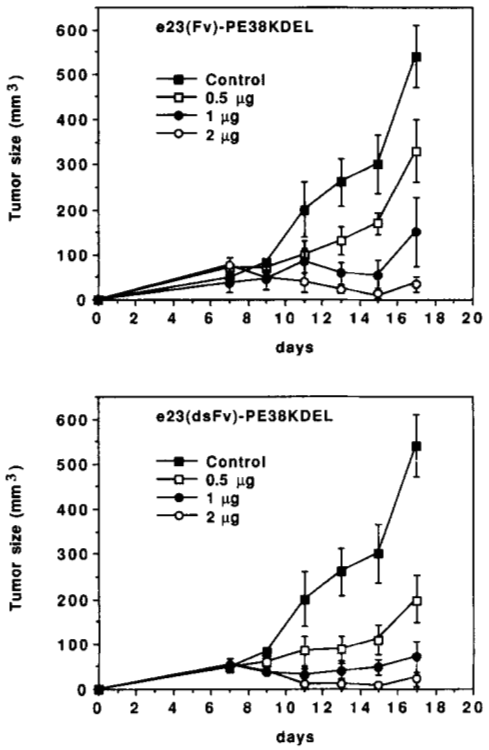


FIG. 4. **Effect of e23(dsFv)-PE38KDEL and e23(Fv)-PE38KDEL on the growth of N87 tumors in nude mice.** Groups of five animals were injected with $5 \times 10^6$ N87 cells and treated intravenously on days 7, 9, and 11. Mice were given 0.5 μg (□), 1 μg (●), or 2 μg (○) of e23(dsFv)-PE38KDEL or e23(Fv)-PE38KDEL. Control groups received PBS, 0.2% human serum albumin (■). *Error bars* indicate S.E.

TABLE II
*Effect of e23(Fv)-PE38KDEL immunotoxins on the regression of N87 tumor xenografts in nude mice*

Tumor size (mean ± S.E.) was measured on day 14–15 after tumor implantation in groups of five mice. The mice were treated with three doses given every other day of e23(Fv)-PE38KDEL (scFv) or e23(dsFv)-PE38KDEL (dsFv).

| | e23(Fv)-PE38KDEL immunotoxin | Mean tumor size after treatment with indicated doses | | | |
|---|---|---|---|---|---|
| | | 0 μg | 0.5 μg | 1 μg | 2 μg |
| | | $mm^3$ | | | |
| Exp. 1 | scFv | 480 ± 75 | 180 ± 38 | 120 ± 34 | 87 ± 31 |
| | dsFv | 480 ± 75 | 120 ± 15 | 68 ± 13 | 31 ± 6 |
| Exp. 2 | scFv | 300 ± 65 | 170 ± 24 | 73 ± 36 | 29 ± 10 |
| | dsFv | 300 ± 65 | 110 ± 32 | 32 ± 17 | 8 ± 5 |

fragment is stabilized by an interchain disulfide bond instead of the linker peptide used to stabilize single-chain Fv fragments. The engineered disulfide bond is at positions within the framework region of the Fv and thus is generally applicable for the stabilization of many Fv fragments, because they are in conserved regions of Fv fragments and are distant from CDRs such that antigen binding should be not affected (13). Some single-chain Fv fragments have a reduced affinity for the antigen when compared with the corresponding IgGs or Fab fragment (7, 9), probably because the peptide linker interferes with binding or the linker does not sufficiently stabilize the Fv heterodimer structure. The single-chain e23(Fv)-PE38KDEL is an example of this; its binding to erbB2 is reduced 4-fold compared with the Fab fragment or whole antibody (Fig. 2 and Ref. 7). In contrast, the disulfide-stabilized immunotoxin, e23(dsFv)-PE38KDEL, is 3–10-fold more active on various cell lines compared with its single-chain counter-

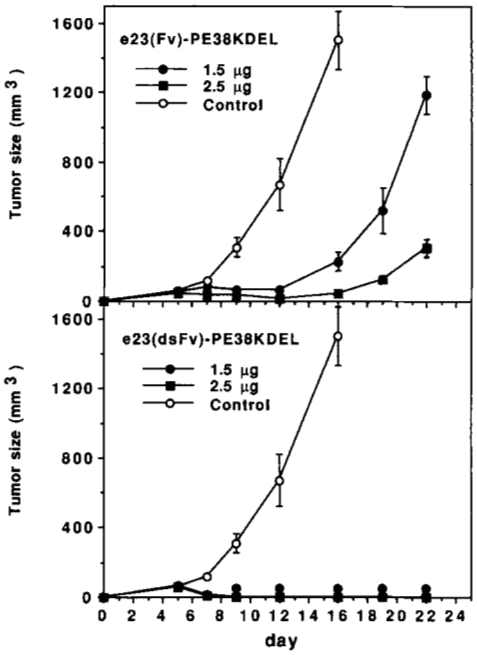


FIG. 5. **Antitumor effect of e23(dsFv)-PE38KDEL and e23(Fv)-PE38KDEL in nude mice bearing A431 tumors.** Groups of five animals were injected with $3 \times 10^6$ A431 cells. The animals were treated intravenously on days 4, 6, and 8 with 1.5 μg (●) or 2.5 μg (■) of e23(dsFv)-PE38KDEL or e23(Fv)-PE38KDEL. Control groups received PBS, 0.2% human serum albumin. *Error bars* indicate S.E.

part. The enhanced activity of e23(dsFv)- over e23(scFv)-immunotoxin is due to improved binding of the dsFv-immunotoxin. The binding of e23(dsFv)-PE38KDEL is almost indistinguishable from the binding of e23 Fab and is improved 4-fold when compared with the single-chain Fv.

*Improved Stability of dsFv-immunotoxins*—The disulfide-stabilized e23(Fv)-immunotoxin is also more stable than the single-chain immunotoxin. Its binding and cytotoxic activity are almost fully retained after incubation in PBS at 37 °C, whereas the single-chain immunotoxin loses all its binding capacity and cytotoxic activity after the same period of incubation. We have shown this increased stability for all disulfide-stabilized Fv immunotoxins produced so far (13–15). The dsFv-immunotoxins are also more stable in human serum (14, 15). The reason for the increased stability is because dsFv-immunotoxins have a decreased tendency to aggregate compared with single-chain immunotoxins (17). The increased stability of the dsFv-immunotoxin should facilitate large scale production and better handling of the molecule in clinical applications.

*Improved Antitumor Activity of e23(dsFv)-PE38KDEL*—To compare the antitumor activity of the single-chain and disulfide-stabilized form, we used two tumor models which allowed a quantitative comparison of e23(Fv)-PE38KDEL and e23(dsFv)-PE38KDEL. One model consists of N87 human gastric tumor xenografts. N87 cells have a gene amplification of the erbB2 gene and express a large amount of erbB2 antigen (7). We compared the extent of tumor regression by measuring mean tumor volume and found that the dsFv-immunotoxin is 2–3-fold more potent in antitumor activity.

In the A431 human epidermoid carcinoma model, we compared the ability of the dsFv- and scFv-immunotoxins to cause complete regressions of the tumors. We found that the scFv-immunotoxin causes significant tumor regression but not complete remissions. However, the disulfide-stabilized immunotoxin caused complete remissions in all animals. Thus, the dsFv-immunotoxin is a more potent antitumor agent. The response of the two *in vivo* tumor models to the same immuno-

toxin was found to be different. The dsFv immunotoxin caused complete regression of A431 tumors with $IC_{50}$ of 1 ng/ml, but not of N87 tumors (0.06 ng/ml $IC_{50}$). Several factors may account for this result. N87 cells might shed antigen more than A431 cells (which express low levels of erbB2) and block immunotoxin activity. Another possibility is poor penetration of immunotoxin into N87 tumors, perhaps because of the relatively poor vascularization of this tumor compared with A431 tumors. We found no significant difference in the animal toxicity of the scFv- and dsFv-immunotoxin. The $LD_{50}$ of a single dose of immunotoxin was 0.175 mg/kg for the scFv-immunotoxin and 0.2 mg/kg for the dsFv-immunotoxin (data not shown). In summary, the results presented here indicate that the improved binding of e23dsFv results in both increased cytotoxic activity *in vitro* and increased antitumor activity in mice.

*Acknowledgments*—We thank E. Lovelace and A. Harris for cell culture assistance and P. Andryszak, A. Jackson, and J. Evans for editorial assistance.

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