Bivalent Disulfide-stabilized Fragment Variable Immunotoxin Directed against Mesotheliomas and Ovarian Cancer

Tapan K. Bera, Juanita Williams-Gould, Richard Beers, Partha Chowdhury, and Ira Pastan[1]

Laboratory of Molecular Biology, National Cancer Institute, NIH, Bethesda, Maryland 20892-4255

Abstract

We have used protein engineering to generate a stable bivalent fragment variable (Fv) molecule from the antimesothelin antibody SS, in which 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 $(Gly_4\text{-}Ser)_3$ linker. The SS $(dsFv)_2$ molecule is fused to a M_r 38,000 truncated form of *Pseudomonas* exotoxin to generate a bivalent, disulfide stabilized, $(dsFv)_2$ immunotoxin. The immunotoxin was expressed in *Escherichia coli*, refolded *in vitro,* and purified to ~95% purity with a high yield of >10%. Binding studies demonstrated that the $(dsFv)_2$ molecule has 40 times higher apparent affinity for recombinant mesothelin than a monovalent dsFv molecule. The $(dsFv)_2$ immunotoxin was 4–10-fold more cytotoxic to three mesothelin antigen-positive cell lines than the monovalent dsFv immunotoxin. However, when tested in mice bearing tumor cells expressing mesothelin, the antitumor activity of the bivalent immunotoxin is very similar to the activity of the lower affinity monovalent immunotoxin. Our data indicate that increasing affinity of an antibody fragment does not necessarily lead to higher antitumor activity of an immunotoxin *in vivo*.

Introduction

Differentiation antigens, which are also expressed on cancer cells, are good targets for immunotherapy, in particular if the cancer arises from dispensable tissues such as ovary, breast, prostate, and B or T cells. Monoclonal antibodies that recognize such antigens on cancer cells are now being used for the targeted therapy of cancer. One of these, BL22 that targets the CD22 molecule on B-cell tumors, has produced many complete remissions in drug-resistant hairy cell leukemia (1).

Fv[2] fragments of antibodies are heterodimers made up of the antibody variable regions of heavy and light chain (V_H and V_L) domains and are the smallest antibody fragments that contain all of 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 (2, 3).

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 with a bivalent IgG or even a decavalent IgM. As a result, the avidity 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. Recently, we have described a new approach to generate a $(dsFv)_2$ of a monoclonal antibody that binds to its antigen with higher affinity (4). In this approach, the V_H and V_L domains of the Fv are linked to each other by a disulfide bond, and two Fvs are connected by a flexible 15-amino acid residue. To determine the applicability of the method to generate bivalent Fv for other antibodies, we used Fvs from the antibody SS directed against the mesothelin antigen expressed on ovarian cancer and mesotheliomas.

Here, we describe the generation of a stable bivalent Fv in which the V_H and V_L domains of the antimesothelin antibody SS (5) are covalently linked to each other by a disulfide bond and two Fvs are associated by a 15-amino acid linker. In contrast to our previous report (4), the final yield of the purified bivalent immunotoxin is much higher. We report here the binding affinity and *in vitro* cytotoxicity activity of the bivalent molecule and compare it with its monovalent counterpart. We also describe the pharmacokinetic behavior and the antitumor activity of the SS bivalent immunotoxin.
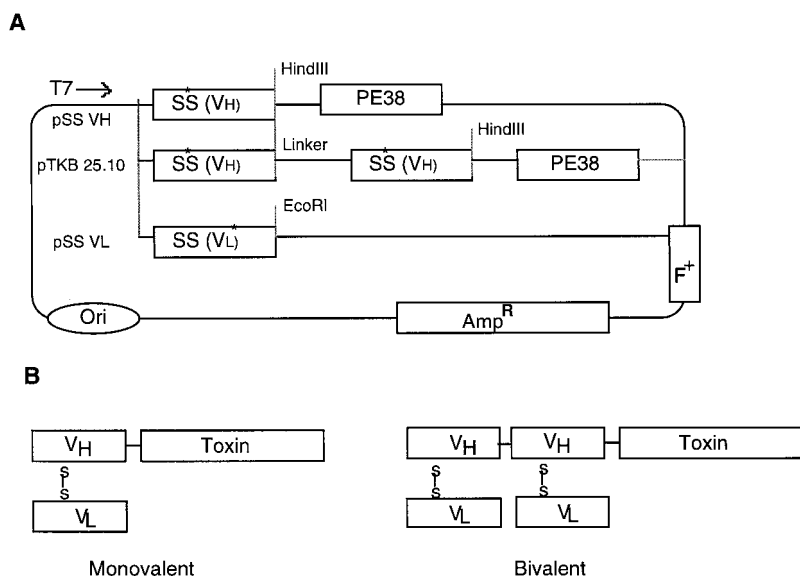
Materials and Methods

Plasmid Constructions. The plasmid pTKB25.10 that encodes two Cys^{45} V_H domains separated by a 15-amino acid linker, fused to PE38, was generated by PCR using the pSS V_H plasmid as the PCR template as described in detail in Fig. 1. The primer T178, 5′-ATC TCT T<u>AA GCT T</u>CA GGC GGA GGC GGA TCA GGT GGT GGC GGA TCT GGA GGT GGC GGA AGC CAG GTA CAA CTG CAG CAG TCT GGG-3′ was used to provide the 15-amino acid linker $(Gly_4\text{-}Ser)_3$ in between two V_H and the cloning site *Hin*dIII (the *Hin*dIII site underlined). The 3′ primer T179, 5′-GGT CC<u>A AGC TTT</u> TGA GGA GAC GGT GAC CGT GGT CCC TTG GCC-3′ was used to provide cloning site *Hin*dIII to the COOH-terminal half of V_H. Then primers T178 and T179 were added to the 100-μl final reaction mix and amplified for 25 cycles to obtain the Cys^{45} V_H domain containing the 15-amino acid linker at the NH_2 terminus. High fidelity polymerase mix (Boehringer Mannheim) was used to avoid PCR errors. The resulting

A

B

Monovalent

Bivalent

Fig. 1. Plasmid maps and schematic representation of immunotoxin. *A,* the parental plasmid pSS V_H encodes for the SS V_H-PE38 fusion protein in which Ser[44] of the SS V_H is mutated to Cys in the framework region. The expression plasmid pTKB25.10 encodes two SS V_H domains that are fused in-frame to PE38 toxin. Both V_H domains have a Ser[44] to Cys mutation and are held together by a 15-amino acid (Gly$_4$-Ser)$_3$ linker. The expression plasmid pSS V_L encodes for the V_L domain of SS, which has a Gly[99] to Cys mutation. *B,* schematic of a monovalent and bivalent disulfide-stabilized Fv immunotoxin.

fragment was cloned into the *Hin*dIII site of pSS V_H,[3] which codes for SS Cys[45] V_H fused to PE38, a truncated form of PE. The *Hin*dIII site fuses the inserted fragment in-frame to the Cys[45] V_H fragment of SS and the truncated toxin (6) in one of two possible orientations. The vector contains the T7 promoter for expression in Studier's *Escherichia coli* BL21(λDE3) expression system (7). The plasmid pSS V_L encodes the V_L domain of SS antibody and contains a Cys[99] mutation.[3] All expression plasmids were confirmed to be correct by DNA sequencing on an ABI 373A sequencer using the dideoxy chain terminator sequencing kit.

Production of Recombinant Protein. The components of SS (dsFv)$_2$ and dsFv immunotoxins were expressed in *E. coli* BL21(λDE3) and accumulated in inclusion bodies (IBs) as described previously for other recombinant immunotoxins (8). IBs were solubilized in guanidine chloride, reduced with dithioerythritol and refolded by dilution in a refolding buffer containing arginine to prevent aggregation and oxidized and reduced glutathione to facilitate redox shuffling (9). Active monomeric protein was purified from the refolding solution by ion exchange and size exclusion chromatography to near homogeneity as described (9). Protein concentrations were determined by Bradford assay (Bio-Rad Coomassie Plus).

Binding and Cytotoxicity Assays. The affinities of SS (dsFv) and (dsFv)$_2$ were assayed and compared by surface plasmon resonance (BIAcore; Pharmacia Biosensor). Recombinant mesothelin protein was coupled to BIAcore sensor chips (CM5, research grade, Pharmacia Biosensor) according to the manufacturer's specifications. The dsFv and (dsFv)$_2$ immunotoxins were applied to the chips, and binding and dissociation (k_{ass} and k_{diss}) were determined from association and dissociation curves of the sensorgrams with the BIAevaluation software package (Pharmacia Biosensor). K_D at equilibrium was calculated as $K_D = k_{diss}/k_{ass}$.

The specific cytotoxicity of the SS (dsFv)$_2$ 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 previously (10). The activity of the molecule is defined by the IC$_{50}$, the toxin concentration that reduces incorporation of radioactivity by 50% compared with cells that were not treated with toxin. The specificity is obtained by comparing the activity toward antigen-positive cells *versus* toxicity against antigen-negative cells.

Pharmacokinetic Analysis of Bivalent SS dsFv Immunotoxin in Mice. BALB/c mice were injected with 10 μg of immunotoxin in the tail vein. At various time intervals, blood samples were collected, and the level of the active immunotoxin was measured by the cytotoxicity assay using A431/K5 cells as described earlier. A standard curve was made with each pure immunotoxin. The data were analyzed by an exponential curve fitting program RSTRIP (version 5; Micro-Math Scientific Software).

Antitumor Activity of Bivalent SS Immunotoxin in Nude Mice. The antitumor activity of bivalent SS immunotoxin was determined in athymic nude mice bearing A431/K5 tumors. A431/K5 cells (3×10^6) were injected s.c. on day 0 into athymic nude mice, and treatment was started at day 7 when the tumors measured ~157 mm^3. Each mouse received three doses of immunotoxin on days 7, 10, and 13 by i.v. injection. Each group consisted of four animals. The control group received only the diluent, which is 0.2% HSA in Dulbecco's PBS. Tumors were measured with a caliper every other day; the volume of the tumor was calculated using the formula: tumor volume (mm^3) = length × (width)^2 × 0.4.

Stability Assays. The stability of the SS (dsFv)$_2$ and dsFv immunotoxins was determined by incubating them at 10 μg/ml at 37°C in HSA (4). Active immunotoxin remaining after incubation was determined by cytotoxicity assays on A431/K5 cells.

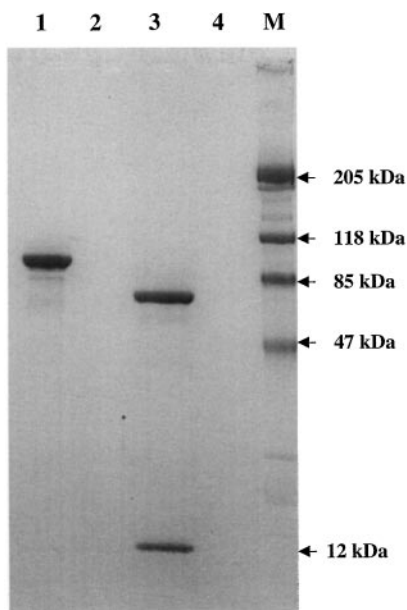[3] P. Chowdhury and I. Pastan, unpublished data.

Fig. 2. Gel analysis of recombinant SS (dsFv)$_2$-immunotoxin. SDS-PAGE gel electrophoresis of purified SS (dsFv)$_2$-PE38 immunotoxins. *Lane 1,* sample in nonreducing loading buffer; *Lane 2,* blank; *Lane 3,* sample in reducing loading buffer; *Lane 4,* blank; *Lane M,* prestain protein molecular weight standard.

Results

The goal of the present study is to make a recombinant bivalent Fv molecule using the antimesothelin antibody SS. Because our laboratory is interested in developing immunotoxins using cancer-specific antibodies, better binding of the antibody to the antigen should translate into better activity of the immunotoxin.

Production of Bivalent SS dsFv Immunotoxin. *E. coli* BL21 (λDE3) cells containing the plasmids pTKB25.10 and pSS VL for expression of the components of SS(dsFv)$_2$ immunotoxin were grown and induced with isopropyl-β-D-thiogalactopyranoside separately (4). The fusion proteins accumulated in insoluble intracellular IBs. These IBs contain almost pure recombinant protein but in an insoluble and aggregated form. Refolded, soluble monomeric protein was purified from improperly folded proteins and minor bacterial contaminants by ion exchange (Q-Sepharose, MonoQ) and size exclusion chromatography to near homogeneity (Fig. 2). The final yield of the purified protein is >10% of the starting inclusion body protein.

Cytotoxicity of Monovalent and Bivalent SS Immunotoxin toward Cell Lines Expressing Mesothelin Antigen. PE38 is a truncated but an enzymatically active form of PE (11). Fusion proteins of antibody fragments with PE38 are cytotoxic to cells that bind and internalize the fusion protein but have very low cytotoxic activity to antigen negative cells. Thus, cytotoxicity reflects specific antigen binding. To test the specificity of the bivalent SS immunotoxin, we analyzed and compared the activity of SS dsFv and (dsFv)2 immunotoxins toward different antigen-positive and antigen-negative cell lines. The cell lines we tested were A431/K5, an epider-

Table 1 Specific cytotoxicity of SS dsFv and (dsFv)$_2$ immunotoxins

Cytotoxicity assays were performed by measuring incorporation of [^{3}H]Leu into cellular proteins as described in "Materials and Methods." IC$_{50}$ is the concentration that causes 50% inhibition of protein synthesis after 20 h incubation with immunotoxin.

Cell line	Meso-antigen[a]	IC$_{50}$, pmoles		
		dsFv	(dsFv)$_2$	Fold increase
A431/K5	++++	18	25	7
N87	++	180	18	10
AGS	++	90	23	4
A1847	+	120	82	
A431	−	>7770	>7770	

[a] Antigen density was measured by immunofluorescence using K1 antibody (5). ++++, high; ++, medium; +, low; −, no expression of mesothelin.

moid carcinoma cell line transfected with full-length mesothelin cDNA (5), and also mesothelin-positive cancer cell lines N87, AGS, and A1847. A431 is an antigen-negative cell line. The bivalent immunotoxin is ~7-fold more active on A431/K5 cells as compared with the monovalent immunotoxin (Table 1). The IC$_{50}$ of the (dsFv)$_2$ immunotoxin is 2.5 pmol, whereas the IC$_{50}$ for the dsFv immunotoxin is 18 pmol. On N87 cells, the (dsFv)$_2$ immunotoxin is 10-fold more active than the dsFv immunotoxin, and the IC$_{50}$s are 18 and 180 pmol, respectively. The (dsFv)$_2$ immunotoxin is also four times more active on AGS cells, where the IC$_{50}$s for (dsFv)$_2$ and dsFv immunotoxin are 23 and 90 pmol, respectively. A1847 cells, which are also mesothelin antigen positive, have similar IC$_{50}$s for both the (dsFv)$_2$ and dsFv immunotoxin, whereas the antigen-negative A431 cells are not affected by either of the two immunotoxins at >7770 pmol (Table 1).

Binding of SS (dsFv)$_2$ on Recombinant Mesothelin Protein. To determine the affinity (or avidity) of the immunotoxins, we used the BioCore assay method. The binding affinities of SS dsFv and SS (dsFv)$_2$ immunotoxin were determined by a surface plasmon resonance assay on recombinant mesothelin protein. For the BiaCore assays, recombinant dsFv or (dsFv)$_2$ immunotoxins were applied to recombinant mesothelin-coated sensor chips. A determination of association and dissociation rates revealed a k_{ass} of 5.21×10^5 M^{-1} s^{-1} for the dsFv and 1.16×10^6 M^{-1} s^{-1} for the (dsFv)$_2$ (Table 2). The dissociation rates of the dsFv is ~18-fold higher than the (dsFv)$_2$ toxin with a k_{diss} 5.49×10^{-3} s^{-1} for the dsFv and 3.13×10^{-4} s^{-1} for the (dsFv)$_2$. The K_D at binding equilibrium, ($K_D = ka/kd$), is 10.5 nM for the dsFv and 0.26 nM for the (dsFv)$_2$ (Table 2). These data indicate that the bivalent dsFv of the SS antibody possesses ~40-fold higher affinity than its monovalent counterpart.

Stability of the SS (dsFv)$_2$ Immunotoxin. Disulfide-stabilized Fv immunotoxins are very stable at 37°C in PBS containing 0.2% HSA (12). To analyze whether the SS (dsFv)$_2$ immunotoxin is stable, we incubated the immunotoxins at 37°C for different periods of time in PBS containing 0.2% HSA at a concentration of 10 μg/ml. Activity of the incubated immunotoxin was determined by a protein synthesis inhibition assay. The (dsFv)$_2$ immunotoxin retains 95% of its activity, even after 24 h incubation at 37°C in human serum
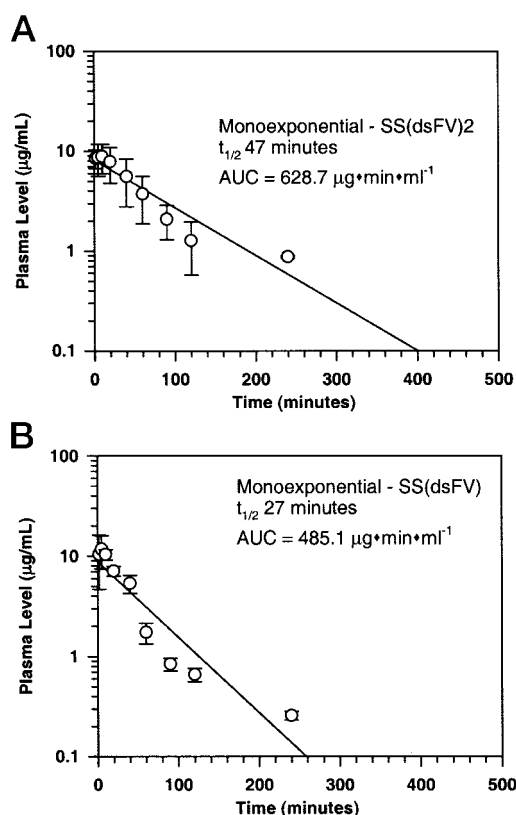
Fig. 3. Pharmacokinetics of monovalent and bivalent SS dsFv-immuno-
toxins in mice. Female BALB/c mice were injected i.v. with a single 10-μg
dose of dsFv or (dsFv)2 immunotoxin. Blood samples were drawn at
specific time points. The level of immunotoxin was measured by bioassay
in which triplicates of diluted serum samples were incubated with
A431/K5 cells. Results are averages of three animals for each point from
two independent assays performed on each immunotoxin; *bars,* SE. *A,*
blood levels of (dsFv)2-PE38. *B,* blood levels for dsFv-PE38.

Fig. 4. Antitumor activity of SS(dsFv) and SS(dsFv)2-PE38 in athymic
nude mice bearing A431/K5 tumors. Groups of five animals were injected
with 3×10^6 A431/K5 cells and treated i.v. on days 7, 10, and 13 days
after cell injection. Mice were given 45 pmol of (dsFv)2-PE38 ($\diamond$) and
dsFv-PE38 ($\bigcirc$) immunotoxin. Control groups received only diluent ($\blacksquare$).

(data not shown), and thus is as stable as the corresponding
monovalent dsFv immunotoxin.[3]

**Pharmacokinetics of Bivalent SS Immunotoxin in
Mouse Blood.** Generally the $t_{1/2}$ for both scFv and dsFv
containing immunotoxins injected i.v. into mice is short and
in the range of 20 min (13). To determine the $t_{1/2}$ of the
bivalent SS immunotoxin in the mouse circulation, BALB/c
mice were injected i.v. with a single dose of 10 μg of SS
(dsFv)2-PE38 or SS dsFv-PE38. The remaining activity of
immunotoxin in mouse blood at different time points after
injection was determined by measuring its ability to inhibit
protein synthesis on A431/K5 cells as described in "Materials
and Methods." In Fig. 3, the plasma clearance curves of SS
(dsFv)2-PE38 and dsFv-PE38 are shown. The clearance of
SS (dsFv)2-PE38 from mouse blood is slower than the dsFv-
PE38 molecule, with a $t_{1/2}$ of 47 min for SS (dsFv)2-PE38 and
$t_{1/2}$ of 27 min for SS dsFv-PE38.

Antitumor Activity. To determine whether the improved
in vitro cytotoxicity data could be translated *in vivo* as anti-
tumor activity, the monovalent and bivalent SS dsFv-PE38
immunotoxins were assayed in tumor xenografts of A431/K5
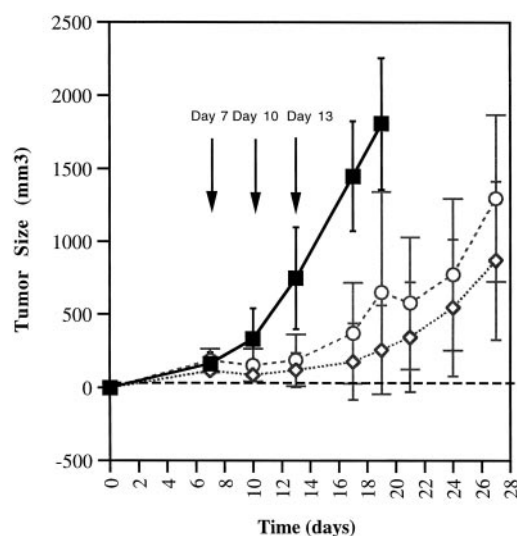cells in athymic nude mice. On day 7 when the tumors

reached ~157 mm³, the animals were injected i.v. with 4 μg
(45 pmol) of SS (dsFv)2-PE38 or 2.9 μg (45 pmol) of SS
dsFv-PE38 every other day $\times$ 3. A dose of immunotoxin was
chosen that would produce regression and regrowth of tu-
mors so that the difference in activity between monovalent
and the bivalent immunotoxin could be assessed. The con-
trol groups received PBS containing 0.2% HSA. As shown in
Fig. 4, control animals treated with 0.2% PBS-HSA devel-
oped large tumors and were sacrificed on day 21, when the
tumors achieved about 1.5×2.0 cm in size. However, the
tumors for both SS dsFv-PE38 and SS (dsFv)2-PE38 groups
decreased to an unmeasurable size after two injections, but
the tumors reappeared on day 11. There is no statistically
significant difference of antitumor activity between the
monovalent and bivalent SS immunotoxins at equimolar
doses.

Discussion

In this report, we have constructed a recombinant bivalent Fv
fragment of antibody SS, in which the V_H/V_L heterodimer is
stabilized by disulfide bond and the two Fvs are covalently
connected by a flexible peptide linker. The bivalent Fv is then
linked to a truncated form of PE to generate a very potent
immunotoxin with significantly improved cytotoxicity and
binding affinity compared with its monovalent counterpart.

Several approaches have been used previously to gener-
ate recombinant bivalent Fvs by different laboratories. Also a
variety of formats and protein designs have been investi-
gated that include miniantibodies, diabodies, and disulfide-
linked fragments (14–20). Our approach to generate a biva-
lent Fv is based on the fact that a dsFv is extremely stable,
and therefore the resulting molecule would be very stable
under physiological conditions. This is supported by our
experimental results.

Table 2   Affinity of SS dsFv and (dsFv)$_2$ immunotoxins

The affinity of SS dsFv and (dsFv)$_2$ immunotoxins was determined by surface plasmon resonance (BiaCore). 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.

Affinity	SS dsFv	(dsFv)$_2$
k_{ass} (M^{-1} s^{-1})	5.21×10^5	1.16×10^6
k_{diss} (s^{-1})	5.49×10^{-3}	3.13×10^{-4}
K_D (M)	10.5×10^{-9}	2.6×10^{-10}

Compared with these other methods, this report further demonstrated that our approach to generate recombinant bivalent Fv fusion proteins is simple, results in homogenous and properly folded bivalent Fvs with a high yield, and can be used to generate bivalent Fv for different antibodies.

Bivalent SS Immunotoxin Has Improved Activity *in Vitro*. The cytotoxicity data described in Table 1 reveal that the bivalent SS immunotoxin has about 4–10-fold higher activity on three of the four mesothelin-expressing cell lines than the monovalent SS immunotoxin. The improved cytotoxicity of the bivalent immunotoxin is not evident on the A1847 mesothelin-positive cancer cell line. This could be attributable to the low number of mesothelin molecules on the surface of the A1847 cell line. However, when tested in mice, the antitumor activity of the bivalent SS immunotoxin against A431/K5 cells is more or less similar to the monovalent immunotoxin, although the bivalent immunotoxin is 7-fold more active than the monovalent immunotoxin on A431/K5 cells *in vitro*. There are several factors that determine the activity of a therapeutic agent *in vivo*. One important factor is the pharmacokinetic behavior of the agent in the circulation. But in this case, the bivalent immunotoxin remains for a longer period of time in the circulation than the monovalent immunotoxin. The most probable explanation for this discrepancy is that the higher affinity of the bivalent immunotoxin creates an affinity barrier (21, 22) and retards its penetration into the tumor cells distant from the capillary, where it enters the tumor mass. Other groups including ours (23–25) have previously observed retardation of tumor penetration attributable to high affinity or avidity of the antibodies. In all cases, the monovalent Fv or Fab fragments were as effective as the bivalent molecules at controlling tumor growth, despite the fact that bivalent molecules have much higher affinity toward their antigen and remained in the mouse circulation (an equal time or) longer than the monovalent Fv or Fab fragments.

In summary, our data indicate that by increasing affinity of an antibody fragment does not necessarily improve the antitumor activity of immunotoxins or other forms of immunotherapeutic agents.

Acknowledgments

References

1. Kreitman, R. J., Wilson, W. H., Bergeron, K., Raggio, M., Stetler-Stevenson, M., FitzGerald, D. J., and Pastan, I. Efficacy of the anti-CD22 recombinant immunotoxin BL22 in chemotherapy-resistant hairy-cell leukemia. N. Engl. J. Med. *345:* 241–247, 2001.

2. Yokota, T., Milenic, D. E., Whitlow, M., and Schlom, J. Rapid tumor penetration of a single-chain Fv and comparison with other immunoglobulin forms. Cancer Res., *52:* 3402–3408, 1992.

3. Kreitman, R., and Pastan, I. Accumulation of a recombinant immunotoxin in a tumor *in vivo*: less than 1000 molecules per cell is sufficient for complete responses. Cancer Res. *58:* 968–975, 1998.

4. Bera, T. K., Onda, M., Brinkmann, U., and Pastan, I. A bivalent disulfide-stabilized Fv with improved antigen binding to erbB2. J. Mol. Biol., *281:* 475–483, 1998.

5. Chowdhury, P. S., Viner, J. L., Beers, R., and Pastan, I. Isolation of a high-affinity stable single-chain Fv specific for mesothelin from DNA-immunized mice by phage display and construction of a recombinant immunotoxin with anti-tumor activity. Proc. Natl. Acad. Sci. USA, *95:* 669–674, 1998.

6. Hwang, J., FitzGerald, D. J., Adhya, S., and Pastan, I. Functional domains of *Pseudomonas* exotoxin identified by deletion analysis of the gene expressed in *E. coli.* Cell, *48:* 129–136, 1987.

7. Studier, F. W., and Moffatt, B. A. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol., *189:* 113–130, 1986.

8. Brinkmann, U., and Pastan, I. Recombinant immunotoxins: from basic research to cancer therapy. Methods: A Companion to Methods Enzymology, *8:* 143–156, 1995.

9. Buchner, J., Pastan, I., and Brinkmann, U. A method to increase the yield of properly folded recombinant fusion proteins: single-chain immunotoxins from renaturation of bacterial inclusion bodies. Anal. Biochem., *205:* 263–270, 1992.

10. Brinkmann, U., Pai, L. H., FitzGerald, D. J., Willingham, M., and Pastan, I. B3(Fv)-PE38KDEL, a single-chain immunotoxin that causes complete regression of a human carcinoma in mice. Proc. Natl. Acad. Sci. USA, *88:* 8616–8620, 1991.

11. Pastan, I., Pai, L. H., Brinkmann, U., and FitzGerald, D. J. Recombinant toxins: new therapeutic agents for cancer. Ann. NY Acad. Sci., *758:* 345–354, 1995.

12. Reiter, Y., Brinkmann, U., Jung, S-H., Lee, B., and Pastan, I. Engineering disulfide bonds into conserved framework regions of Fv fragments: recombinant immunotoxins containing disulfide-stabilized Fv with improved biochemical characteristics. Protein Eng., *7:* 697–704, 1994.

13. Reiter, Y., Pai, L. H., Brinkmann, U., Wang, Q-C., and Pastan, I. Antitumor activity and pharmacokinetics in mice of a recombinant immunotoxin containing a disulfide-stabilized Fv fragment. Cancer Res., *54:* 2714–2718, 1994.

14. De-Jonge, J., Brissinck, J., Heirman, C., Demanet, C., Leo, O., Moser, M., and Thielemans, K. Production and characterization of bispecific single-chain antibody fragments. Mol. Immunol., *32:* 1405–1412, 1995.

15. Holliger, P., Brissinck, J., Williams, R. L., Thielemans, K., and Winter, G. Specific killing of lymphoma cells by cytotoxic T-cells mediated by a bispecific diabody. Protein Eng., *9:* 299–305, 1996.

16. Gruber, M., Schodin, B. A., Wilson, E. R., and Kranz, D. M. Efficient tumor cell lysis mediated by a bispecific single chain antibody expressed in *Escherichia coli*. J. Immunol., *152:* 5368–5374, 1994.

17. Hayden, M. S., Linsley, P. S., Gayle, M. A., Bajorath, J., Brady, W. A., Norris, N. A., Fell, H. P., Ledbetter, J. A., and Gilliland, L. K. Single-chain mono- and bispecific antibody derivatives with novel biological properties and antitumour activity from a COS cell transient expression system. Ther. Immunol., *1:* 3–15, 1994.

18. Mallender, W. D., Ferreira, S. T., Voss, E. J., and Coelho, S. T. Inter-active-site distance and solution dynamics of a bivalent-bispecific single-chain antibody molecule. Biochemistry, *33:* 10100–10108, 1994.

19. Haunschild, J., Faro, H-P., Pack, P., and Pluckthun, A. Pharmacokinetic properties of bivalent miniantibodies and comparison to other immunoglobulin forms. Antibody Immunoconj. Radiopharm., *8:* 111–128, 1995.

20. Cumber, A. J., Ward, E. S., Winter, G., Parnell, G. D., and Wawrzynczak, E. J. Comparative stabilities *in vitro* and *in vivo* of a recombinant

mouse antibody FvCys fragment and a bisFvCys conjugate. J. Immunol., *149*: 120–126, 1992.

21. Malik, J., Neumann, R., Paik, C., Perez-Bacete, M. J., Sato, J., Van Osdol, W., and Weinstein, J. N. Micropharmacology of monoclonal-antibodies in solid tumors—direct experimental evidence for a binding-site barrier. Cancer Res., *52:* 5144–5153, 1992.

22. Saga, T., Neumann, R. D., Heya, T., Sato, J., Kinuya, S., Le, N., Paik, C. H., and Weinstein, J. N. Targeting cancer micrometastases with mono-clonal antibodies—a binding-site barrier. Proc. Natl. Acad. Sci. USA, *92:* 8999–9003, 1995.

23. Behr, T. M., Memtsoudis, S., Sharkey, R. M., Blumenthal, R. D., Dunn, R. M., Gratz, S., Wieland, E., Nebendahl, K., Schmidberger, H., Golden-berg, D. M., and Becker, W. Experimental studies on the role of antibody fragments in cancer radio-immunotherapy: influence of radiation dose and dose rate on toxicity and anti-tumor efficacy. Intl. J. Cancer, *77:* 787–795, 1998.

24. Bera, T. K., Viner, J., Brinkmann, M., and Pastan, I. Pharmacokinetics and antitumor activity of a bivalent disulfide-stabilized Fv immunotoxin with improved antigen binding to erbB2. Cancer Res., *59:* 4018–4022, 1999.

25. Adams, G. P., Schier, R., McCall, A. M., Simmons, H. H., Horak, E. M, Alpaugh, R. K., Marks, J. D., and Weiner, L. M. High affinity restricts the localization and tumor penetration of single-chain Fv antibody molecules. Cancer Res., *61:* 4750–4755, 2001.