0022-1767/92/1491-0120$02.00/0
THE JOURNAL OF IMMUNOLOGY
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Vol. 149, 120–126, No. 1, July 1, 1992
Printed in U.S.A.

COMPARATIVE STABILITIES IN VITRO AND IN VIVO OF A RECOMBINANT MOUSE ANTIBODY FvCys FRAGMENT AND A bisFvCys CONJUGATE[1]

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A murine antibody FvCys fragment with a single additional cysteine residue at the C terminus of the V_H domain was expressed in *Escherichia coli* from a modified expression plasmid containing the structural genes for the V_H and V_L domains derived from the anti-lysozyme hybridoma D1.3. Chemical cross-linking between the introduced sulfhydryl groups of two FvCys fragments by means of bis-maleimidohexane was used to generate a bisFvCys conjugate. The stability of the bisFvCys conjugate and an FvCys analogue that had been reacted with *N*-ethylmaleimide to block the free sulfhydryl group, FvCys(BL), were compared after ^{125}I-labeling. The bisFvCys conjugate was completely stable to incubation in solution at 37°C for 24 h whereas only 60% of the FvCys(BL) fragment remained soluble. After i.v. administration to normal Wistar rats, both Fv proteins were rapidly cleared from the circulation with biphasic kinetics that were best fitted to a two-compartment open pharmacokinetic model. The α-phase half-life of the bisFvCys conjugate, 0.32 h, was significantly longer than that of the FvCys(BL) fragment, 0.15 h ($p < 0.001$) whereas there was no significant difference between the β-phase half-lives, 1.4 to 1.6h. No chain cleavage or covalent attachment to serum protein was detected by SDS-PAGE analysis of serum samples. However, gel permeation HPLC revealed that both Fv proteins associated with serum proteins in vivo and in vitro.

The ability of mAb to bind selectively to tumor-associated Ag expressed on the surface of malignant cells makes them valuable agents for the diagnosis and therapy of cancer (1). In practice, the effectiveness of antibodies in vivo is limited by their molecular and biologic properties. In tumor imaging, the aim of maximizing the discrimination between the tumor target and the background field is confounded by the long half-life of intact antibody. For the delivery of cytotoxic agents, the relatively large molecular mass of antibody inhibits the rate of immunoconjugate extravasation and the extent of tumor penetration. These limitations can be minimized in part by the use of antibody fragments, such as F(ab) or $F(ab')_2$ generated by proteolysis of intact Ig, that retain the ability to bind the target Ag. However, univalent F(ab) fragments usually associate less well with target cells than bivalent $F(ab')_2$ fragments, which are endowed with a higher avidity of binding.

Developments in the techniques for manipulating and expressing Ig genes allow the generation of functional Fv fragments that comprise the variable domains only of the H and L chains and are approximately one-half the size of a F(ab) fragment (2). Such Fv fragments can be secreted from myeloma cells or from *Escherichia coli* as heterodimers formed by the association of independently expressed V_H and V_L domains (3–5). Alternatively, single chain Fv fusion proteins in which the V_H domain is connected by a peptidyl linker to the V_L domain can either be expressed as intracellular inclusion bodies or secreted from *Escherichia coli* (6, 7). In the case of a mouse anti-phosphocholine Fv fragment, the spontaneous separation of the V_H and V_L chains, which are in dynamic equilibrium in solution, was shown to be detrimental to stability, apparently because the isolated V_H chain was highly susceptible to denaturation (8).

In this report, we have investigated the stability of a bivalent Fv construct comprising two murine anti-lysozyme Fv fragments. The study involved three stages: first, the production of an FvCys fragment by genetic manipulation to introduce an additional cysteine residue at the C terminus of the V_H domain; second, the construction of a bivalent bisFvCys conjugate by chemical cross-linking of the introduced cysteine residues of two FvCys fragments; and third, a comparison of the stabilities of the bisFvCys construct and a sulfhydryl-blocked FvCys analogue both in vitro and in vivo.

MATERIALS AND METHODS

Construction of plasmid for the expression of rFvCys. The expression plasmid was derived from pSW1-V_HD1.3-V_kD1.3 (5), which contains the structural genes for the V_H and V_L domains derived from the anti-lysozyme murine hybridoma D1.3 (9, 10). The genes were linked in translational frame to the pelB leader to direct secretion of the expressed protein into the periplasmic space, and the backbone vector was pUC19 (11). Several minor modifications have been made to insert unique restriction sites for the cloning of other Ig genes. *Pst*I and *Bst*EII sites have been inserted into the 5′ and 3′ ends, respectively, of the V_HD1.3 gene, and a *Sac*I site at the 5′ end of the V_L gene. To insert an additional cysteine residue at the 3′ end of the V_H gene, the plasmid was restricted with *Bst*EII and *Sma*I, purified using Geneclean (United States Biochemical Corp., Cleveland, OH) and ligated with the annealed, complementary oligonucleotides, Cys1 and Cys2. (Cys1: 5′-GTC ACC GTC TCC TCA

Received for publication January 27, 1992.
Accepted for publication April 13, 1992.
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TGT TAA TAA-3'; Cys2: 5'-TTA TTA ACA TGA GGA GAC G-3').

The correct insertion of the Cys residue was checked by nucleotide sequencing. The nucleotide sequence of the insert of plasmid $V_HCysD1.3\text{-}V_kD1.3$ and the amino acid sequence of the encoded FvCys are shown in Figure 1.

Expression and purification of FvCys. $V_HCysD1.3\text{-}V_kD1.3$ plasmid DNA was transformed into *E. coli* BMH71-18 (12) and recombinants grown up and induced for expression using similar methods to those described previously by Ward et al. (5). Briefly, 1-liter cultures using 2 × YT (13) plus 100 μg/ml ampicillin and 1% (w/v) glucose were grown up for 16 h with shaking at 37°C and then pelleted by centrifugation. Cell pellets were washed in 50 mM NaCl and resuspended in 1 liter of 2 × YT plus 100 μg/ml ampicillin and 1 mM IPTG. Induced cultures were grown with shaking at 37°C for a further 24 to 48 h, stood on ice for 1 h, and then pelleted by

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                                        M  K  Y  L  L  P  T  A  A
GCATGCAAATTCTATTTCAAGGAGACAGTCATAATGAAATACCTATTGCCTACGGCAGCC
        10        20        30        40        50        60
A  G  L  L  L  L  A  A  Q  P  A  M  A  Q  V  Q  L  Q  E  S
GCTGGATTGTTATTACTCGCTGCCCAACCAGCGATGGCCCAGGTGCAGCTGCAGGAGTCA
        70        80        90       100       110       120
G  P  G  L  V  A  P  S  Q  S  L  S  I  T  C  T  V  S  G  F
GGACCTGGCCTGGTGGCGCCCTCACAGAGCCTGTCCATCACATGCACCGTCTCAGGGTTC
       130       140       150       160       170       180
S  L  T  G  Y  G  V  N  W  V  R  Q  P  P  G  K  G  L  E  W
TCATTAACCGGCTATGGTGTAAACTGGGTTCGCCAGCCTCCAGGAAAGGGTCTGGAGTGG
       190       200       210       220       230       240
L  G  M  I  W  G  D  G  N  T  D  Y  N  S  A  L  K  S  R  L
CTGGGAATGATTTGGGGTGATGGAAACACAGACTATAATTCAGCTCTCAAATCCAGACTG
       250       260       270       280       290       300
S  I  S  K  D  N  S  K  S  Q  V  F  L  K  M  N  S  L  H  T
AGCATCAGCAAGGACAACTCCAAGAGCCAAGTTTTCTTAAAAATGAACAGTCTGCACACT
       310       320       330       340       350       360
D  D  T  A  R  Y  Y  C  A  R  E  R  D  Y  R  L  D  Y  W  G
GATGACACAGCCAGGTACTACTGTGCCAGAGAGAGAGATTATAGGCTTGACTACTGGGGC
       370       380       390       400       410       420
Q  G  T  T  V  T  V  S  S  C  *  *
CAAGGCACCACGGTCACCGTCTCCTCATGTTAATAAGGGAGCTTGCATGCAAATTCTATT
       430       440       450       460       470       480
                                  M  K  Y  L  L  P  T  A  A  A  G  L  L  L
TCAAGGAGACAGTCATAATGAAATACCTATTGCCTACGGCAGCCGCTGGATTGTTATTAC
       490       500       510       520       530       540
L  A  A  Q  P  A  M  A  D  I  E  L  T  Q  S  P  A  S  L  S
TCGCTGCCCAACCAGCGATGGCCGACATCGAGCTCACTCAGTCTCCAGCCTCCCTTTCTG
       550       560       570       580       590       600
A  S  V  G  E  T  V  T  I  T  C  R  A  S  G  N  I  H  N  Y
CGTCTGTGGGAGAAACTGTCACCATCACATGTCGAGCAAGTGGGAATATTCACAATTATT
       610       620       630       640       650       660
L  A  W  Y  Q  Q  K  Q  G  K  S  P  Q  L  L  V  Y  Y  T  T
TAGCATGGTATCAGCAGAAACAGGGAAAATCTCCTCAGCTCCTGGTCTATTATACAACAA
       670       680       690       700       710       720
T  L  A  D  G  V  P  S  R  F  S  G  S  G  S  G  T  Q  Y  S
CCTTAGCAGATGGTGTGCCATCAAGGTTCAGTGGCAGTGGATCAGGAACACAATATTCTC
       730       740       750       760       770       780

L  K  I  N  S  L  Q  P  E  D  F  G  S  Y  Y  C  Q  H  F  W
TCAAGATCAACAGCCTGCAACCTGAAGATTTTGGGAGTTATTACTGTCAACATTTTTGGA
       790       800       810       820       830       840
S  T  P  R  T  F  G  G  G  T  K  L  E  I  K  R  *  *
GTACTCCTCGGACGTTCGGTGGAGGCACCAAGCTGGAAATCAAACGGTAATAAGAGCTCGAATTC
       850       860       870       880       890       900
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Figure 1. Nucleotide sequence of $V_HCysD1.3\text{-}V_kD1.3$. The *PstI*, *BstEII*, and *SacI* sites are indicated by underlining of the nucleotide sequence. The amino acid sequence of the open reading frames, corresponding to pelB-$V_HCysD1.3$ and pelB-$V_kD1.3$, is shown in one-letter code. The amino acid sequence of the pelB leader sequence is shown *underlined*. Stop codons are indicated by *asterisks*.

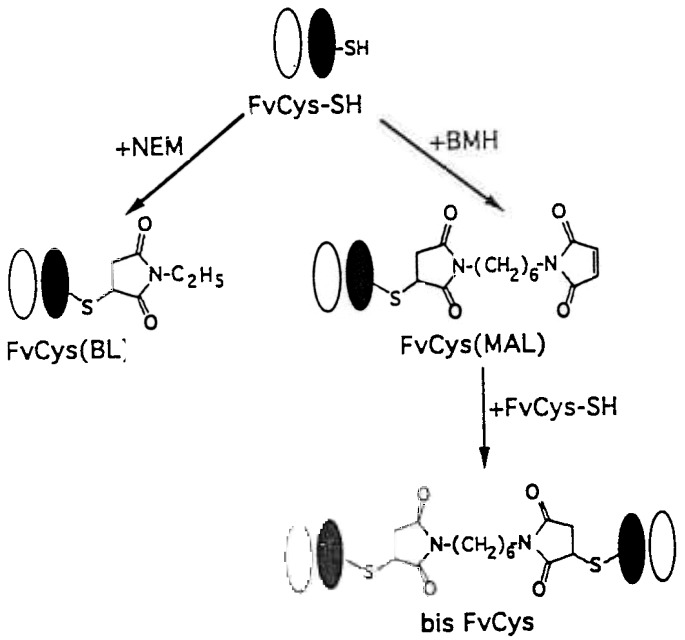

Figure 2. Scheme for the synthesis of FvCys(BL) and bisFvCys.

centrifugation. The culture supernatant was passed through a Nalgene 0.45-μm filter, and then through a lysozyme-Sepharose affinity column prepared by coupling hen-egg lysozyme to CNBr-activated Sepharose 4B. After passage of the supernatant, the column was washed with 10-column volumes of PBS followed by 10-column volumes of 0.1 M Tris-HCl, 0.5 M NaCl, pH 8.5. The FvCys protein was batch eluted using 50 mM diethylamine. The eluting solution was neutralized immediately using a one-tenth volume of 1 M Tris-HCl buffer, pH 7.4. The yield of FvCys was approximately 10 mg/liter of culture.

The extinction coefficient of FvD1.3, determined by quantitative amino acid hydrolysis, is $2.5 \times 10^4\ M^{-1}cm^{-1}$ at 280 nm. This value was assumed for the determination of the concentration of FvCys from OD measurements at this wavelength.

Synthesis of FvCys(BL) and bisFvCys. The synthetic scheme is shown in Figure 2. The FvCys fragment at 1 mg/ml in 0.1 M Tris-HCl buffer, containing 50 mM diethylamine, pH 7.4, was treated with dithiothreitol at a final concentration of 15 mM for 30 min at room temperature to reduce disulfide-linked FvCys dimers to monomers. The resulting FvCys-SH was separated from the reducing agent by gel filtration on Sephadex G-25(M) (Pharmacia) equilibrated with 20 mM sodium phosphate, 0.1 M sodium sulfate, pH 6.8. A 1.5-fold molar excess of NEM[4] (Aldrich, Milwaukee, WI) was added to the solution of FvCys-SH by slow addition and stirring of a solution of NEM at 5 mg/ml in dimethylformamide. After reaction for 30 min at room temperature, the modified fragment, FvCys(BL), was separated from excess reagent by gel filtration on Sephadex G-25(M) and was purified finally by gel permeation HPLC on a Superdex75 HR10/30 column (Pharmacia, Piscataway, NJ).

To prepare the bisFvCys conjugate, FvCys-SH generated as described above was treated with a 1.5-fold molar excess of BMH (Pierce) by addition of a solution of BMH at 2 mg/ml in dimethylformamide. After reaction for 30 min, the substituted FvCys(MAL) fragment was separated from excess BMH by gel filtration on Sephadex G-25(M), mixed immediately with an equimolar quantity of freshly reduced FvCys-SH, and left to react overnight. The bisFvCys conjugate was exposed to 15 mM dithiothreitol to reduce any disulfide-linked FvCys conjugates that might have formed during the reaction and was then purified by gel permeation HPLC as described above.

The final reaction products were analyzed by analytic gel permeation HPLC on Superdex75, by SDS-PAGE and by adsorption to lysozyme-Sepharose.

Radiolabeling of the Fv proteins. FvCys(BL) and bisFvCys were radiolabeled with ^{125}I by the method of Bolton and Hunter (14). The protein at 100 μg/ml in 0.3 ml 50 mM sodium borate buffer, containing 50 mM sodium chloride, pH 8.1, was added to 0.5 ml of Bolton and Hunter reagent (74 MBq/μmol) (Amersham International) dried in a glass vial according to the manufacturer's recommendations. After 15 min at 4°C with gentle agitation, 50 μl of 1 M glycine in

[4] Abbreviations used in this paper: NEM, *N*-ethylmaleimide; BMH, 1,6-bismaleimidohexane.

borate buffer was added and left for 5 min. The ^{125}I-labeled Fv protein was separated from reactants by gel filtration on Sephadex G-25(M) in PBS. The gel filtration column was pretreated with the appropriate unlabeled Fv protein to minimize losses of labeled material because of adsorption. In addition, eluting fractions of ^{125}I-labeled protein were collected directly into sample tubes containing a solution of unlabeled Fv protein in PBS. The final products contained Fv protein at a concentration of 60 to 80 μg/ml with the specific activity of different batches ranging between 10 and 25 MBq/ng.

Measurement of stability in vitro. Samples of [^{125}I]FvCys(BL) and [^{125}I]bisFvCys, approximately 1 to 2 mg containing 18,000 cpm at a final concentration of approximately 1 μM in PBS, were prepared under sterile conditions in Eppendorf microcentrifuge tubes sealed with O-rings. The samples were incubated at 37°C or kept at 4°C and at various times up to 24 h, samples were removed and ultracentrifuged before analysis by i) SDS-PAGE, autoradiography using preflashed film at −70°C, and densitometry on a Bio-Rad model 620 Video Densitometer, ii) gel permeation HPLC on Superdex75 and gamma counting of fractions in an LKB Compugamma 1282. Parallel experiments were conducted following the addition of ^{125}I-labeled Fv protein to freshly prepared serum from the normal Wistar rat.

In a separate experiment, samples of [^{125}I]bisFvCys were incubated in the presence of an excess of unlabeled Fv under sterile conditions either at 37°C or at 4°C. At various times up to 24 h, samples were analyzed for the distribution of radioactivity between the two Fv proteins, which had distinct elution positions on gel permeation HPLC.

Measurement of stability in vivo. Sterile solutions of [^{125}I]FvCys(BL) and [^{125}I]bisFvCys prepared in PBS were injected i.v. into groups of four normal rats of the Wistar/CBI strain using the methodology previously described by Wawrzynczak et al. (15). In brief, a single sample containing a total of 20 to 24 μg ^{125}I-labeled protein (7 to 10 × 10^6 cpm), which had been checked for the absence of aggregated material by gel permeation HPLC, was administered to each rat. Blood samples (0.1 to 0.2 ml) were obtained at various times up to 24 h after injection. Individual serum samples were subjected to TCA precipitation to determine the protein-associated radiolabel. Pooled serum samples from the earlier time points were also analyzed by SDS-PAGE and by gel permeation HPLC after microcentrifugation.

RESULTS

Molecular properties of FvCys(BL) and bisFvCys. A single cysteine residue was introduced into the anti-lysozyme Fv fragment by genetically engineering an additional codon at the 3′ end of the gene encoding the V_H domain (Fig. 1). The sulfhydryl group so introduced served as a specific site for the attachment of the homobifunctional cross-linking reagent BMH and allowed the production by a two-step procedure of a bivalent bisFvCys conjugate in which the two V_H chains were linked via a nonreducible thioether bond (Fig. 2). To allow a comparison of the properties of the bivalent conjugate with an analogous univalent Fv fragment, the rFvCys fragment was reacted with the monofunctional reagent NEM to give the FvCys(BL) fragment in which the sulfhydryl group is permanently blocked.

The two Fv proteins could be demonstrated to possess the expected molecular properties. On gel permeation HPLC, FvCys(BL) and bisFvCys eluted at distinct positions slightly retarded relative to the predicted elution positions based on M_r of 25 kDa and 50 kDa, respectively (Fig. 3). The elution positions of parental Fv and FvCys(BL) were indistinguishable (not shown). By SDS-PAGE under reducing conditions (Fig. 4), FvCys(BL) showed two bands identical in size to those of the parental Fv: the apparent M_r of the V_L and V_H chains were approximately 15 kDa and 16 kDa, respectively, indicating that the two chains migrated anomalously slowly. The bisFvCys conjugate also consisted of two bands, one corresponding to the V_L chain, and one with an apparent M_r of approximately 30 kDa consistent with the predicted

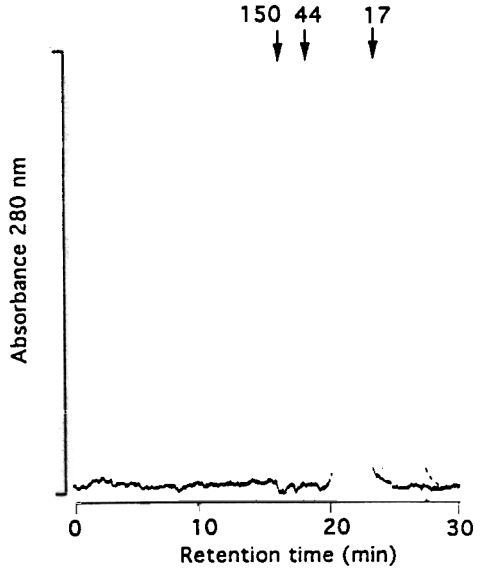

Figure 3. Gel permeation HPLC analysis of Fv proteins. Samples of FvCys(BL) (– – –) and bisFvCys (——) were applied to a column (30 cm × 1 cm diameter) of Superdex75HR equilibrated with 50 mM sodium borate buffer, containing 50 mM potassium chloride, pH8.1, and eluted at a flow rate of 0.5 ml/min. The *arrows* indicate the elution positions of molecular mass standards in kDa.

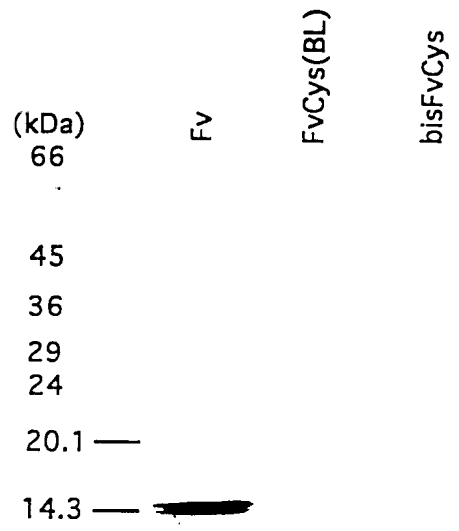

Figure 4. SDS-PAGE analysis of Fv proteins. Samples of Fv, FvCys(BL) and bisFvCys were run on a 5 to 20% polyacrylamide gradient gel under reducing conditions. Protein bands were visualized using Coomassie brilliant blue staining. The running positions of standard proteins of known M_r are shown.

size of the covalently cross-linked V_H chain dimer. There was no evidence of any unconjugated V_H chain in the preparation consistent with the absence of any disulfide-linked conjugate. The ^{125}I-labeled FvCys(BL) and bisFvCys appeared identical to the unlabeled Fv proteins by gel permeation HPLC and SDS-PAGE (see below). The [^{125}I]bisFvCys conjugate retained its selective binding ability as judged by its capacity to adhere to a column of lysozyme-Sepharose and to be eluted quantitatively with diethylamine buffer solution.

Stability in vitro. The susceptibility of the V_H and V_L chains to separation and denaturation was investigated by incubating [^{125}I]FvCys(BL) and [^{125}I]bisFvCys in PBS at 37°C. In the case of the FvCys(BL) fragment, analysis by

SDS-PAGE and autoradiography revealed a progressive loss of material over the 24-h time course of the experiment (Fig. 5). Densitometric analysis of the autoradiogram indicated that the protein remaining in solution at 24 h was about 60% of that present in the starting sample and that the relative amounts of V_H and V_L were similar throughout suggesting that the denaturation of the two chains may have occurred at a similar rate. In contrast, the bisFvCys fragment was completely stable over the 24-h incubation period (Fig. 5). Analysis by gel permeation HPLC confirmed that the [^{125}I]bisFvCys conjugate eluted with the M_r expected for the monomeric form and that no aggregation had occurred (not shown).

The incubation experiment was repeated in the presence rat serum at 37°C. Gel permeation HPLC indicated the presence of radiolabel eluting as a discrete peak at the void volume of the column as well as with material corresponding to the authentic Fv proteins (not shown). The proportion of this high M_r material increased with time and to a similar extent in each case (Fig. 6). After

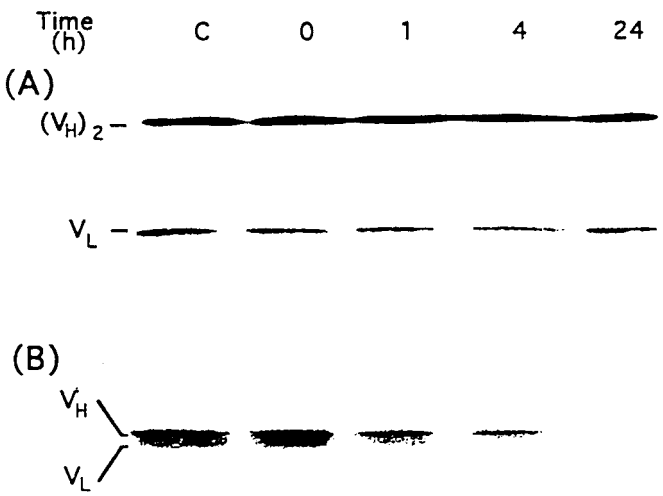

Figure 5. Stability of ^{125}I-labeled Fv proteins to incubation at 37°C. [^{125}I]bisFvCys (*A*) and [^{125}I] FvCys(BL) (*B*) were incubated at 37°C in PBS. Samples incubated for different lengths of time were run on SDS-PAGE and the bands visualized by autoradiography. The starting material was also prepared for analysis without centrifugation as control (*track C*).

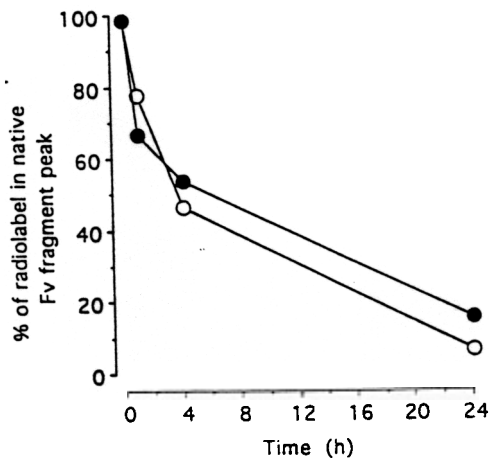

Figure 6. Rate of association of ^{125}I-labeled Fv proteins with serum proteins in vitro. [^{125}I]FvCys(BL) (●) and [^{125}I]bisFvCys (○) were incubated at 37°C in the presence of rat serum for different lengths of time. Samples were analyzed by gel permeation HPLC on Superdex75HR, as in the legend to Figure 2, to determine the proportion of radiolabeled protein associated with the Fv monomer fraction or with higher M_r fractions.

24 h, less than 20% of radiolabel was present in the M_r fraction corresponding to the authentic Fv protein. By comparison, when [^{125}I]bisFvCys was incubated for 24 h in the presence of rat serum at 4°C, greater than 85% of the radiolabel remained associated with the native conjugate fraction.

The findings of the experiments in the presence and absence of rat serum suggest that the redistribution of ^{125}I-labeled FvCys(BL) and bisFvCys into higher M_r fractions upon incubation is the result of an interaction between the Fv protein and one or more serum proteins, which occurs preferentially at 37°C. The simplest hypothesis to explain this finding is that the V_H and V_L chains can separate readily at 37°C and can then associate stably with serum proteins, which are present in excess. In support of this interpretation, exchange of radiolabel between [^{125}I]bisFvCys and unlabeled Fv occurred readily at 37°C and had virtually reached equilibrium after 4 h of incubation. The exchange occurred to a much lesser extent at 4°C: after 24 h of incubation, the transfer of radiolabel to the Fv fraction was equivalent to that seen within 1 h at 37°C.

Stability in vivo. The ^{125}I-labeled Fv proteins were administered i.v. to normal rats and serum samples were taken at intervals up to 24 h after injection. The samples were analyzed in three ways. First, the protein-associated radiolabel was measured by gamma counting of TCA precipitates. Second, the presence of radiolabel in protein bands of the appropriate apparent M_r was confirmed by SDS-PAGE and autoradiography. Third, the interaction of the ^{125}I-labeled Fv proteins with serum proteins in vivo was monitored by gel permeation HPLC and gamma counting.

Figure 7 shows the comparative pharmacokinetics of the two ^{125}I-labeled Fv proteins up to 4 h after injection. Both FvCys(BL) and bisFvCys were rapidly cleared from the circulation of the rat with biphasic kinetics that were best fitted to a two-compartment open pharmacokinetic model. The α- and β-phase half-lives of [^{125}I]FvCys(BL) were 0.15(±0.02) h and 1.41(± 0.20) h, respectively. For the [^{125}I] bisFvCys conjugate, the α- and β-phase half-lives were 0.32(±0.01) h and 1.55(±0.08) h, respectively.

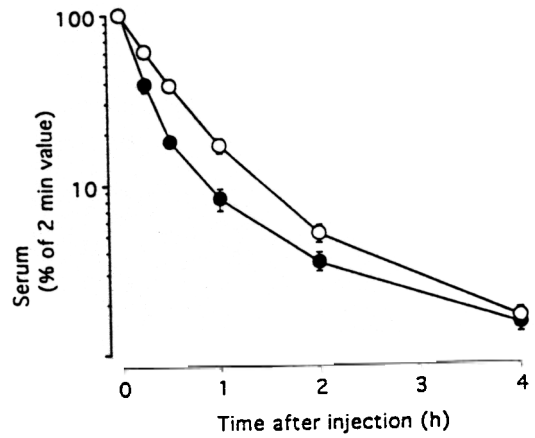

Figure 7. Serum clearance of ^{125}I-labeled Fv proteins in the normal rat. Serum samples were recovered from normal rats after a single i.v. injection of [^{125}I]FvCys(BL) (●) or [^{125}I]bisFvCys (○). Each point represents the mean value of protein-associated counts in serum from four animals in each group. The experimental data are expressed as the percentage of the protein-associated counts per gram relative to the value measured at 2 min after injection. The *error bars* denote the SD from the mean values, unless smaller than the symbols used.

The difference between the α-phase half-lives was highly significant ($p < 0.001$) by Student's t-test: there was no significant difference between the β-phase half-lives. By 4 h, less than 1% of the Fv protein originally injected remained in the bloodstream. The percentages of the two Fv proteins remaining in circulation at 8 h and at 24 h were indistinguishable (not shown). The rapid clearance of the Fv proteins was accompanied by rapid catabolism: less than 50% of the radiolabel in blood at 4 h could be precipitated with TCA.

SDS-PAGE followed by autoradiography (Fig. 8) revealed that all the TCA-precipitable radiolabel in serum was associated with bands having the characteristic apparent M_r of the V_H and V_L chains, or in the case of the bisFvCys conjugate, of the V_H chain dimer and V_L chain. Although the samples were matched for total content of radioactivity, there was an apparent loss of material at the later time points. This may have been caused by association of ^{125}I-labeled Fv protein with aggregates removed by microcentrifugation of the samples before loading on the gel. There was no evidence of chain cleavage or extensive covalent attachment to serum protein in the circulation.

Analysis of serum samples by gel permeation HPLC revealed a complicated picture. Figure 9 shows a comparison of the radioactivity elution profiles of the starting samples of [^{125}I]FvCys(BL) and [^{125}I]bisFvCys and of serum samples taken at 15 min after injection of the two Fv proteins. In the case of FvCys(BL), the radiolabel was distributed throughout all the fractions of the chromatogram suggesting that the components of the Fv fragment associate with many different serum proteins in vivo. This association must be noncovalent in nature because, under dissociating conditions in SDS, all the radiolabel was present in protein bands corresponding to the V_H and V_L chains (see above). In contrast, virtually all the

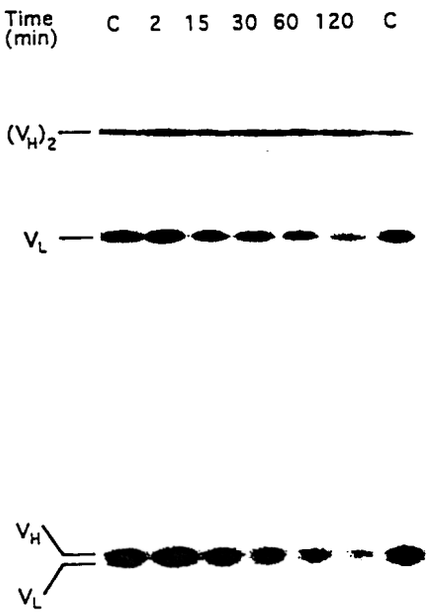

Figure 8. SDS-PAGE analysis of serum samples containing ^{125}I-labeled Fv proteins. Serum samples removed from normal rats at different times after injection of [^{125}I]bisFvCys (*A*) and [^{125}I]FvCys(BL) (*B*) were run on a 5 to 20% polyacrylamide gradient gel under reducing conditions. The samples were matched for the total number of counts and volume. The labeled protein bands were visualized by autoradiography. The tracks labeled *C* are control samples prepared in rat serum.

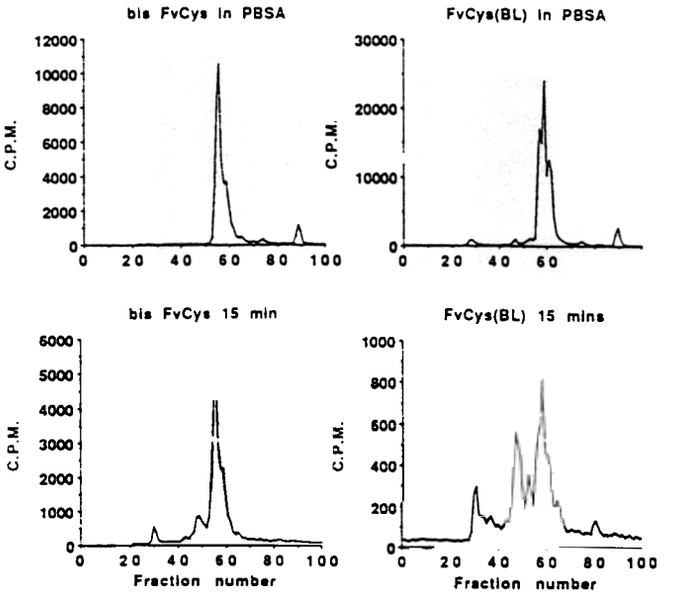

Figure 9. Gel permeation HPLC analysis of serum samples containing ^{125}I-labeled Fv proteins. The distribution of radiolabel in fractions with different M_r at 15 min after injection into rats was compared with the original preparations of [^{125}I]FvCys(BL) and [^{125}I]bisFvCys. Samples were analyzed by chromatography on a column (30 cm × 0.78 cm diameter) of TSK-G3000SWXL equilibrated with 20 mM sodium phosphate, 0.1 M sodium sulfate, pH 6.8, at a flow rate of 0.4 ml/min and gamma counting of individual 0.2-ml fractions.

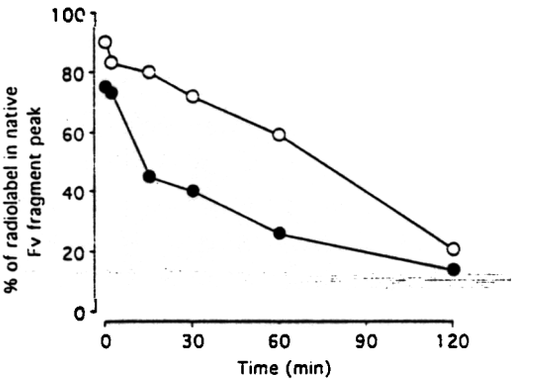

Figure 10. Decrease of circulating monomeric ^{125}I-labeled Fv proteins with time. Serum samples taken at different times after injection of [^{125}I]FvCys(BL) (●) or [^{125}I]bisFvCys (○) were analyzed by gel permeation HPLC and gamma counting as described in the legend to Figure 8. The percentage of Fv monomer at each time point was obtained by comparing the radiolabel recovered in the monomer fraction and that in fractions with higher M_r. The zero time point represents the dosing sample mixed with fresh rat blood and treated in identical fashion to the serum samples from rats injected with ^{125}I-labeled Fv proteins.

[^{125}I]bisFvCys conjugate present in the circulation at 15 min eluted with an M_r corresponding to that of the authentic conjugate monomer. A progressive decrease in the proportion of the monomeric form occurred with time for both Fv proteins and was most rapid in the case of the FvCys(BL) fragment (Fig. 10). The apparent rates at which the ratio of Fv monomer diminished were faster than those determined in vitro (see Fig. 5): the rate in vivo is influenced by the rate of elimination as well as by the intrinsic rate of complex formation (see *Discussion*).

DISCUSSION

In this report, we have described the production of a novel Fv fragment that contains an additional cysteine residue at the C-terminal end of the V_H chain designed to

introduce a single sulfhydryl group allowing site-specific chemical manipulation. The FvCys fragment was used to construct a bivalent bisFvCys conjugate by a simple chemical cross-linking strategy. The stabilities of the univalent and bivalent Fv proteins were compared in vitro and in vivo. The major findings of this study were: first, that the bisFvCys conjugate was more stable than the FvCys(BL) fragment at 37°C in vitro; second, that both Fv proteins associated noncovalently with serum proteins to a similar extent at 37°C in vitro; and third, that the bisFvCys conjugate was cleared less rapidly from the bloodstream than the analogous FvCys fragment.

Studies of an Fv fragment derived from a mouse phosphocholine-binding Ig determined that, under physiologic conditions, the fragment had poor stability (half-life, 1 h) showing preferential loss of the V_H chain presumably as a result of denaturation (8). Our experiments with the anti-lysozyme FvCys(BL) fragment indicate that under comparable conditions this fragment was much more stable (half-life >24 h) and there was no preference for loss of the V_H chain. The apparent stability of this fragment probably reflects a relatively stable interaction between the V_H and V_L chains compared with the anti-phosphocholine Fv fragment. The stability of V_H-V_L association will probably vary from one Fv protein to another as a result of differences in the residues at the V_H-V_L interface. In particular, the highly variable sequences of the CDR3s of both V_H and V_L domains may affect the stability of this interaction because the CDR3 residues are found to be in close proximity in the three-dimensional structures of antibodies solved to date (9, 16, 17). In contrast to its relative stability in the absence of any other protein, FvCys(BL) was found to associate with proteins present in rat serum in a time- and temperature-dependent fashion. It is likely that in the presence of an excess of serum, V_H and V_L chains that dissociate become free to engage in stable noncovalent contacts with serum proteins, possibly by means of hydrophobic interactions, and so are prevented from reforming the native Fv protein.

The rate of clearance of [^{125}I] FvCys(BL) from the circulation of the rat was very rapid with an initial half-life of only 9 min and a second phase with a half-life of about 1.5 h. A major component of this rapid elimination from the circulation was probably renal filtration given the relatively small molecular size of the Fv fragment. However, the interpretation of the pharmacokinetic data is complicated by the progressive decrease in the proportion of radiolabeled protein present in the circulation as the native Fv monomer. At the later time points, the [^{125}I] FvCys(BL) in the circulation was virtually completely associated with serum proteins suggesting that the protein complexes had a longer serum half-life than the Fv monomer and that the measured β-phase half-life overestimated the true half-life of the authentic Fv fragment. Several studies have reported in vivo half-lives of different types of Fv fragment which ranged between 2 min and 4.5 h (18–21). Although the half-lives we report are clearly comparable, the other studies were conducted using different experimental systems and did not address the possibility of Fv association with serum protein.

The bisFvCys conjugate prepared by chemical cross-linking of two FvCys fragments displayed molecular properties consistent with that of a stably linked bivalent Fv protein. The conjugate was completely stable at 37°C in contrast with the FvCys(BL) fragment. The reason for the higher stability of the bisFvCys conjugate is unclear because chemical cross-linking of two Fv domains would not be expected to enhance the affinity between the V_H and V_L chains. In the presence of rat serum, bisFvCys nevertheless associated with serum proteins in vitro at the same rate as FvCys(BL). This suggests that the interaction with serum protein must occur more rapidly than chain denaturation. In vivo, [^{125}I]bisFvCys was cleared less rapidly than the ^{125}I-labeled FvCys analogue with an initial half-life of 19 min. This longer half-life may reflect a slower rate of renal filtration of the larger bisFvCys conjugate compared with that of the FvCys(BL) fragment. At earlier time points, a higher proportion of the bisFvCys conjugate was present in the monomeric form than FvCys(BL) although the in vitro incubation experiments indicated that the rates of complex formation by both Fv proteins were similar. This difference is most easily explained by considering the difference between the serum clearance rates of the two ^{125}I-labeled Fv proteins. The more rapid elimination of FvCys(BL) compared with bisFvCys leaves less of the Fv monomer in the circulation relative to the less quickly cleared background of complexed protein. At later time points, little distinction could be drawn between the circulating ^{125}I-labeled proteins that were present predominantly as protein complexes.

The studies described with the model anti-lysozyme Fv proteins indicate that Fv proteins in which the V_H-V_L association is relatively stable are nevertheless unlikely to be suitable for use in vivo. This is caused by the tendency of the V_H and V_L chains to associate with serum proteins, giving rise to relatively long lived complexes that would undermine the intended advantages of the fragments for targeting. The problem of chain dissociation has been addressed by engineering single chain Fv fragments (6, 7). However, Glockshuber et al. (8) found that although a single chain anti-phosphocholine Fv fragment was more stable than the two-chain version, it was still significantly less stable than internally cross-linked Fv or the natural F(ab) fragment. It is possible, therefore, that even single chain Fv fragments will be able to bind to serum proteins when the close noncovalent contacts between the V_H and V_L components of the fusion protein are momentarily disrupted by flexing of the protein structure at 37°C. Such an association with serum proteins will be less likely to occur the stronger the non covalent interactions between the V_H and V_L domains.

In conclusion, we have demonstrated that a bivalent bisFvCys conjugate produced by a combination of rDNA and protein chemical techniques had higher stability than a univalent Fv analogue and a significantly longer serum half-life. Such bivalent Fv proteins should have superior properties to conventional univalent F(ab) fragments in tumor imaging and drug targeting because the increased avidity of the bivalent Fv conjugate for target cells would be predicted to improve the extent of localization in tumor. However, novel recombinant antibody constructs intended for therapy will require rigorous analysis of their stability under experimental conditions relevant to their use.

Acknowledgments. We thank Drs. Ellen Vitetta and Philip Thorpe, University of Texas, Dallas, and Dr. Chris-

topher Dean, Institute of Cancer Research, for their comments and criticisms of the manuscript. We thank R. V. Henry for assistance with preparation of the manuscript.

REFERENCES

1. Baldwin, R. W., V. S. Byers, and R. D. Mann (eds.). 1990. Monoclonal antibodies and immunoconjugates. Parthenon, Carnforth, U. K.
2. Winter, G., and C. Milstein. 1991. Man-made antibodies. *Nature 349:293.*
3. Riechmann, L., J. Foote, and G. Winter. 1988. Expression of an antibody Fv fragment in myeloma cells. *J. Mol. Biol. 203:825.*
4. Skerra, A., and A. Pluckthun. 1988. Assembly of a functional immunoglobulin Fv fragment in *Escherichia coli. Science 240:1038.*
5. Ward, E. S., D. Gussow, A. D. Griffiths, P. T. Jones, and G. Winter. 1989. Binding activities of a repertoire of single immunoglobulin variable domains secreted from *Escherichia coli. Nature 341:544.*
6. Bird, R. E., K. D. Hardman, J. W. Jacobsen, S. Johnson, B. M. Kaufman, S-M. Lee, T. Lee, S. H. Pope, G. S. Riordan, and M. Whitlow. 1988. Single-chain antigen-binding proteins. *Science 242:423.*
7. Huston, J. S., D. Levinson, M. Mudgett-Hunter, M-S. Tai, J. Novotny, M. N. Margolies, R. J. Ridge, R. E. Bruccoleri, E. Haber, R. Crea, and H. Oppermann. 1988. Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli. Proc. Natl. Acad. Sci. USA 85:5879.*
8. Glockshuber, R., M. Malia, I. Pfitzinger, and A. Pluckthun. 1990. A comparison of strategies to stabilize immunoglobulin Fv fragments. *Biochemistry 29:1362.*
9. Amit, A. G., R. A. Mariuzza, S. E. V. Phillips, and R. J. Poljak. 1986. Three-dimensional structure of an antigen-antibody complex at 2.8A resolution. *Science 233:747.*
10. Boulot, G., J-L. Eisele, G. A. Bentley, T. N. M. Bhat, E. S. Ward, G. Winter, and R. J. Poljak. 1990. Crystallization and preliminary X-ray diffraction study of the bacterially expressed Fv from the monoclonal anti-lysozyme antibody D1.3 and of its complex with the antigen, lysozyme. *J. Mol. Biol. 213:617.*
11. Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene 33:103.*
12. Ruther, U., M. Koenen, K. Otto, and B. Muller-Hill. 1981. pUR322, a vector for cloning and rapid chemical sequencing of DNA. *Nucl. Acids Res. 9:4087.*
13. Bankier, A. T., and B. G. Barrell. 1989. Sequencing single-stranded DNA using the chain-termination method. In *Nucleic Acids Sequencing: A Practical Approach.* C. J. Howe and E. S. Ward, eds. IRL Press, Oxford, U. K., p. 37.
14. Bolton, A. E., and W. H. Hunter. 1973. The labelling of proteins to high specific radioactivities by conjugation to a ^{125}I-containing acylating agent. *Biochem. J. 133:529.*
15. Wawrzynczak, E. J., A. J. Cumber, G. D. Parnell, P. T. Jones, and G. Winter. 1992. Blood clearance in the rat of a recombinant mouse monoclonal antibody lacking the N-linked oligosaccharide side-chains of the C_H2 domains. *Mol. Immunol. 29:213.*
16. Colman, P. M., W. G. Laver, J. N. Varghese, A. T. Baker, P. A. Tulloch, G. M. Air, and R. G. Webster. 1987. Three-dimensional structure of a complex of antibody with influenza virus neuraminidase. *Nature 326:358.*
17. Padlan, E. A., E. W. Silverton, S. Sheriff, G. H. Cohen, S. J. Smith-Gill, and D. R. Davies. 1989. Structure of an antibody-antigen complex: crystal structure of the HyHEL-10 Fab-lysozyme complex. *Proc. Natl. Acad. Sci. USA 86:5938.*
18. Colcher, D., R. Bird, M. Roselli, K. D. Hardman, S. Johnson, S. Pope, S. W. Dodd, M. W. Pantoliano, D. E. Milenic, and J. Schlom. 1990. *In vivo* tumor targeting of a recombinant single-chain antigen-binding protein. *J. Natl. Canc. Inst. 82:1191.*
19. Milenic, D. E., T. Yokota, D. R. Filpula, M. A. J. Finkelman, S. W. Dodd, J. F. Wood, M. Whitlow, P. Snoy, and J. Schlom. 1991. Construction, binding properties, metabolism, and tumor targeting of a single-chain Fv derived from the pancarcinoma monoclonal antibody CC49. *Cancer Res. 51:6363.*
20. Shealy, D., M. Nedelman, M-S. Tai, J. S. Huston, H. Berger, J. Lister-James, and R. T. Dean. 1990. Characterization and biodistribution of Tc-99m labeled single chain antibody Fv fragment. *J. Nucl. Med. 31:776 (Abstr.).*
21. King, D., A. Mountain, A. Harvey, N. Weir, R. Owens, K. Proudfoot, A. Phipps, J. Adair, H. Lisle, S. Bergin, A. Lawson, S. Rhind, B. Pedley, J. Boden, R. Begent, and G. Yarranton. 1991. Tumour localisation of engineered antibody fragments. *Antibody Immunoconj. Radiopharm. 4:201 (Abstr.).*