Improved Tumor Targeting with Chemically Cross-Linked Recombinant Antibody Fragments


ABSTRACT

The construction and use of recombinant chimeric and later fully humanized (CDR-grafted) antibodies to tumor-associated antigens has reduced the immune response generated to these antibodies in clinical studies. However, their long circulating half-life is a disadvantage for tumor imaging and therapy. Fragments such as F(ab')2, Fab', Fv and single chain Fv (scFv) offer faster blood clearance but also lower overall tumor doses. We have examined the tumor targeting of several novel fragments produced by chemical cross-linking of Fab' or scFv to dimeric and trimeric species. To facilitate cross-linking of Fab'-linking fragments, a chimeric B72.3 Fab' fragment has been expressed with a hinge sequence containing a single cysteine residue. B72.3 scFv was also produced with a similar hinge region peptide attached to the COOH terminus to allow cross-linking. These fragments, Fab'Acys and scFv'Acys were cross-linked with linkers containing two or three maleimide groups to produce dimeric and trimeric molecules with increased avidity for antigen. Cross-linkers were also designed to contain a 12-N-4 macrocycle capable of stable radiolabeling with 111In. This allowed the production of site-specifically labeled, fully immunoreactive proteins. Biodistribution studies in the nude mouse LS174T xenograft model with scFv, di-scFv, and tri-scFv demonstrated that these fragments clear extremely rapidly from the circulation and give rise to only low levels of activity accumulated at the tumor. Di-Fab (DFM) and tri-Fab (TFM) however, accumulated relatively high levels of activity at the tumor with high tumor: blood ratios generated, demonstrating improved targeting compared to IgG. cB72.3 111In-labeled tri-Fab was found not to accumulate in the kidney or the bone, resulting in an attractive antibody fragment for tumor therapy.

INTRODUCTION

The efficacy of radiolabeled monoclonal antibody conjugates in cancer therapy is often limited by their inability to reach their target tumor in adequate quantities without delivering a toxic dose to bone marrow (1, 2). The two factors of key importance are the dose delivered to the tumor and the rate of clearance from the rest of the body. Optimization of these two parameters may allow the administration of effective therapeutic doses with minimal associated toxicity.

Several recombinant chimeric and later fully humanized (CDR-grafted) antibodies recognizing tumor-associated antigens have been produced, and these appear to offer reduced immunogenicity in humans (reviewed in Ref. 3). This allows attempts at therapy with repeat dosing to increase the activity delivered to the tumor. However, the circulating half-life of chimeric and humanized antibodies in humans is considerably longer than that of murine antibodies; thus, problems of bone marrow toxicity due to circulating activity may be accentuated (4, 5).

Several approaches to reduce circulating activity have been attempted including chemical modification of antibodies (6), in vivo or

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2 To whom requests for reprints should be addressed.
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plasmid pAl15. The Fab' produced from this gene was termed cB72.3 Fab' ΔCys.

The expression plasmid pAl15 was introduced by electroporation into the CHO cell line cL18 described previously (23). Cell lines resistant to myco-

Phenolic acid and expressing functional cB72.3 Fab' ΔCys were identified by

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Addition of the recombinant gene to cL18 resulted in the expression of the

phenolic acid and expressing functional cB72.3 Fab' ΔCys were identified by

screening culture supernatants in an antigen-binding enzyme-linked immu-

norsorbent assay format as described previously (23).

CHO cell lines expressing cB72.3 Fab' ΔCys were also isolated using gene

amplification procedures. A plasmid vector comprising the chimeric light

chain and modified Fab' heavy chain gene, each expressed separately from the

HCMV promoter, and the glutamine synthetase minigene (26) were con-

structed and introduced into CHO-K1 cells by the calcium phosphate precipi-

tation procedure (26). Transfectants were selected (26) in GMEM medium

containing 20 μM MSX. Cell lines expressing functional cB72.3 Fab' ΔCys

were identified as before and cultured in medium containing MSX concentra-

tions from 30 to 5000 μM. Cell lines able to grow in medium containing 200

μM MSX and which expressed cB72.3 Fab' ΔCys to higher levels than the

parent line were identified, and these were selected for further studies.

Construction of scFvΔCys and Expression in Escherichia coli. The construction and expression of B72.3 scFv has been reported previously (27).

To allow production of scFv multimers a version of B72.3 scFv was produced

using the construct Vc-(Gly, Ser)2-VH with the ΔCys hinge region used for

Fab' ΔCys on the COOH-terminal end of VH. A DNA sequence encoding the

hinge region was assembled from eight oligonucleotides (ranging from 20 to

36 base pairs in length) by incubating annealed oligomers with DNA ligase for

16 h at room temperature. The assembled sequences were purified by electropho-

resis on 5% acrylamide gels, restricted with BglII/EcoRI, and ligated together

with an EcoRI/BglII fragment of Vαι into the unique EcoRI site of the

pEE6.6CMV expression vector (26). An EcoR/ EcoRI restriction fragment

containing the VαιΔCys-hinge gene was isolated from this plasmid and inserted

downstream of the ompA signal sequence in the plasmid pSkompA (28).

The ompA-VαιΔCys-hinge gene was inserted into pTTQ9 (Amer
data) and the scFvΔCys-hinge expression plasmid was constructed by substitution of a

SfiI/BstHI fragment from this plasmid into the scFv pTTQ9 plasmid (27).

Expression of scFvΔCys-hinge (termed scFv' ΔCys) in E. coli was achieved as
described previously for scFv (27).

Cross-Linkers. The synthesis of 12-N-4-lysine-bis-maleimide, CT52 (structure 4) is summarized in Fig. 1. N,N'-bis-(benzyloxy carbonyl) lysine,

N-hydroxysuccinimide ester (structure 1) in DMF was added to the 12-N-4
derivative (structure 2) described previously (29). N-methyloxopholine and water (metal free) were then added, followed by dimethylaminopyridine in

DMF, and the mixture was heated at 60°C. Couplings were also carried out at

room temperature with 1,4-piperazinediethanesulfonic acid buffer:1,4-dioxan

(1:1) to give the same products in similar yield. The reaction was monitored by

reverse phase HPLC, and the crude mixture was purified by reverse phase HPLC

to yield structure 3. The structure (structure 3) was initially deprotected by

stirring in acetonitrile under nitrogen for 5–10 minutes, followed by adding

freshly distilled trimethylsilyl iodide. After stirring overnight the reaction was

quenched with water and extracted with dichloromethane. The resultant

material was then reacted with SMP ester in DMF in the presence of N-

methylmorpholine, and the reaction was monitored by reverse phase HPLC.

When the reaction was complete, the mixture was purified by polymer reverse

phase HPLC to yield 12-N-4-lysine-bis-(maleimide) (structure 4). The

synthesis of the tri-maleimide linker CT998 (structure 10) is summarized in

Fig. 2. BOC-(εZ)-lysyllysyllysine amide (structure 5) was dissolved in dry THF under

nitrogen. The temperature of the reaction mixture was lowered to −20°C and ethylchloroformate and N-methylmorpholine were added. The temperature

was maintained at −20°C for 30 min. Then ammonia was added, and the reaction

was allowed to warm to room temperature. The reaction mixture was washed with saturated sodium bicarbonate solution and then extracted with ethylaceta-

ete, dried with magnesium sulfate, and evaporated to give BOC-(εZ)-lysyllysyllysine amide (structure 6) as a fine white solid. The white solid (structure 6) was

recovered and without further purification dissolved in a 1:1 solution of trifluoroacetic acid:dichloromethane. The mixture was stirred at room tempe-

rature for 30 min. The solvent was evaporated and the residue triturated with ether and dried to give εZ)-lysyl-(εZ)-lysyllysine amide (structure 9) as a white solid. BOC-(εZ)-lysyllysine amide (structure 8) was recovered and dissolved in a 1:1 solution of trifluoro-

acetic acid:dichloromethane without further purification, and the mixture was stirred at room temperature for 30 min. The solvent was evaporated and the

residue triturated with ether and dried to give (εZ)-lysyl-(εZ)-lysyllysine amide

(structure 9) as a white solid. BOC-(εZ)-lysyllysine amide (structure 5) was dissolved in dry THF under nitrogen. The temperature of the reaction mixture was lowered to −20°C and ethylchloroformate and N-methylmorpholine were added. After 30 min (εZ)-lysyl-(εZ)-lysine amide (structure 9) was added without prior purification, and the reaction mixture was allowed to warm to room temperature.

The organic layers were washed with saturated sodium bicarbonate solution and the aque-

ous layer was extracted with ethylacetate, dried, and evaporated to give

BOC-(εZ)-lysyl-(εZ)-lysine amide (structure 8). BOC-(εZ)-lysyl-(εZ)-lysyllysine amide (structure 8) was recovered and dissolved in a 1:1 solution of trifluoro-

acetic acid:dichloromethane without further purification, and the mixture was stirred at room temperature for 30 min. The solvent was evaporated and the

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ature. The organic layers were washed with saturated sodium bicarbonate solution and the aque-

ous layer was extracted with ethylacetate, dried, and evaporated to give

BOC-(εZ)-lysyl-(εZ)-lysyllysine amide (structure 10) as a white solid. BOC-(εZ)-lysyllysine amide (structure 10) was recovered and dissolved in methanol without further purification, and the solution was degassed for 15 min with nitrogen. The solution was then hydrogenated at room temperature using 10% palladium on carbon under a hydrogen atm-

osphere. The catalyst was filtered off, and the solution was concentrated under reduced pressure to give BOC-lysyllysyllysine amide (structure 11) as a pale

yellow oil. THF was added to dried N-maleoyl-β-alanine, and the mixture was

stirred at −20°C for 10 min. Ethyl chloroformate and N-methylmorpholine were then added, and the reaction mixture stirred at −20°C for 30 min. BOC-lysyllysyllysine amide (structure 11) was used without prior purification by dissolving in dry DMF and adding to the mixture maintaining the reaction

line were added. After 30 min, (εZ)-lysyllysyllysine amide (structure 7) was added and the reaction mixture was allowed to warm to room temperature. The organic layers were washed with saturated sodium bicarbonate solution and the aque-

ous layer was extracted with ethylacetate, dried, and evaporated to give

BOC-(εZ)-lysyllysyllysine amide (structure 8). BOC-(εZ)-lysyllysyllysine amide (structure 8) was recovered and dissolved in a 1:1 solution of trifluoro-

acetic acid:dichloromethane without further purification, and the mixture was stirred at room temperature for 30 min. The solvent was evaporated and the

residue triturated with ether and dried to give (εZ)-lysyllysyllysine amide

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Fig. 2. Schematic diagram of the synthesis of the trivalent linker CT998 (structure 16).
The tri-maleimide of BOC-lysyllysyllysine amide (structure 12) was dissolved in a mixture at —20°C. The mixture was allowed to warm to room temperature and dried to give lysyllysyllysine amide (structure 13) as a white solid. The crude material (structure 15) was then dissolved in DMF and the tri-maleimide of BOC-lysyllysyllysine amide (structure 12) was dissolved in a 1:1 solution of trifluoroacetic acid:dichloromethane, and the mixture was stirred at room temperature for 30 min. The solvent was evaporated and the residue triturated with ether to precipitate the trifluoroacetate salt and freeze-dried to give lysyllysyllysine amide (structure 13) as a white solid. The 12-N-4 macrocycle derivative (structure 2) was reacted with bis-(p-nitrophenyl)succinate in dimethylsulphoxide in the presence of N-methylmorpholine at 20°C for 3 h to yield the active ester (structure 15) as a brown solid. The signal remaining was expressed as a percentage of that seen when only excess free antibody had been washed away, and plotted against time for dissociation.

Radiolabeling and Animal Studies. Labeling with 125I was carried out with Bolton-Hunter reagent (Amersham) to a specific activity of 0.5—1 μCi/μg. For studies with 99mTc-labeled IgG, the macrocyclic chelator 12-N-4 was conjugated onto cB72.3 as described previously (30). DFM and TFM for 99mTc studies contained macrocycle in the cross-linker and were not further modified. Proteins containing macrocycle were prepared under metal-free conditions to minimize any contamination of the macrocycle before labeling. 99mTc labeling was carried out on preparations dialysed previously into 0.1 M potassium acetate buffer (pH 6) at concentrations of 1 mg/ml or greater. [99mTc]Cl3 (Amersham) was added to the required specific activity, ensuring that the buffer present was sufficient to neutralize the acidic [99mTc]Cl3, and the preparation incubated at 37°C for 15 min. The labeling was then quenched by the addition of 10 mM DTPA, followed by further incubation at 37°C for 10 minutes. The extent of labeling was assessed by HPLC gel filtration as described above and any free 99mTc removed by HPLC or desalting.

Proteins labeled with 125I or 99mTc were analyzed by SDS-PAGE/autoradiography and immunoassay to determine the quality of the labeled preparations.

Nude mouse studies were carried out using the LS174T xenograft system as described previously (14).

RESULTS

Expression of cB72.3 Fab' ΔCy and Preparation of DFM and TFM. cB72.3 Fab' ΔCy was expressed in gene-amplified CHO cells with a yield of approximately 200 mg/liter after purification. The material recovered from cell supernatants was found to be almost exclusively Fab' with very little F(ab')2 produced, consistent with results obtained with cB72.3 Fab' with two hinge disulphides (23). Thiol titrations, performed by reaction with diithiodyridine, demonstrated that the hinge thiol was not readily available, although a free thiol could be generated by mild reduction with β-mercaptoethanol under conditions which were shown not to liberate free light and heavy chains (data not shown), suggesting that heavy-light disulphide bonds were not disrupted. After reduction with these conditions, thiol titrations reproducibly gave values in the range 0.9—1.2 thiol per Fab', again suggesting generation of the hinge cysteine in the free thiol form.

Initial experiments were carried out at various cross-linker:Fab' ratios to determine the optimum protocol for the preparation of DFM and TFM; these experiments are described in "Materials and Meth-
binding experiments, the relative potency of TFM compared to IgG was improved by a mean value of 1.85-fold (SD, 0.34-fold).

The improved avidity of TFM over IgG was also demonstrated by analysis of dissociation after binding to antigen-coated plates. Results from this experiment (Fig. 4b) demonstrated considerably slower dissociation from the antigen for the trivalent TFM compared to divalent IgG.

A tetravalent maleimide linker has also been synthesized and used to produce cB72.3 tetra-Fab. Neither avidity nor targeting were improved over TFM; therefore, the data are not presented here. A recent report has revealed similar findings for a tetra-Fab of the antibody CC49 (31).

Expression of scFv'@Cys and Preparation of Di- and Tri-scFv. scFv'@Cys was recovered from shaker flask E. coli supernatant at a yield of approximately 20 mg/liter, similar to the yield of unmodified scFv (27). Initial experiments demonstrated that some cleavage of the hinge peptide was apparently taking place, but this was simply overcome by the inclusion of a cocktail of protease inhibitors in the culture medium prior to purification. Cross-linking of scFv'@Cys was difficult due to problems with aggregation and precipitation of the protein, probably due to the tendency of scFv to aggregate (27); however, cross-linking yields of approximately 44% for di-scFv and 25% for tri-scFv were achieved, which were sufficient to purify enough of the multi-scFv proteins for further analysis. SDS-PAGE analysis of the purified scFv proteins demonstrated that proteins of the correct size were produced (Fig. 5). Antigen-binding analysis again demonstrated that di-scFv and tri-scFv were more potent than the monovalent scFv, again showing the increase in valency giving rise to improved antigen binding (data not shown).

Evaluation of 125I-cB72.3 Fragments in Nude Mice Bearing LS174T Xenografts. The biodistribution of cB72.3 IgG compared to F(ab')2 and smaller fragments in nude mice bearing LS174T xenografts has been reported previously (14). The F(ab')2 used in these previous experiments was produced with the native IgG4 hinge region and thus contained two hinge cysteine residues forming two inter-heavy chain disulphide bonds. cB72.3 Fab'@Cys in the present study, however, contained a single hinge cysteine residue. The two types of F(ab')2 were compared for in vivo stability in a mouse LS174T xenograft biodistribution experiment with time points at 6 and 24 h (data not shown). The biodistribution of cB72.3 F(ab')2 proved to be very similar to that observed previously (14) with relatively rapid

**Fig. 4.** Antigen-binding analysis of cB72.3 IgG and cross-linked fragments. (a) Typical results from an assay measuring direct binding to a mucin coated plate comparing Fab'@Cys, DFM, TFM, and IgG. (b) Dissociation assay from mucin-coated plates comparing IgG and TFM. Points, mean of four estimations; bars, SD.

**Fig. 5.** SDS-PAGE analysis of purified B72.3 scFv'@Cys and cross-linked forms under reducing conditions. Lane 1, marker proteins (relative molecular mass indicated); Lane 2, scFv'@Cys; Lane 3, di-scFv; Lane 4, tri-scFv. kDa, molecular weight in thousands.
blood clearance and the accumulation of only approximately 1.2% injected dose/g in the tumor at 24 h. The biodistribution of F(\text{ab}')\text{2} \Delta\text{Cys} was very similar to cB72.3 F(\text{ab}')\text{2}, suggesting that there is no significant difference in the in vivo stability of these two species.

The divalent species cB72.3 IgG, F(\text{ab}')\text{2} \Delta\text{Cys}, and DFM were then compared for in vivo behavior. Chemically cross-linked F(\text{ab}')\text{2} fragments, DFM, showed blood clearance slower than F(\text{ab}')\text{2} but still significantly faster than IgG (Table 1). Tumor levels with DFM were intermediate between IgG and F(\text{ab}')\text{2}, probably as a result of the intermediate blood activity levels (Fig. 6). However, the net result of this altered biodistribution was the generation of greatly improved tumor:blood ratios for DFM at later time points compared to both IgG and F(\text{ab}')\text{2} (Table 2). With iodinated DFM there was no accumulation of activity in any nonspecific tissue.

In subsequent experiments the biodistribution of IgG was compared with both DFM and TFM (Fig. 7). The biodistribution of IgG and DFM was very similar to that achieved in the previous experiment (Fig. 6), with slight differences observed in the rate of clearance from the blood responsible for any slight differences, as evidenced by the very similar tumor:blood ratios seen between experiments. The blood clearance of TFM was surprisingly rapid considering that the molecular weight of the trivalent fragment is very similar to that of IgG at approximately M, 150,000 (Table 1). The slower \( \alpha \) phase clearance of TFM compared to DFM resulted in a higher level of tumor activity for TFM. This was achieved at a very similar tumor:blood ratio (Table 3), suggesting that there is little difference in the targeting ability of these cross-linked fragments in vivo, the level of tumor activity being determined by the blood level achieved (Fig. 7).

Iodinated scFv was compared to di-scFv and tri-scFv in a separate

Table 1 Blood clearance half-lives of \( ^{125}\text{I}-\text{labeled cB72.3 IgG and fragments in nude mice} \)

<table>
<thead>
<tr>
<th></th>
<th>( t_{1/2} \alpha ) (hours)</th>
<th>( t_{1/2} \beta ) (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>scFv</td>
<td>0.23</td>
<td>4.00</td>
</tr>
<tr>
<td>F(\text{ab}')\text{2} \Delta\text{Cys}</td>
<td>0.87</td>
<td>26.10</td>
</tr>
<tr>
<td>DFM</td>
<td>3.14</td>
<td>21.20</td>
</tr>
<tr>
<td>TFM</td>
<td>7.20</td>
<td>22.50</td>
</tr>
<tr>
<td>IgG</td>
<td>11.10</td>
<td>56.40</td>
</tr>
</tbody>
</table>

Table 2 Tumor:blood ratios of percentage injected dose/g figures from the biodistributions of \( ^{125}\text{I}-\text{labeled cB72.3 IgG, F(\text{ab}')\text{2} \Delta\text{Cys} and DFM in nude mice bearing s.c. LS174T tumor xenografts at 6, 24, 48, 72, and 144 h (no data available for F(\text{ab}')\text{2} \Delta\text{Cys at 48 and 144 h)} \)

<table>
<thead>
<tr>
<th>Hours</th>
<th>IgG</th>
<th>F(\text{ab}')\text{2} \Delta\text{Cys}</th>
<th>DFM</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>0.48 ± 0.14</td>
<td>0.96 ± 0.14</td>
<td>1.11 ± 0.22</td>
</tr>
<tr>
<td>24</td>
<td>0.77 ± 0.27</td>
<td>2.45 ± 0.32</td>
<td>3.33 ± 0.88</td>
</tr>
<tr>
<td>48</td>
<td>0.57 ± 0.24</td>
<td>1.70 ± 0.23</td>
<td>4.72 ± 1.66</td>
</tr>
<tr>
<td>72</td>
<td>0.57 ± 0.24</td>
<td>1.70 ± 0.23</td>
<td>4.72 ± 1.66</td>
</tr>
<tr>
<td>144</td>
<td>0.57 ± 0.24</td>
<td>1.70 ± 0.23</td>
<td>4.72 ± 1.66</td>
</tr>
</tbody>
</table>

Table 3 Tumor:blood ratios of percentage injected dose/g figures from the biodistributions of \( ^{125}\text{I}-\text{labeled cB72.3 IgG, DFM, and TFM in nude mice bearing s.c. LS174T tumor xenografts at 6, 24, 48, 72, and 168 h (no data available for DFM at 168 h) \)

<table>
<thead>
<tr>
<th>Hours</th>
<th>IgG</th>
<th>DFM</th>
<th>TFM</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0.20 ± 0.05</td>
<td>0.88 ± 0.09</td>
<td>1.30 ± 0.45</td>
</tr>
<tr>
<td>24</td>
<td>0.57 ± 0.18</td>
<td>1.61 ± 0.23</td>
<td>3.41 ± 0.34</td>
</tr>
<tr>
<td>48</td>
<td>0.57 ± 0.18</td>
<td>1.61 ± 0.23</td>
<td>3.41 ± 0.34</td>
</tr>
<tr>
<td>72</td>
<td>0.23 ± 0.08</td>
<td>1.75 ± 0.36</td>
<td>4.41 ± 0.68</td>
</tr>
<tr>
<td>168</td>
<td>0.23 ± 0.08</td>
<td>1.75 ± 0.36</td>
<td>4.41 ± 0.68</td>
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</table>

Fig. 6. Biodistribution of \( ^{125}\text{I}-\text{labeled cB72.3 IgG ( ), F(\text{ab}')\text{2} \Delta\text{Cys ( ) and DFM ( )} in nude mice bearing s.c. LS174T tumor xenografts at (a) 6, (b) 24, and (c) 72 h. Columns, mean of groups of four animals at each time point; bars, SD.\)
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(a) 4 hours

(b) 24 hours

(c) 72 hours

Fig. 8. Biodistribution of ¹²⁵I-labeled B72.3 scFv'ΔCys (■), di-scFv (□), and tri-scFv (▲) in nude mice bearing s.c. LS174T tumor xenografts at (a) 4 and (b) 24 h. Columns, mean of groups of four animals at each time point; bars, SD.

Evaluation of ⁹⁰Y-cB72.3 Fragments in Nude Mice Bearing LS174T Xenografts. Radiolabeling of IgG-12-N-4, DFM, and TFM with ⁹⁰Y was achieved to specific activities of 5 μCi/μg with incorporation efficiencies of >90%. To ensure high purity for the material tested in vivo, all conjugates were purified by HPLC before injection, and the purity was tested using SDS-PAGE/autoradiography. High purity preparations were obtained (data not shown); in addition, enzyme-linked immunosorbent assays of the labeled preparations demonstrated that immunoreactivity after labeling was equivalent to that before labeling (data not shown).

The biodistribution in normal mice of ⁹⁰Y-labeled B72.3 chelated through the 12-N-4 macrocycle has been reported previously, and dem-
The low femur levels reported for \(^{90}\)Y-labeled B72.3-12-N-4 conjugates demonstrated the stability of the 12-N-4 conjugate (30). The biodistribution of \(^{90}\)Y-labeled cB72.3 IgG4(12-N-4) in nude mice bearing LS174T xenografts at 24, 48, and 144 h is shown in Fig. 9. Comparison of this data with that for the \(^{125}\)I-labeled IgG (Figs. 6 and 7) revealed similar blood activity levels, and similar activity levels accumulated in the tumor. The low femur levels reported for \(^{90}\)Y-labeled B72.3-12-N-4 conjugates reported by Harrison et al. (30) were reproduced for cB72.3 in this model, emphasizing the stability of the \(^{90}\)Y-labeled 12-N-4 complex. Relatively high levels of \(^{90}\)Y were seen in the kidney with this antibody as expected from a chimeric IgG4 antibody labeled with a metallic radionuclide. This high kidney level has been shown to be due to the proportion of human IgG4 molecules which do not completely form inter-heavy chain disulphide bonds (34).

\(^{90}\)Y-labeled cB72.3 DFM and TFM were also evaluated in nude mice bearing LS174T xenografts. The biodistribution of \(^{90}\)Y-labeled cB72.3 DFM is shown in Fig. 10. Blood levels of activity for \(^{90}\)Y-labeled cB72.3 DFM were similar to those achieved with iodinated DFM (Fig. 7), but tumor levels accumulated were higher (for example, 3.25 ± 0.25% injected dose/g on the tumor for \(^{125}\)I-labeled DFM at 24 h compared to 9.41 ± 0.27 for \(^{90}\)Y-labeled DFM). The most prominent feature of the biodistribution of DFM was the very high accumulation of activity in the kidney, consistent with results seen previously with metal-labeled antibody fragments including F(ab')2, Fab', and scFv fragments (15, 32, 33). In contrast \(^{90}\)Y-labeled TFM was not accumulated in the kidney but cleared effectively and gave tumor levels similar to those seen with \(^{90}\)Y-labeled DFM (Fig. 11). Indeed, for \(^{90}\)Y-labeled TFM there was no apparent accumulation in any nonspecific tissue. As for DFM, the blood levels observed for TFM were similar to those of the molecule labeled with iodine, and again activity persisted on the tumor for longer.

**DISCUSSION**

Over the last few years many conjugates of antibodies to tumor-associated antigens have been prepared and evaluated for their ability to target a cytotoxic dose of a radioisotope, drug, or protein toxin to tumor cells. Clinical effectiveness has largely been restricted to easily accessible tumors such as leukemias and lymphomas (reviewed in Ref. 35), and several major problems have been identified which limit usefulness for therapy of solid tumors. We have embarked on a comprehensive program to attempt to develop a new generation of effective antibody molecules for tumor imaging and therapy and have addressed some of these problems. Since the levels of antibody targeted to solid tumors in clinical studies are low, it is unlikely that a single dose of antibody conjugate will be sufficient to give sustained antitumor effects. The development of conjugates of low immunogenicity is therefore important to allow repeated administration. This has been addressed by the development of recombinant chimeric and humanized antibodies which offer reduced immunogenicity in the clinic (3). However, the problem of extended circulating half-life of these antibodies leading to bone marrow toxicity also needs to be addressed. The fragments developed here offer one approach to this problem by improving tumor:blood ratios over the whole antibody, together with improving total tumor dose over conventional F(ab')2 fragments. Previous work comparing IgG with conventional F(ab')2 and Fab' fragments for radioimmunotherapy, both theoretically (18) and practically (13, 36), has indicated a potential advantage for the F(ab')2 fragment, particularly if stably labeled with \(^{90}\)Y and if the problem of kidney accumulation could be overcome (18).

Early studies with \(^{90}\)Y-labeled antibodies and fragments used the weak chelator DTPA, which led to extensive leakage of \(^{90}\)Y from the
conjugate and subsequent deposition of the isotope in bone (37, 38). Since then more stable chelators have been developed including the macrocyclic ligand 12-N-4, which avoids $^{90}$Y leakage and reduces bone uptake (30). The linkers used in this study incorporate the 12-N-4 macrocycle and have the additional advantage of being attached to antibody fragments in a site-specific manner, away from the antigen-binding site, such that high specific activity conjugates can be prepared with no loss of immunoreactivity. There have been some preliminary reports using a related macrocyclic structure (DOTA) that macrocyclic ligands can be immunogenic in patients (39). The DOTA used in this study varies from the 12-N-4 structure used here in the presence of an aromatic ring in the linkage to antibody. The immunogenicity of the 12-N-4 ligand used in these studies is unknown either when attached to IgG randomly or when attached in a site-specific manner to DFM or TFM.

The mechanisms of metabolism and clearance of antibody fragments are poorly understood. Our initial rationale for cross-linking fragments to increase their circulating half-life was based on the idea that larger fragments may be filtered more slowly through the kidney, which is thought to have a size-related cutoff (40). This was apparently successful in that clearance from the blood was slowed by stabilizing the linkage between Fab' fragments from a relatively labile disulphide bond in F(ab')$_2$ to the thioether linkage in DFM. We also have evidence that stabilization of the F(ab')$_2$ inter-heavy chain bonds by the introduction of three extra inter-heavy chain disulphide bonds in the hinge region can also slow clearance in the same way as chemical cross-linking. The biodistribution of this F(ab')$_2$ with multiple disulphides was almost identical to DFM. Similarly, an engineered F(ab')$_2$ produced with multiple disulphides in the hinge region has recently been produced by Rodrigues et al. (41), and this also resulted in increased in vivo stability and slower clearance kinetics. However, clearance from the circulation is obviously a complex process which is not dependent simply on size, as evidenced by the dramatic difference in blood levels seen with the two $M_r$ 150,000 molecules cB72.3 IgG and TFM.

Bis-maleimide cross-linkers have been used previously in the preparation of bispecific di-Fab molecules (42, 43) and also in the preparation of di-Fabs of both an antibody to ferritin and a humanized anti-p185HER2 antibody (24, 44). Also, both bi- and tri-specific tri-Fab' has been prepared previously using bismaleimide linkers coupled to Fab' fragments with multiple hinge thiols (45, 46); a similar strategy has also been adopted by others to produce divalent scFv's (47, 48). Cross-linking of fragments with multiple cysteine residues in the hinge region proved to result in comparatively low yields in these studies, demonstrating the benefit of designing the antibody hinge region specifically to contain one cysteine residue for cross-linking. In addition, because there is no need for further chemical modification of DFM or TFM to achieve radiolabeled conjugates, a very homogeneous reagent is produced which may have advantages in terms of batch to batch variation and regulatory requirements.

The domain structure of IgG facilitates the production of a wide range of engineered antibody fragments, and we have examined a number of such antibody fragments for their ability to target tumors effectively in the nude mouse LS174T xenograft system using chimeric B72.3 as a model antibody. Chimeric F(ab')$_2$ and Fab' gave only a small improvement in tumor:blood ratio compared to IgG with reduced tumor loading (14). Smaller fragments such as Fv (14, 49) cleared extremely rapidly from the circulation, leading to very low levels of tumor accumulation, but nevertheless increased tumor:blood ratios. We have demonstrated that B72.3 scFv also clears very rapidly, with low levels of tumor accumulation observed. Similar findings have been reported for other scFv fragments (12, 48). Thus, these fragments are potentially useful for tumor-imaging strategies but are unlikely to be capable of delivering therapeutic doses of a cytotoxic agent. Multivalent forms of B72.3 scFv still cleared from the circulation extremely quickly, although clearance appeared to be size-related at least at the early time point (tri-scFv was slower than di-scFv, which was slower than monomeric scFv). This resulted in low tumor doses achieved, even with tri-scFv. In addition, we have found that all of these fragments result in significant accumulation in the kidney when labeled with metallic isotopes such as $^{90}$Y or $^{111}$In (data not shown). This is also the experience of other groups (15, 33). Small antibody fragments such as scFv may have a significant advantage in terms of penetration into a tumor mass (50), and it will be important to determine the size limits for effective penetration of particular antibody fragment conjugates into their respective tumor types. Overall, our conclusions from these studies are that antibody fragments should be tailored differently for different clinical applications. For example, in tumor imaging a very rapidly clearing fragment which may localize to only a small extent but penetrate well to give an even distribution throughout the tumor and generate high tumor to background ratios may be ideal. However, for tumor therapy a larger absolute dose is required, which could be delivered using a molecule such as the DFM or TFM reported here. In particular the lack of kidney accumulation of $^{90}$Y with TFM may allow the first attempts at therapy with an $^{90}$Y-labeled antibody fragment in cancer patients.

We have developed this cross-linking and labeling technology using cB72.3, but the methodology can be easily transferred to other recombinant antibodies. Experiments are currently under way to test the applicability of DFM and TFM for tumor targeting with other recombinant humanized antibodies.

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Improved Tumor Targeting with Chemically Cross-Linked Recombinant Antibody Fragments

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