Pharmacokinetics and Biodistribution of Engineered Single-Chain Antibody Constructs of MAb CC49 in Colon Carcinoma Xenografts

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Monoclonal antibodies (MAbs) have been proven useful in clinical studies for both diagnostic and therapeutic applications. The single-chain Fv (scFv) construct made from MAbs has potential applications for improved cancer diagnosis and therapy. A new CC49 scFv construct recognizing a tumor-associated mucin, TAG-72, was engineered and evaluated by immunological, pharmacokinetic and biodistribution analysis. **Methods:** The CC49 scFv construct was generated in which the V_L and V_H variable region genes were joined together with a 25-amino acid helical linker (205C). The new CC49 scFv(205C) was expressed as a monomer as well as a stable noncovalent dimer ($[scFv]_2$). The pharmacokinetic, biodistribution and tumor targeting characteristics of radiolabeled CC49 scFv were compared with CC49 IgG and enzymatically derived fragments $F(ab')_2$ and Fab', using the athymic mice bearing human colon cancer xenografts. **Results:** The association constant (K_A) for the intact CC49, dimeric scFv $(scFv)_2$ and monomeric scFv were 1.7×10^9, 1.99×10^9 and 0.52×10^9 M^{-1} by Scatchard analysis and 1.14×10^8, 4.46×10^7 and 1.5×10^7 M^{-1}, respectively, by BIAcore analysis. Pharmacokinetic studies showed that more than 50% of monomeric scFv (~27 kDa) was cleared from the blood in less than 10 min. The CC49 Fab' generated enzymatically from the parent murine Mab' (50 kDa) had a blood clearance that was faster than that of the $(scFv)_2$ (60 kDa) with half of the activity cleared from the serum within 30 and 50 min, respectively. The CC49 dimeric scFv(205C) showed a two-fold higher tumor uptake (than scFv or Fab') reaching 10 %ID/g at 60 min after injection. The scFv dimer also showed an excellent stability and increased avidity in vivo compared with the monomer, as demonstrated by the longer retention in tumor with 3 %ID/g remaining at 48 h. **Conclusion:** The rapid clearance from the blood, higher tumor uptake and longer retention of the stable dimer of CC49 scFv make it an important agent for potential imaging and therapeutic applications.

Key Words: monoclonal antibodies; single chain antibodies; radioimmunodiagnosis; colon carcinoma xenografts; antibody engineering

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Maximal tumor targeting with minimal background or minimal exposure of normal organs is the goal for the clinical application of monoclonal antibodies (MAbs) for cancer diagnosis and therapy. Genetic engineering provides powerful tools for manipulating the structure and properties of antibodies. One useful strategy has been production of single-chain antibody fragments (scFv) comprised the variable regions of the immunoglobulin heavy and light chain, covalently connected by a flexible peptide linker (*1*). Several anticarcinoma scFvs have been evaluated for their specific in vivo tumor targeting to antigens such as TAG-72, carcinoembryonic antigen (CEA) and the c-erbB-2 receptor. They demonstrated more rapid clearance and higher tumor-to-normal tissue ratios than corresponding IgG or Fab' fragments (*1–4*). Furthermore, the excellent penetration of scFv into a tumor from the vasculature, as demonstrated by microscopic autoradiography, was superior to that of corresponding intact IgG, $F(ab')_2$ or Fab' (*5*). However, because of a rapid clearance of these 25- to 30-kDa proteins from the blood pool, the absolute amount of the tumor uptake by the monomeric scFvs is limited.

Biodistribution studies have demonstrated that scFv dimers showed improved tumor targeting compared with the corresponding monomeric forms. Therefore, different strategies are being explored for the formation of scFv dimers to optimize tumor uptake and clearance properties. Adams et al. (*3*) described a two-fold improvement in in vivo tumor targeting using divalent forms of the anti-erbB-2 scFv with a C-terminal Gly_4Cys joined by a disulfide bond. Another novel engineered antibody fragment-minibody was produced by fusion of T84.66 anti-CEA scFv to the human IgG1 C_H3 domain (*6*). Others have fused scFvs to protein domains capable of multimerization, e.g., leucine zipper proteins (*7*), streptavidin (*8*) or the κ-constant region (*9*), to promote dimer formation. The easiest approach for the production of dimeric scFvs is based on spontaneous formation of noncovalent dimers such as 50-kDa diabodies (*10*). Biodistribution studies by Wu et al. (*4*) showed improved targeting by a noncovalent dimeric form of T84.66 scFv, with tumor uptake reaching the range of 5 to 15 %ID/g compared with their monomeric form (1–5 %ID/g).

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MAb CC49 recognized a unique Sialyl-Tn antigen present on tumor-associated mucin, TAG-72, expressed by most human adenocarcinomas. Radiolabeled MAb CC49 showed excellent tumor localization in the imaging of colorectal and ovarian carcinomas as well as in passive and active immunotherapy trials. We have developed a new CC49 scFv construct that forms a monomeric scFv that associates into a stable high-affinity noncovalent dimer [$(scFv)_2$]. The biochemical and binding properties of CC49 scFv(205C) and $(scFv)_2$(205C) were compared with CC49 IgG and enzymatically derived fragments.

MATERIALS AND METHODS

Purification of Monoclonal Antibody CC49 IgG and Enzymatic Fragments

MAb CC49 IgG was developed by the immunization of mice with purified TAG-72 as previously described (*11,12*). CC49 IgG was purified from ascitic fluid obtained from pristane-primed BALB/c mice. The resulting ascitic fluid was diluted 1:1 with 20 mmol/L sodium phosphate (pH 7.0) and loaded on Protein G Sepharose 4 Fast Flow (Pharmacia Biotech, Piscataway, NJ). The antibody was eluted with 100 mmol/L glycine (pH 2.7) and immediately adjusted to a neutral pH. Fab′ and $F(ab')_2$ fragments were generated by the digestion of the purified IgG with pepsin, as previously described (*13*). Briefly, to generate Fab′ fragments, CC49 IgG was incubated with 1,4-dithiothreitol and then iodoacetamide. The dialyzed IgG was digested with pepsin at 37°C for 18 h. The Fab′ fragments were separated from the other digestion products by gel filtration through a Sephacryl S-200 HR column (Pharmacia Biotech). $F(ab')_2$ fragments were similarly prepared without the use of 1,4-dithiothreitol or the iodoacetamide treatment. The protein concentration of purified Ab and fragments was determined by the method of Lowry et al. (*14*).

Construction of Expression Vector

The CC49 scFv was constructed through a collaborative effort of the National Cancer Institute Laboratory of Tumor Immunology (Bethesda, MD) and the Dow Chemical Company (Midland, MI). The CC49 scFv gene (V_L-linker-V_H) was constructed using the polymerase chain reaction method by combining the V_L sequence and the V_H sequence by a linker sequence (*15*). The previously published 205C linker (*16*) was modified to accommodate restriction sites by changing the terminal amino acids, resulting in the sequence LSADDAKKDAAKKDDAKKDDAKKDL, and used for scFv construction. The sequences of the variable domains of CC49 have been reported by Rixon et al. (*17*). The sequence of CC49 scFv(205C) was confirmed by deoxyribonucleic acid sequencing. The fragment was cloned into the pRW83 vector that contains a chloramphenicol resistance gene for selection, a *penP* gene with a penP promotor and terminator, and a pelB signal peptide that directs the recombinant protein to the periplasm resulting in a biologically active protein (*18*).

CC49 Single-Chain Fv Protein Purification

The expression vector containing the CC49 scFv sequence was transformed into *Escherichia coli* strain AG1 using Strategene competent cells (La Jolla, CA) according to the manufacturer's protocol. The bacteria were grown at 37°C in Luria-Bertani medium containing 34 μg/mL chloramphenicol. The CC49 scFv protein was prepared from periplasmic fraction of 1.0 L overnight cultures. The cells were centrifuged at 5000*g* for 20 min, washed with 10 mmol/L Tris-HCl (pH 7.3), 30 mmol/L NaCl and recentrifuged. The cell pellets were resuspended in 100 mL 30 mmol/L Tris-HCl (pH 7.3) and 100 mL 30 mmol/L Tris-HCl, 40% w/v sucrose, 10 mmol/L ethylenediaminetetraacetic acid (EDTA) was added. After 10 min incubation at room temperature, the hypertonic cells were centrifuged, resuspended in ice cold 0.5 mmol/L $MgCl_2$ and recentrifuged as described above. The supernatant containing the periplasmic fraction was filtered on a 0.2-μm filter (Nalgene Co., Rochester, NY) and dialyzed against 20 mmol/L bis-Tris propane (pH 9.1) and applied to a Mono-Q HR 16/10 column (Pharmacia Biotech). The CC49 scFv was eluted with 40 mmol/L sodium chloride in 20 mmol/L bis-Tris propane (pH 9.1). Fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 15% polyacrylamide gel (*19*) and by enzyme-linked immunoabsorbent assay (ELISA) (*20*). Dimeric scFv was separated from the monomeric form by size exclusion chromatography using a Superdex 75 column (1.6 × 70 cm; Pharmacia Biotech). The concentration of purified fragments as determined by the method of Lowry et al. (*14*).

Labeling of CC49 Ig Forms and Single-Chain Fv

MAb CC49 IgG, $F(ab')_2$, Fab′ and the scFv forms were labeled with $Na^{125}I$ or $Na^{131}I$ using Iodo-Gen (Pierce Chemical, Rockford, IL) as described by Colcher et al. (*13*). The iodination produced specific activities of approximately 111–333 MBq/mg (3–9 mCi/mg).

High-Performance Liquid Chromatography

Samples were injected onto TSK G3000SW and TSK G2000SW (Toso Haas, Tokyo, Japan) size exclusion columns connected in series and eluted with 0.067 mol/L phosphate and 0.1 mol/L KCl buffer (pH 6.8) at a flow rate of 0.5 mL/min. Elution was monitored by absorption at 280 nm and by measuring the radioactivity of the eluted fractions in a gamma scintillation counter.

Solid Phase Competition ELISA

A competition ELISA was used to detect and quantify the specific immunoreactivity of MAb CC49 IgG, $F(ab')_2$, Fab′ and scFv forms at various steps in the purification process. Plates were coated with 50 ng/well of bovine submaxillary gland mucin (BSM) (Sigma, St. Louis, MO), which contains the epitopes recognized by MAb CC49 (*21*). Five microliters of the test samples were added to prepared plates in three-fold serial dilutions and incubated with 6 ng/well of biotinylated CC49 IgG for 2 h at room temperature. After washing with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA), the plates were incubated for 1.5 h with alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch Lab, West Grove, PA) and developed with p-nitrophenyl phosphate substrate. The optical density was determined using the Dynatech MR 5000 reader (Chantilly, VA). The percent of inhibition was compared on a molar basis. Relative affinity constants were calculated by a modification of the Scatchard method (*22,23*). The final concentration for each of the dilutions of the various CC49 Ig forms was calculated and slopes were determined by linear regression of the (Ab_{bound}) versus (Ab_{bound})/(Ab_{free}).

Solid Phase Radioimmunoassay

The quality control test of the radiolabeled CC49 forms was performed using an antibody capture assay with BSM as a positive control and BSA as a negative control. The proteins (1.66 mg/mL gel at 0.2 mol/L Na_2CO_3 pH 10.0) were attached to a solid-phase matrix (Reacti-Gel HW-65F) (Pierce) for 16 h. The coated beads were centrifuged at 475*g* for 5 min, washed with 1% BSA, 0.1% Tween 20 in PBS (pH 7.2) and suspended in 0.5 mL binding buffer (PBS containing 1% BSA). The radiolabeled samples (20 μL) were added to each tube containing 0.5 mL of beads, and the samples were vortexed every 10 min to ensure complete suspension. After a 1-h incubation at room temperature, the unbound radiolabeled protein was removed by washing with PBS containing 1% BSA, and the pellet was counted for 1 min in a gamma scintillation counter.

The binding sites of the CC49 forms were compared using an antibody competitive radioimmunoassay on BSM coated plates. The plates were prepared as described above for ELISA using 10 ng/well BSM. Three-fold serial dilution of each unlabeled sample was incubated overnight at 4°C with the appropriate radiolabeled CC49 form (100,000 cpm in 50 μL). The plates were washed with PBS containing 1% BSA, the individual wells were cut out and the bound radioactivity was measured in a gamma counter.

Surface Plasmon Resonance

The BIAcore biosensor (Pharmacia Biosensor, Uppsala, Sweden), which uses surface plasmon resonance detection and permits real-time kinetic analysis of two interacting species (*24*), was used to measure the binding kinetics of CC49 IgG, $F(ab')_2$, scFv monomers and dimers.

BSM was immobilized on a CM5 dextran sensor chip in 100 mmol/L sodium acetate (pH 3.0) using the Amine Coupling kit (Pharmacia Biosensor). The dextran layer of the sensor chip was activated by injecting 35 μL 0.05 mol/L N-hydroxysuccinimide and 0.2 mol/L N-ethyl-N-(3-diethylaminopropyl) carbodiimide. Next, 200 μg/mL BSM in 0.1 mol/L acetate buffer (pH 3.0) were injected. BSM was injected until a surface of 700 resonance units was realized. Excess reactive groups were then blocked by injection of 35 μL 1 mol/L ethanolamine/HCl (pH 8.5). A similar surface was made using BSA as a control. Binding analyses were performed in (HBS) buffer (10 mmol/L (N-[2-hydroxyethyl]piperazine-N'-[2-ethane sulfonic acid]) [HEPES] [pH 7.4], 0.15 mol/L NaCl, 3.4 μmol/L EDTA, 0.005% surfactant P20) at a flow rate of 30 μL/min at 25°C. The surface was regenerated with 6 mol/L guanidine, 0.2 mol/L acetic acid, at a flow rate of 5 μL/min with no loss of activity. Samples for binding analysis were diluted into the binding buffer (HBS). The association constant (K_A) and dissociation constant (K_D) were evaluated using the BIAevaluation 3.0 software (Biacore, Inc., Piscataway, NJ) supplied by the manufacturers where the experimental design correlated with the Langmuir 1:1 interaction model (*24*).

Biodistribution Analysis

Female athymic mice (nu/nu) obtained from Charles River (Wilmington, MA) at 4–6 wk old were injected subcutaneously on the back with 4×10^6 human colon carcinoma cells (LS-174T) (*25*). Tumor xenograft bearing animals were used for biodistribution study approximately 10 d after injection. Dual-label biodistribution studies were performed with mice injected through the tail vein with radiolabeled CC49 Ig forms (0.185 MBq [5 μCi] ^{125}I-labeled MAb and 0.0925 MBq [2.5 μCi] ^{131}I-labeled MAb). The CC49 scFv forms and Fab' fragments were coinjected in three sets of experiments: ^{125}I-scFv versus ^{131}I-Fab', ^{125}I-$(scFv)_2$ versus ^{131}I-$F(ab')_2$ and ^{125}I-scFv versus ^{131}I-$(scFv)_2$. At specific times, mice (groups of six) were killed, dissected and major organs were wet weighed and counted in a gamma scintillation counter. The percentage of the injected dose per gram of tissue (%ID/g) was calculated. The pharmacokinetic studies were conducted by obtaining blood samples (5 μL) by tail bleeds at various time points after injections of 0.37 MBq (10 μCi) of the radioiodinated CC49 forms (six mice/group). For the whole body retention studies mice bearing the LS-174T xenograft (three/group) were injected through a tail vein with 0.056 MBq (1.5 μCi) of radiolabeled CC49 forms and counted using a custom built NaI crystal at various times after injection. Data were analyzed using Graphpad Prism software (Graph Pad Software, Inc., San Diego, CA). A one-phase exponential decay curve fit was used for pharmacokinetic analyses of scFv fragments and Fab'. The data for $F(ab')_2$ and IgG were analyzed by two-phase exponential decay curve fit.

RESULTS

Characterization of Monoclonal Antibody CC49 Single-Chain Fv

The CC49 scFv(205C) was secreted as soluble, active protein using the pRW83 expression vector and purified by ion-exchange chromatography as described in the Materials and Methods section. The purified scFv was first analyzed by SDS-PAGE. The CC49 scFv was 99% pure and migrated consistently with its theoretical molecular weight (M_r) (Fig. 1). Additional high-performance liquid chromatography (HPLC) analysis of the purity of the CC49 scFv showed two peaks corresponding to the expected M_r 27,749 monomeric form (scFv) and a 60-kDa peak consistent with a dimeric form of the CC49 scFv [$(scFv)_2$]. The CC49 $(scFv)_2$ was

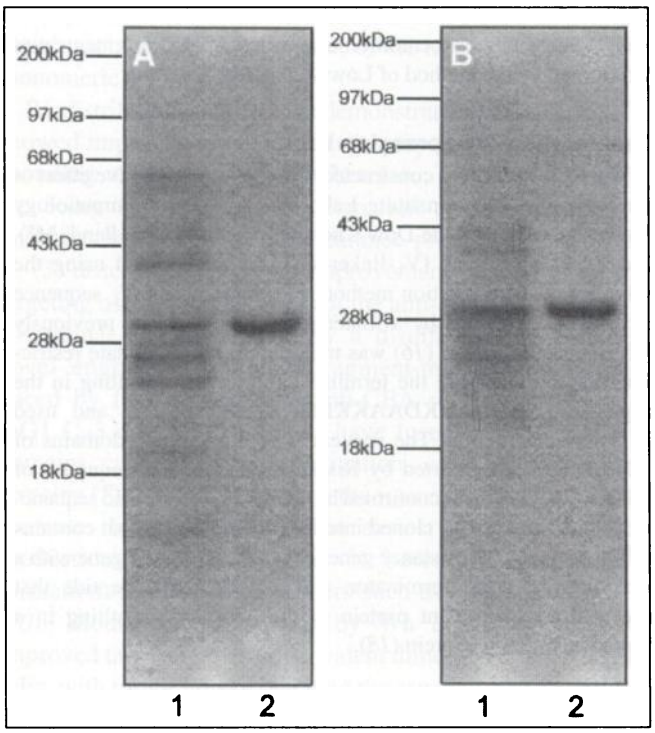

FIGURE 1. SDS-PAGE analysis of purified CC49 scFv under nonreducing (A) and reducing (B) conditions. Lane 1, crude periplasmic preparation; lane 2, purified scFv. Positions and relative molecular weight of marker proteins are indicated.

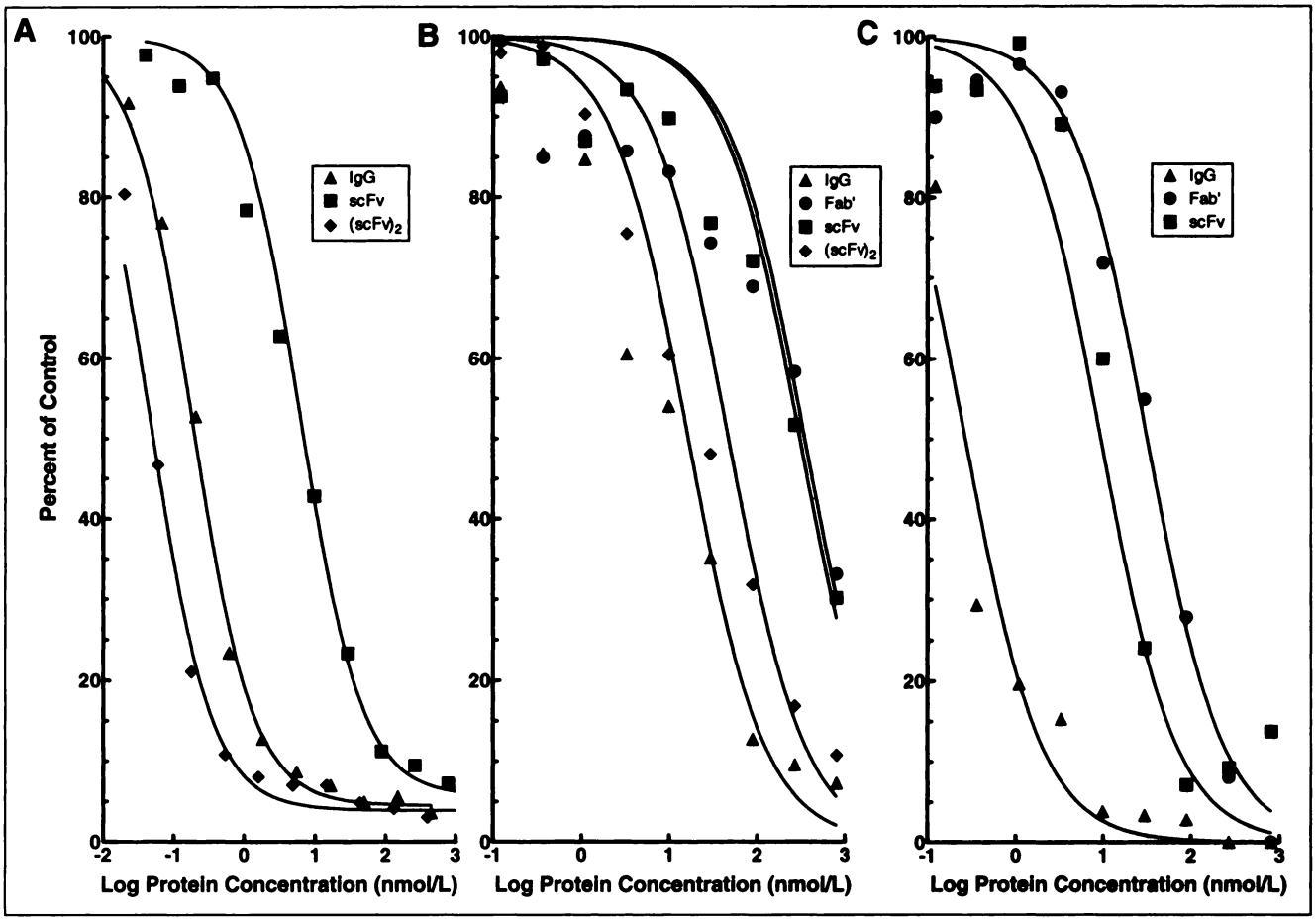

FIGURE 2. Competition assays for MAb CC49 and related constructs. (A) Biotinylated CC49 IgG binding to BSM in competitive ELISA. (B)^{125}I-labeled CC49 IgG binding to BSM in competitive radioimmunoassay. (C)^{125}I-CC49 scFv binding to BSM in competitive radioimmunoassay.

successfully separated from scFv by gel filtration, as described in the Materials and Methods section. The immunoreactivity and the binding properties of the scFv forms in each peak were tested in a competition ELISA. Both species competed with biotinylated CC49 IgG for the binding to BSM (Fig. 2). The dimeric $(scFv)_2$ and IgG were able to compete with the IgG at 10-fold lower molar ratios compared with the monomeric scFv. However, both scFv and $(scFv)_2$ were able to compete for 100% of the binding.

The apparent rate constants of the CC49 antibody constructs for the BSM antigen were measured using surface plasmon resonance. This technique allowed measurement of real-time interactions of the apparent rates of association and dissociation. The results presented in Table 1 show the apparent on (k_{on}) and off (k_{off}) rates for various antibody constructs. The affinity of the antibody constructs was also determined by Scatchard analysis (Table 1).

The purified scFv forms were radiolabeled with Na^{125}I to evaluate the effect of labeling on the immunoreactivity and the stability of the molecules. A single band representing approximately 98% of total incorporated radioactivity and corresponding to the molecular weight of the scFv molecule (28 kDa) was detected by SDS-PAGE. The dimeric and

TABLE 1
Apparent Kinetic Constants for Binding of CC49 Ig Forms to Immobilized BSM Determined in BIAcore and Scatchard Analyses

	BIAcore*			Scatchard†
	k_{on} [$M^{-1}\,s^{-1}$]	k_{off} [s^{-1}]	K_A [M^{-1}]	K_A [M^{-1}]
IgG	2.37×10^5	2.07×10^{-3}	1.14×10^8	1.70×10^9
$F(ab')_2$	6.44×10^4	6.20×10^{-4}	1.04×10^8	ND
scFv	7.64×10^3	4.35×10^{-4}	1.5×10^7	0.52×10^9
$(scFv)_2$	1.23×10^5	2.8×10^{-3}	4.46×10^7	1.99×10^9

*Kinetic constants were analyzed from association and dissociation phase using BIAcore biosensor and evaluated with BIAevaluation 3.0 software. $K_A = k_{on}/k_{off}$.

†Relative affinity constants were also calculated from competition ELISA using Scatchard method. Final concentration for each dilution of Ig forms and slopes was determined by linear regression.

BSM = bovine submaxillary mucin; ND = not determined.

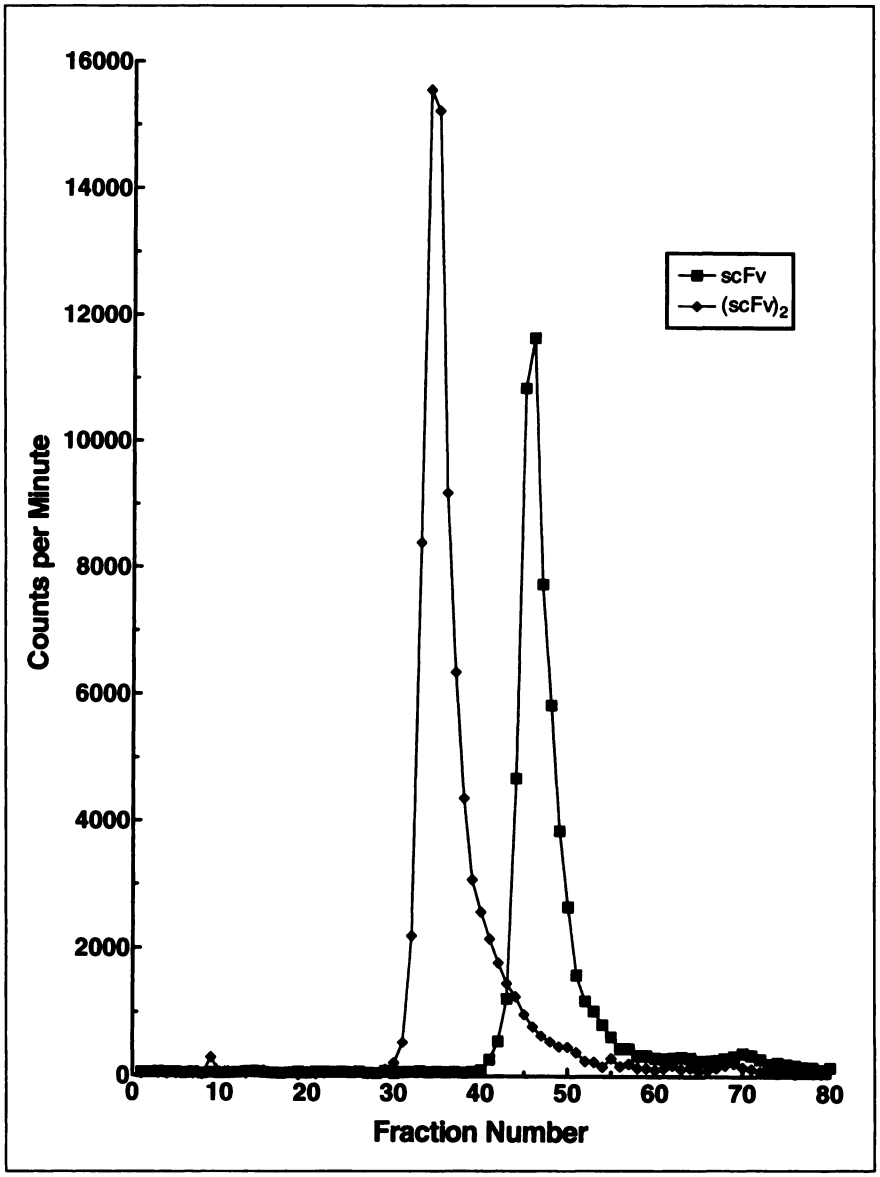

FIGURE 3. HPLC size-exclusion profiles of radiolabeled CC49 scFv and $(scFv)_2$. After radiolabeling ^{125}I-scFv and ^{125}I-$(scFv)_2$ were analyzed using TSK G3000SW and TSK G2000SW size-exclusion columns connected in series. The CC49 scFv was eluted as single peak of approximately 25 kDa, whereas CC49 $(scFv)_2$ was eluted as single peak of approximately 60 kDa, with no evidence of unassociated monomeric scFv.

monomeric scFvs, after radiolabeling, were also analyzed by HPLC (Fig. 3). Both scFv forms eluted as single peaks without any detectable aggregates, demonstrating that they were not altered by iodination. A small peak present corresponding to less than 2% free iodine was observed.

The immunoreactivity of the labeled forms was determined by solid phase radioimmunoassay using proteins attached to Reacti-Gel beads. BSM, which contains the epitope recognized by MAb CC49 on the human tumor-associated antigen (TAG-72), was used as a positive control, and BSA was used as a negative control. The monomeric scFv showed 70%–83% binding to BSM with only 1.2%–3% binding to BSA. The $(scFv)_2$ showed 88%–94% binding to BSM, which was similar to that obtained with intact antibody (87%–90%) or an antibody fragment with divalent binding, i.e., $F(ab')_2$ (81%).

Radiolabeled samples of the CC49 dimeric and monomeric scFv were stable for at least 35 d when stored at 4°C as determined by HPLC and radioimmunoassay. Furthermore, the immunoreactivity of both labeled forms was evaluated over this time using the direct binding assay as described above. The binding of the monomeric and dimeric scFv exhibited only minimal loss in immunoreactivity (~2%–3%) over this period. Radiolabeled scFv forms were also analyzed to determine their stability in serum. scFvs were mixed with murine serum or 1% BSA and incubated at 37°C for up to 4 d. Samples were taken at various times and were analyzed by HPLC size-exclusion chromatography. The monomer scFv and noncovalent dimeric scFv maintained their full integrity and stability throughout the testing period (data not shown). The serum samples analyzed from later time points, 24 and 48 h, demonstrated a small increase of higher molecular weight forms. This was probably a result of aggregation with serum proteins, as shown by the lack of detectable aggregates in the control samples. Unlabeled samples were stored at −70°C for more than 10 mo without

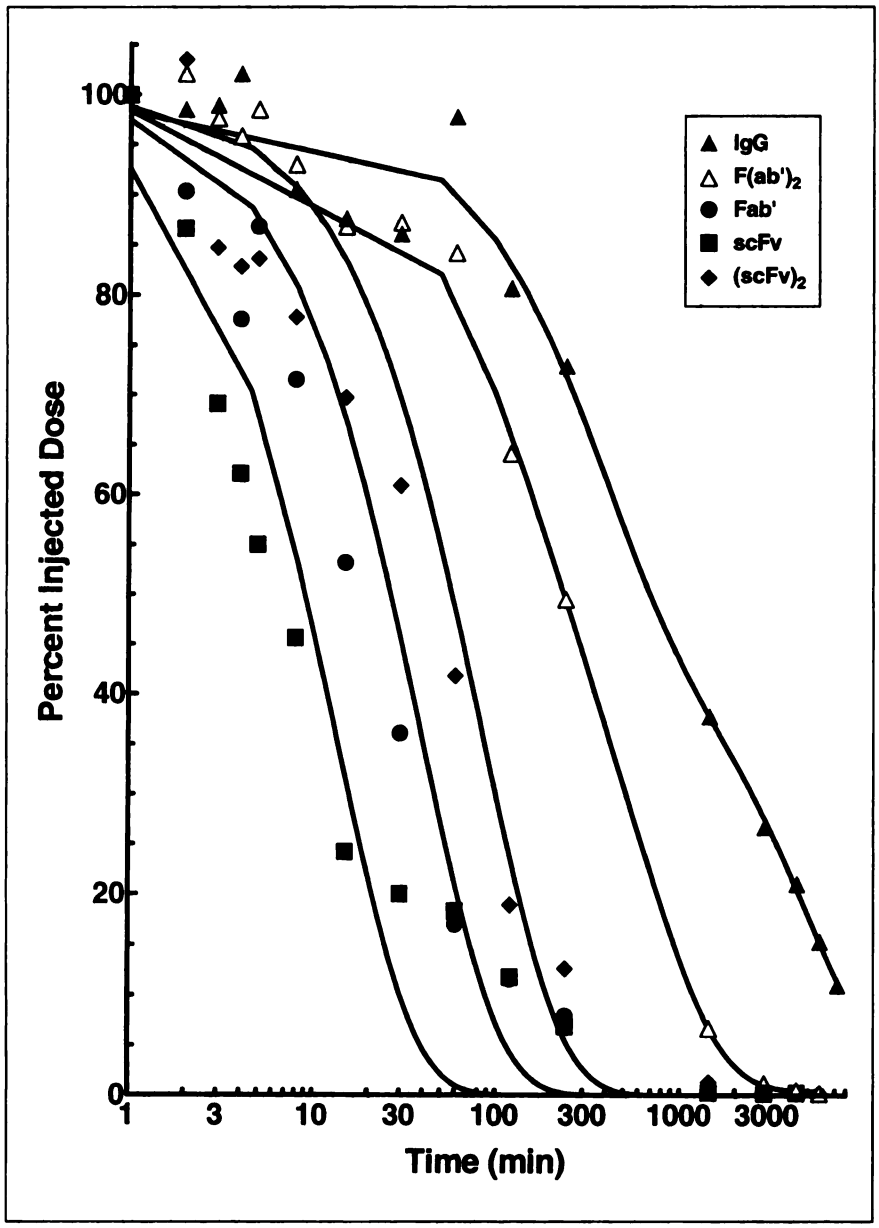

FIGURE 4. Blood clearance of CC49 IgG, $F(ab')_2$, Fab', scFv and $(scFv)_2$. Female athymic mice (six/group) bearing LS-174T colon carcinoma xenografts were injected with various radiolabeled CC49 forms and blood samples were obtained at specific times.

the loss of immunoreactivity or change in the ratio of monomeric to dimeric forms.

Biodistribution, Pharmacology and Tumor Targeting of CC49 Single-Chain Fv Forms

Pharmacokinetic studies were conducted to determine the blood clearance rates of radiolabeled CC49 scFv forms and to compare them with the blood clearance of the intact IgG and enzymatically derived fragments [Fab' and $F(ab')_2$]. As seen in Figure 4, the scFv showed a rapid blood clearance with more than 50% cleared from the blood pool in less than 10 min. The CC49 Fab' (50 kDa) clearance was faster than the $(scFv)_2$ clearance with half of activity cleared from serum within 30 and 50 min, respectively.

Whole-body clearance analyses also displayed a rapid scFv clearance, suggesting that scFv is not being retained in the extravascular space or in any specific organ. As seen in Figure 5, the whole-body clearance of the $(scFv)_2$ was slower than that of the Fab' fragment. The relative rates of clearance observed in the whole-body experiments were similar to those observed with the blood clearance.

Colcher et al. (*12*) showed in biodistribution competition studies that 250–500 μg CC49 IgG were needed to reduce the binding of the radiolabeled CC49 IgG to the antigen on LS-174T colon carcinoma xenograft. Therefore, the biodistribution dual-label studies can be performed to directly compare the antigen-binding ability of the scFvs in vivo and their efficiency to target human colon carcinoma xenograft (using less than 10 μg radiolabeled protein).

At various times after injection, blood, tumor and normal organs were analyzed to determine the amount of each radionuclide retained per gram of tissue. The Fab', $(scFv)_2$ and scFv %ID/g levels in tissues were generally lower

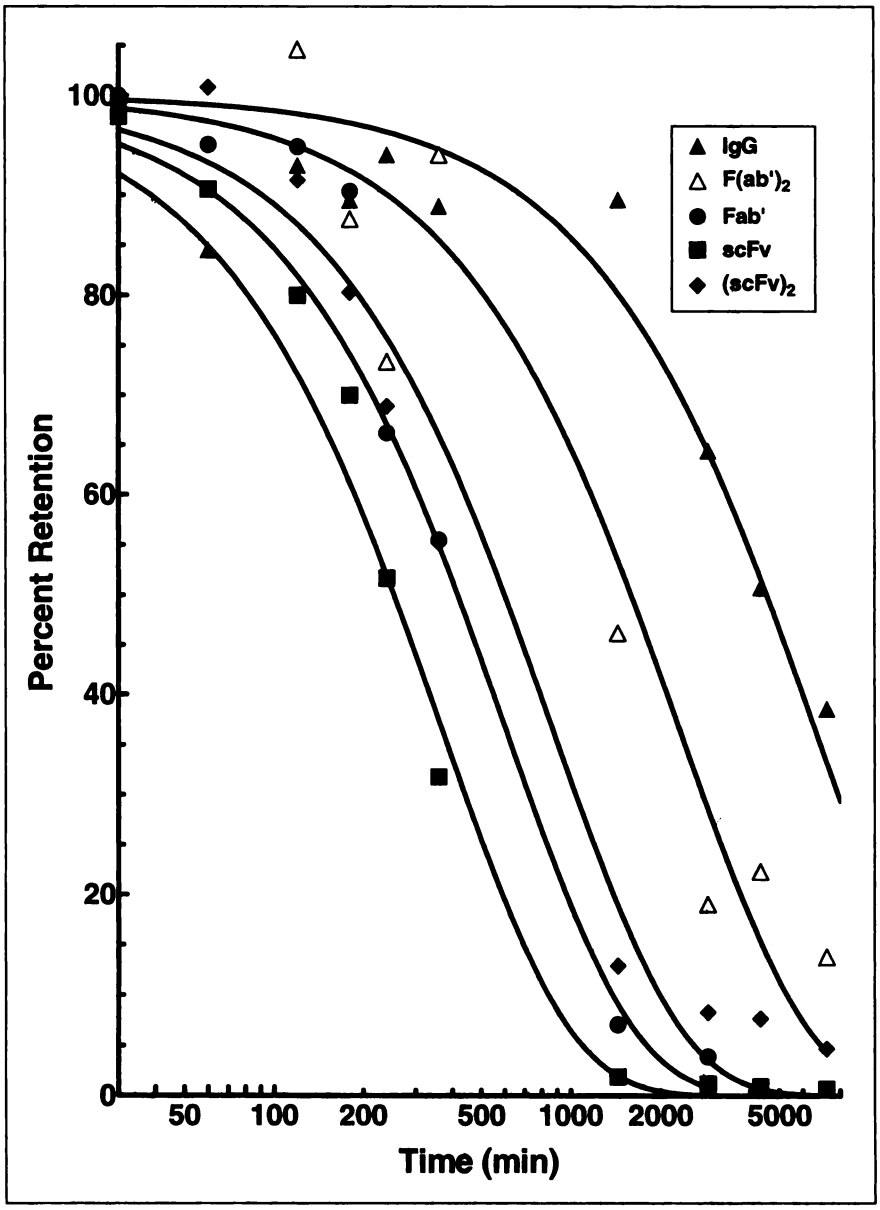

FIGURE 5. Pharmacokinetics of whole-body retention of CC49 Ig forms. CC49 IgG, $F(ab')_2$, Fab', scFv and $(scFv)_2$ were injected into athymic mice (three/group) bearing LS-174T colon carcinoma xenografts. Radioactivity was measured using custom-built NaI gamma scintillation counter at indicated times, as described in Materials and Methods section.

compared with $F(ab')_2$ %ID/g because of a more rapid blood and whole-body clearance (Table 2). As expected, Fab' and scFv forms showed higher levels in the kidneys at the earliest times evaluated. The Fab' uptake by kidneys was much higher than that observed for CC49 monomeric scFv (eight-fold higher at 1 h). The level of uptake of $(scFv)_2$ was elevated in kidneys at the early time points. However, the observed uptake was four-fold lower at 30 min and six-fold lower at 1 h than that seen with the Fab' fragment. The %ID/g in the kidneys observed with the $(scFv)_2$ was similar to that found in other organs at 4 h.

Differences in the tumor binding of CC49 $F(ab')_2$, Fab', scFv and $(scFv)_2$ were revealed in the analysis of the in vivo tumor targeting (Table 2). A higher level of tumor uptake was observed with $(scFv)_2$ compared with monomeric scFv and Fab' fragments, in which 5.6 %ID/g was seen for targeting to the LS-174T xenograft compared with 1.1 %ID/g for monomeric scFv and 3 %ID/g for Fab' at 24 h after administration. The CC49 $F(ab')_2$ showed elevated uptake in the liver, spleen and kidneys compared with $(scFv)_2$. Dimeric scFv appeared to remain intact in vivo, as shown by the higher tumor uptake than that with the monovalent forms of CC49, scFv and Fab'.

An equally important parameter is the radiolocalization index ([RI] ratio of the %ID/g in tumor to the %ID/g in normal tissue). The scFv monomer exhibited a higher RI value (tumor-to-blood ratio of 23.2:1 at 24 h) than intact IgG (3.4:1; Table 3). Extremely high RIs were obtained with $(scFv)_2$ with tumor-to-blood and tumor-to-liver ratios of 80.3:1 and 25.5:1, respectively, compared with 34.0:1 and 16.3:1 for Fab' at 24 h.

Whole-body clearance data showed that 90% of the scFvs had cleared from the body by 24 h. To investigate whether there was any specific or nonspecific accumulation of scFv

TABLE 2
Comparative Biodistribution Studies with CC49 scFv, $(scFv)_2$, Fab' and $F(ab')_2$

		Time (h)							
	Tissue	0.5	1	4	6	24	48	72	120
$F(ab')_2$	Tumor	14.63	12.48	25.82	35.74	28.06	19.42	13.11	12.55
	Blood	30.15	27.88	16.32	10.09	1.68	0.36	0.16	0.15
	Liver	7.47	6.79	7.84	6.03	2.24	1.21	1.21	1.49
	Spleen	7.13	5.56	7.09	5.64	1.37	0.42	0.48	0.62
	Kidneys	11.48	11.31	9.78	7.44	2.10	0.52	0.25	0.23
	Heart	5.54	4.87	4.16	3.01	0.47	0.11	0.06	0.05
	Lungs	8.06	7.12	5.94	3.93	0.95	0.26	0.16	0.13
Fab'	Tumor	4.87	6.29	5.91	3.94	2.96	2.15	ND	ND
	Blood	9.63	5.17	2.38	1.68	0.1	0.06	ND	ND
	Liver	2.59	2.06	1.29	0.93	0.14	0.12	ND	ND
	Spleen	3.44	2.72	1.78	1.16	0.11	0.07	ND	ND
	Kidneys	138.34	132.35	21.50	9.58	0.37	0.16	ND	ND
	Heart	3.1	2.36	0.83	0.57	0.05	0.02	ND	ND
	Lungs	3.4	2.68	1.32	0.96	0.11	0.05	ND	ND
scFv	Tumor	4.74	4.81	2.93	2.01	1.06	0.72	0.27	ND
	Blood	4.66	3.78	1.32	0.77	0.06	0.04	0.05	ND
	Liver	1.79	1.41	0.59	0.37	0.07	0.05	0.05	ND
	Spleen	2.73	2.17	0.84	0.45	0.06	0.04	0.03	ND
	Kidneys	41.24	15.82	2.65	1.48	0.15	0.07	0.06	ND
	Heart	2.05	1.35	0.42	0.24	0.03	0.02	0.04	ND
	Lungs	3.25	2.33	0.83	0.59	0.06	0.04	0.04	ND
$(scFv)_2$	Tumor	9.77	10.00	9.25	10.87	5.62	3.32	2.77	1.69
	Blood	17.16	10.66	1.99	1.09	0.07	0.04	0.06	0.03
	Liver	5.03	4.24	2.80	1.42	0.22	0.10	0.07	0.04
	Spleen	7.31	5.86	2.80	1.40	0.18	0.08	0.05	0.06
	Kidneys	34.56	21.48	2.63	1.54	0.26	0.09	0.08	0.04
	Heart	4.51	2.91	0.71	0.41	0.03	0.02	0.03	0.01
	Lungs	6.13	4.69	1.32	0.96	0.07	0.05	0.05	0.03

ND = not determined.

Iodinated CC49 $F(ab')_2$, Fab', scFv, and $(scFv)_2$ were injected into athymic mice (six/group) bearing LS-174T tumors. The mice were killed at indicated times, and %ID/g for each organ was determined. Values presented are average of multiple studies. SEM for samples was less than 20 %ID/g of corresponding tissue.

forms in tissues, the tissue-to-blood ratios were calculated (Fig. 6). Tumor-to-blood ratios increased over time with scFv, Fab', $F(ab')_2$ and $(scFv)_2$, reaching approximately 23:1, 34:1, 17:1 and 80:1, respectively, at 24 h after administration. The concentrations of Fab' fragments and scFv forms were low in most normal tissues. However, the spleen and liver showed higher accumulation with the $F(ab')_2$ fragment at 120 h, with liver-to-blood and spleen-to-blood ratios of 10:1 and 4:1, respectively, whereas the tissue-to-blood ratios of the $(scFv)_2$ were only 1 and 2:1 in the same tissues.

TABLE 3
Comparative Biodistribution Studies of CC49 IgG, $F(ab')_2$, Fab', scFv and $(scFv)_2$ (Radiolocalization Index) at 24 Hours after Administration

Tissue	IgG	$F(ab')_2$	Fab'	scFv	$(scFv)_2$
Blood	3.4	16.7	34.0	23.2	80.3
Liver	6.1	12.5	16.3	16.6	25.5
Spleen	9.1	20.5	24.9	19.3	31.2
Kidneys	17.3	13.4	9.1	7.3	21.6
Lungs	7.2	29.5	31.2	16.6	80.3

Iodinated CC49 IgG, $F(ab')_2$, Fab', scFv, and $(scFv)_2$ were injected into athymic mice (six/group) bearing LS-174T tumors. Mice were killed at indicated times, and radiolocalization index (%ID/g of tumor divided by %ID/g of normal tissue) for each organ was determined.

DISCUSSION

The scFv molecules demonstrated better tumor-to-normal tissue ratios than enzymatically derived fragments in animal studies, and therefore they may be useful in clinical applications for cancer diagnosis and therapy. Major advantages of scFv molecules are their rapid clearance from the blood pool and their excellent penetration into tumor tissue in comparison with intact antibody. However, the short persistence of scFv in the circulation limits the exposure of tumor cells, thus limiting the accumulation of the scFv in the tumor. Dimeric scFvs combine a longer biologic half-life as a result of higher molecular weight and increased avidity as a result of bivalency of their molecule. They were also shown to

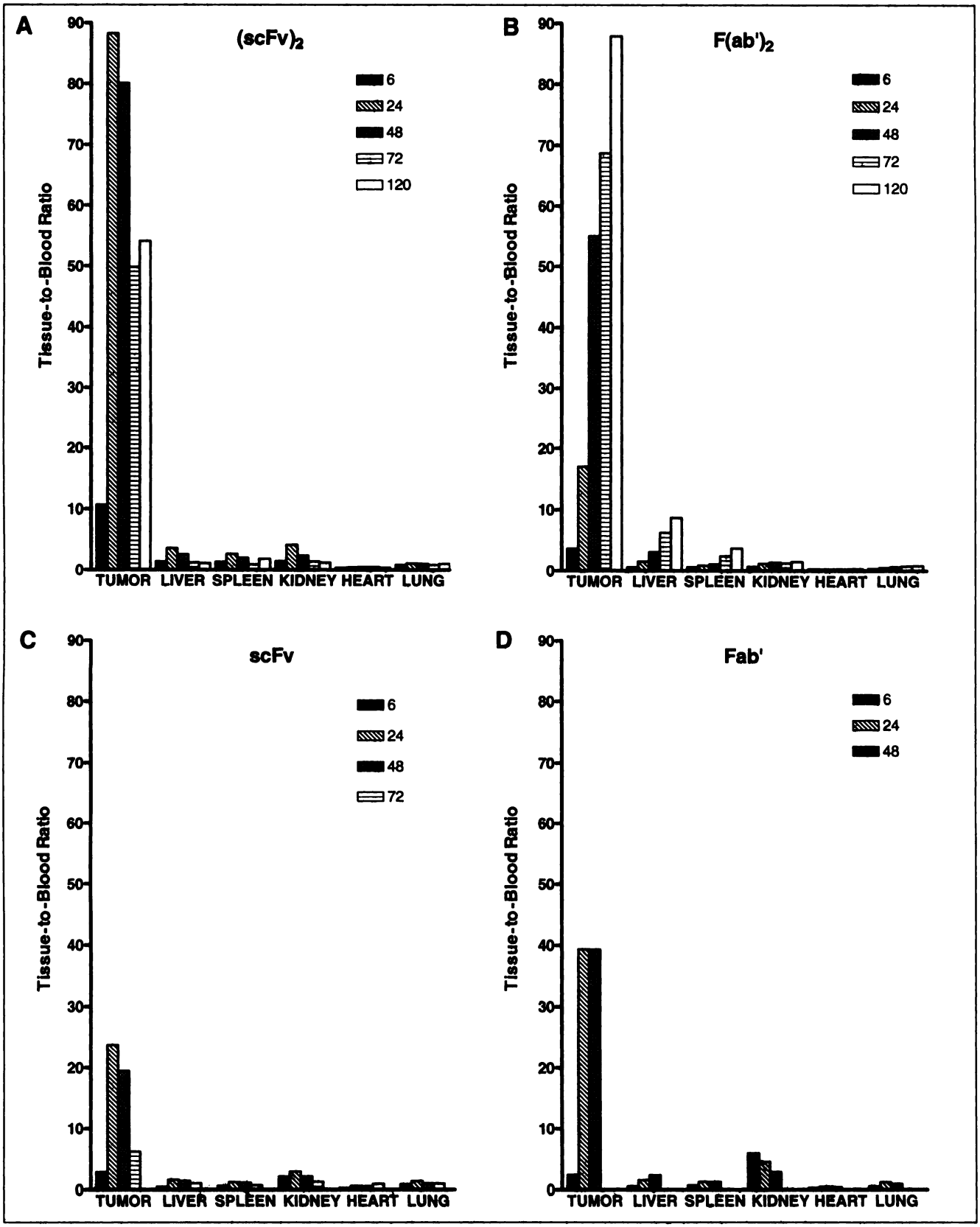

FIGURE 6. Biodistribution studies (tissue-to-blood ratio) with CC49 $F(ab')_2$, Fab', scFv and $(scFv)_2$. Iodinated CC49 Ig forms were injected into athymic mice (six/group) bearing LS-174T tumors. Mice were killed at indicated times, and ratio of %ID/g of tissue to %ID/g of blood was calculated.

have more favorable tumor targeting and biodistribution than corresponding monomeric scFv (*4*).

MAb CC49 reacts with Sialyl-Tn related epitope, present on tumor-associated mucin, TAG-72. Radiolabeled MAb CC49 has shown excellent tumor localization in several ongoing clinical trials. In this study, a new CC49 scFv(205C) construct was engineered, and its in vitro and in vivo properties were analyzed. Interestingly, when CC49 scFv was expressed and processed in the intracellular space of *E. coli,* the majority (60%–70%) of scFv was folded into stable noncovalent dimers. The CC49 noncovalent dimer was purified, and a direct comparison of monomeric and dimeric scFvs was performed as well as a comparison with intact IgG, Fab′ and $F(ab')_2$. The noncovalent dimeric scFv showed an excellent binding affinity similar to IgG. The dimers did not dissociate on dilution and demonstrated excellent stability and immunoreactivity at elevated temperatures (37°C). Furthermore, the monomeric and noncovalent dimeric CC49 scFvs retained their immunoreactivity and their integrity after radiolabeling. The dimeric form of scFv showed an association constant (K_A) for the BSM antigen comparable with the other bivalent forms [IgG and $F(ab')_2$] as determined in both the BIAcore and Scatchard analyses. The monovalent form of scFv showed an apparent affinity constant lower than IgG due to its lower association constant (k_{on}).

The central issue in practical clinical applications of scFv is an appropriate pharmacology and distribution. In biodistribution studies, we found that the monomeric form of CC49 scFv reached a maximal tumor uptake of 4–5 %ID/g at 30–60 min after intravenous injection. These results were comparable with those reported by other investigators using a variety of scFv molecules (*1,2,4,26*). Purified noncovalent dimers of CC49 scFv(205C) showed two- to three-fold higher uptake, reaching 9–13 %ID/g in tumors at 60 min after injection. Furthermore, the %ID/g was more than twice as high as that observed with the CC49 scFv(212) dimers as the maximal levels reported by Whitlow et al. (*26*). For example, they published that the CC49 scFv(212), CC49 scFv(212) dimer and CC49 Fab′ had 1.5, 2.5 and 3.7 %ID/g in tumor at 24 h, respectively. Also their dimeric scFv showed elevated uptake in the spleen and liver compared with the monomer. Our biodistribution data demonstrated that the CC49 $(scFv)_2$(205C) had higher tumor uptake than Fab′ fragment with 5.6 and 3.0 %ID/g, respectively. The CC49 $(scFv)_2$(205C) did not show higher uptake in any normal tissue compared with the $F(ab')_2$ fragment. The slightly higher uptake in the spleen and liver, compared with other tissues at early time points, could be a result of the clearance by the reticuloendothelial system.

The method of production and purification of the dimeric form could explain the excellent stability and immunoreactivity of the CC49 $(scFv)_2$(205C) construct. The CC49 scFv dimer was expressed and folded in *E. coli* as an active, soluble protein. Whitlow et al. (*26*) generated the CC49 scFv(212) dimers by addition of 0.5 mol/L GuHCl and 20% ethanol after refolding of insoluble protein with maximal yields of 18% dimer, 78% monomer and 4% multimers. The CC49 scFv(205C) dimer showed excellent stability and increased avidity in vivo compared with the monomer scFv as seen by the longer retention in tumor, 3.3 and 0.7 %ID/g at 48 h, respectively. Additionally the tumor retention values of the $(scFv)_2$ were higher than those reported by Wu et al. (*4*) and Whitlow et al. The enhanced conformational stability of the CC49 scFv(205C) dimer could be explained by the 205C linker potentially packing up against the V domains and introducing specific stabilizing interactions as described by Pantoliano et al. (*16*).

CONCLUSION

The stable dimer of CC49 scFv was produced and characterized in vitro and in vivo. In biodistribution studies, the dimeric scFv molecule exhibited excellent tumor targeting properties and some characteristics similar to those of the monomeric form of scFv, e.g., the rapid clearance, low kidney uptake (compared with the enzymatically derived fragments) and small size for rapid penetration through tumor tissue. Increased $(scFv)_2$ dimer tumor targeting was probably a result of its higher molecular weight and increased avidity as a divalent binding molecule compared with monomeric CC49 scFv(205C). High tumor-to-blood ratios at early times and its rapid clearance make the CC49 scFv dimer an excellent candidate for imaging applications. Higher tumor uptake and longer retention seen in these studies are important factors for potential therapeutic applications.

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