Pharmacokinetics of an Anti-Carcinoembryonic Antigen Monoclonal Antibody Conjugated to a Bifunctional Transition Metal Carborane Complex (Venus Flytrap Cluster) in Tumor-Bearing Mice

Barbara G. Beatty, Raymond J. Paxton, M. Frederick Hawthorne, Lawrence E. Williams, Karen J. Rickard-Dickson, Tulan Do, John E. Shively and J. David Beatty

Department of General Oncologic Surgery and Division of Radiologic Sciences, City of Hope National Medical Center, Duarte, Canada; Division of Immunology, Beckman Research Institute of the City of Hope, Duarte, Canada; Department of Chemistry and Biochemistry, University of California at Los Angeles, Los Angeles, California

An anticarcinoembryonic antigen (CEA) monoclonal antibody, T84.66, has been conjugated to a metallocarborane complex (Venus flytrap cluster, VFC) containing ^{57}Co. This radioimmunoconjugate, ^{57}Co-VFC-T84.66, retained >90% immunoreactivity, was stable in serum (7 days) and demonstrated good localization in LS174T tumor xenografts. Pharmacokinetics of ^{57}Co-VFC-T84.66 in tumor-bearing mice were compared to T84.66 Mab conjugated with either DTPA or its benzylisothiocyanate derivative (BzDTPA) labeled with ^{111}In. Whole-body half-life for VFC-T84.66 was less ($t_{1/2}$ = 62 hr) than that for either DTPA-T84.66 ($t_{1/2}$ = 157 hr) or BzDTPA-T84.66 ($t_{1/2}$ = 167 hr). Blood clearance was similar for all three radioimmunoconjugates ($t_{1/2}$ = 22 hr). Hepatic uptake of the radiolabel was rapid and remained constant for 7 days for both DTPA radioimmunoconjugates (DTPA radioimmunoconjugate = 13.7 ± 1.5 %ID/g; BzDTPA radioimmunoconjugate = 10.4 ± 1.7 %ID/g). For VFC, however, liver radioactivity decreased from 19.1 ± 0.6 %ID/g at 1 hr to 0.9 ± 0.1 %ID/g 7 days postinjection, suggesting a possible role for VFC radioimmunoconjugate in the imaging and therapy of liver metastases.

J Nucl Med 1993; 34:1294–1302

Radiometals attached to monoclonal antibodies directed against tumor-associated antigens have shown promise as imaging and therapeutic agents for human cancers. The chelation techniques for attaching radiometals such as ^{111}In and ^{90}Y to antibodies or their fragments have most frequently employed bifunctional chelates of the aminocarboxylate family. The most commonly used of these reagents have been diethylenetriaminepentaacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA) (*1–4*) and their derivatives (*5–8*). A consistent concern with the use of these radiometal-antibody complexes has been the high uptake of the radionuclide in normal liver, observed in both the mouse model (*9–11*) and in patients (*12–14*). Modifications of chelation methodology from mixed anhydride, cyclic anhydride and active ester (*1–4*) techniques, to isothiocyanato and para-amino benzyl derivatives (*5,8*) in order to improve antibody-radiometal chelate stability, have demonstrated variable results with respect to lowering liver radioactivity levels (*5,14,15*). The use of metabolizable diester linkers (*16*) for enhancing blood clearance of radiolabeled chelate demonstrated limited improvement in decreasing normal liver retention of radioactivity. Work is continuing on the production of other metabolizable linkers which may show more promise (*17*).

For more effective use of radiometal-antibody conjugates for clinical diagnosis and treatment, a ligand that will produce a stable complex with both the antibody and the radiometal is essential. A ligand that is not retained in normal tissues, such as liver, is also a high priority. A prototype *commo*-bisdicarbollide bifunctional reagent specifically designed to hold transition metals has recently been described and characterized by Hawthorne et al. (*18–20*). The radiometal-dicarbollide complex, Venus flytrap cluster (VFC), is chemically very stable due to the cluster-bonding between the transition metal and the two covalently linked carborane ligands. Also, the chelate is inorganic in nature and therefore not catabolizable by organic enzymes. Conjugation of the VFC reagent containing ^{57}Co to an anti-carcinoembryonic antigen (CEA) murine monoclonal antibody (Mab) T84.66 has demonstrated the ability to localize CEA-producing xenografts in nude mice (*21*).

In the present study, we have examined the immunore-

Received Nov. 24, 1992; revision accepted Apr. 1, 1993.
For correspondence and reprints contact: Barbara G. Beatty, PhD, Toad Hall, 7 Hermit Ct., Toronto, Ontario, Canada M3B 1R4.

activity, serum stability and pharmacokinetics of T84.66 conjugated with ^{57}Co-VFC in tumor-bearing mice. The results were compared to those obtained for ^{111}In-DTPA and ^{111}In-BzDTPA conjugated T84.66. A potential advantage of the VFC conjugate for imaging tumors in or near the liver was indicated due to a lower retention of radiolabel in normal tissues, especially the liver.

MATERIALS AND METHODS

Monoclonal Antibody

The murine anti-CEA Mab, T84.66, was produced in large quantities by Damon Biotechnology (Needham, MA) and characterized as described previously (*22*). This IgG_1 has been shown to be specific for the 3A domain of CEA, has a high affinity constant of $2.6 \times 10^{10} M^{-1}$ and does not cross-react with normal tissues, including granulocytes (*22,23*).

Preparation of the ^{57}Co-VFC Mab Conjugate

The synthesis and characterization of the VFC ligand has been described previously by Hawthorne et al. (*18*). Briefly, 2 moles of closo-1,8-$C_2B_9H_{11}$ were reacted with 1 mole of 4-carbomethoxypyrazole anion to form dl and meso bis-(dicarbollide) anion intermediates. Addition of $^{57}Co^{3+}$ in the presence of aqueous KOH followed by acidification and diethyl ether extraction resulted in the production of dl and meso isomers of the commo and bis(dicarbollide) ^{57}Co complexes. Removal of the protecting pyrazole carboxyl group of the functionalized metallocarborane cluster (^{57}Co-VFC) during this process enabled the cluster to be conjugated to the T84.66 Mab. Carrier-free ^{57}Co was obtained from ICN (Costa Mesa, CA).

The active N-hydroxysulfosuccinimide ester of ^{57}Co-VFC was prepared as described by Paxton et al. (*21*) and purified by reverse-phase HPLC (Applied Biosystems, Foster City, CA). Sulfosuccinimido-^{57}Co-VFC was reacted with T84.66 Mab and the resulting conjugate was purified using a Superose 12-FPLC column (1 × 30 cm) (Pharmacia, Uppsala, Sweden).

Preparation of Radiolabeled DTPA and Benzylisothiocyanate(Bz)DTPA Mab Conjugates

T84.66 was conjugated with DTPA using the N-hydroxysuccinimide active ester method described by Paxton et al. (*4*). The conjugation of T84.66 with the benzylisothiocyanate derivative of DTPA (BzDTPA) (Abbott Laboratories, Abbott Park, IL) was performed as described by Westerberg et al. (*8*). Briefly, the BzDTPA chelater in 0.1 *M* KH_2PO_4/0.1 *M* $NaHCO_3$ with a pH of 8.5 was added to T84.66 Mab (10 mg/ml) in the same buffer and incubated at 37°C for 3 hr. The mixture was then dialyzed against a 0.1 *M* solution of DTPA in 0.05 *M* citrate buffer with a pH of 6.0 for 48 hr at 4°C. This was followed by a 3-day dialysis against 0.05 *M* citrate buffer with a pH of 6.0 alone. The number of chelates per molecule of Mab was obtained by a thin-layer chromatography (TLC) ^{57}Co binding assay (*6*). Both conjugates were labeled with ^{111}In citrate (Hybritech Inc., San Diego, CA) to a specific activity of 5 μCi/μg. Labeling efficiency was also determined by TLC. The DTPA-T84.66 conjugate was labeled with ^{57}Co following the same procedure substituting ^{57}Co for ^{111}In.

Immunoreactivity and Affinity

Immunoreactivity of the VFC-T84.66 conjugate was measured using CEA-coated plates and a solid-phase enzyme immunoassay (EIA). Plates were coated overnight (4°C) with 5 μg/ml of CEA. Serial dilutions (1:4) of the T84.66 Mab-conjugate or the unconjugated T84.66 Mab were then added to the wells beginning with a concentration of 10 μg/ml. Plates were incubated at room temperature for 2 hr and washed five times, after which a double-sandwich assay (utilizing rabbit anti-mouse followed by alkaline phosphatase-conjugated goat anti-rabbit antisera) was performed and the binding curves compared (*24*).

Affinity of the conjugated Mab was estimated using a solid-phase EIA method described previously (*25*).

Stability of the Radioimmunoconjugates

Stability of the three radioimmunoconjugates was determined in vitro by incubating a sample of each radioimmunoconjugate in normal human plasma (pH 7.4) at 37°C for 168 hr. In order to approximate the in vivo conditions, 5 μg (4 μl) of conjugated Mab in phosphate buffered saline (PBS) were incubated in 500 μl of plasma and samples were removed at 24 hr and 168 hr. Each sample was analyzed on a size exclusion Superose-12 FPLC column (Pharmacia) equipped with an on-line gamma detector (Beckman Inst., Fullerton, CA) and integrator (Medi-Physics, Arlington Heights, IL). Using the integrator, the percent of the label remaining associated with the Mab conjugate peak at each time point was compared to that present in the conjugate peak prior to incubation in the serum.

Animal Tumor Model and Biodistribution Studies

Female, 6-to-8-wk-old BALB/c athymic (nu/nu) mice (20 g) were obtained from Simonsen Laboratories (Gilroy, CA). LS174T, a CEA-producing human colon carcinoma cell line (ATCC) was maintained in continuous culture in a supplemented RPMI medium (*26*). The tumor cells were trypsinized and resuspended in PBS at a concentration of 5×10^6 cells per ml. Each nude mouse was injected with 1×10^6 cells (0.2 ml) subcutaneously in the hind flank on day 0. On days 10–14, 20 μg of ^{57}Co-VFC-Mab (2 μCi), ^{111}In-DTPA-Mab (100 μCi) or ^{111}In-BzDTPA-Mab (100 μCi) were administered intravenously in 0.2 ml of PBS. From 0.5 to 168 hr following labeled Mab injection, the animals were euthanized and tissues were removed, weighed and counted on a GammaTrac well counter (TM Analytic, Elk Grove Village, IL). Tissue content of ^{57}Co or ^{111}In was expressed as percent injected dose per gram (%ID/g) or percent injected dose per organ (%ID/organ). Animals were imaged at various times postinjection using a Siemens Pho-Gamma V camera (Siemens Medical Systems, Inc., Hoffman Estates, IL). The camera used a Siemens pinhole collimater with a 4-mm insert at 9.8 cm from the imaging table. The gamma camera energy window was set at 20% around the 122 keV photon energy. At 24 and 48 hr, 10,000 counts were acquired. At 148 hr, 8,500 counts were acquired.

The effect of increasing the dose of Mab on liver uptake for each radioimmunoconjugate was examined in tumor-bearing animals by injecting the mice intravenously with 10, 20, 50, 100 or 200 μg of antibody and biodistributions performed at 48 hr. The amount of radiolabeled antibody was kept constant at each level.

Total body retention of the radiolabeled Mab was measured by counting a constrained animal placed approximately midway between two parallel opposed NaI probes (Ludlum Instruments, Sweetwater, TX). Average uptake was evaluated using the geometric mean of the two measurements. Evaluations were made immediately (0 hr) and at 24-hr intervals following Mab injection. The injected dose was similarly counted at each time point to correct for radiodecay. Total body retention (%) at each time point was expressed as the ratio of the geometric mean (corrected for radiodecay) divided by the geometric mean obtained for the same animal at 0 hr. Retention of radiolabeled Mab was first

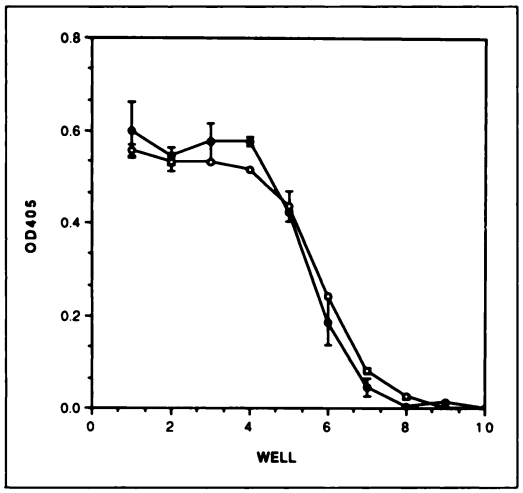

FIGURE 1. Enzyme immunoassay of T84.66 (●) and VFC-T84.66 conjugate (○). The assay was performed on CEA-coated plates. The initial concentration of the antibody and the antibody conjugate was 10 μg/ml and the serial dilutions were 1:4. Each curve represents the average of duplicate runs ± s.d.

calculated for each individual animal and results for the group of animals at each timepoint were expressed as the mean ± s.e.m.

Two control experiments to assess the in vivo behavior of the radiometals alone (^{57}Co and ^{111}In) or in the chelated form (^{57}Co-VFC, ^{111}In-Bz-DTPA and ^{111}In-DTPA) were performed in BALB/c mice. Total body half-time and biodistribution were determined in each case.

RESULTS

Immunoreactivity and Stability of ^{57}Co-VFC-T84.66 Radioimmunoconjugate

Conjugation of T84.66 to ^{57}Co-VFC resulted in 0.03–0.05 molecules ^{57}Co-VFC per molecule of antibody and a specific activity of 0.1 μCi/μg Mab. As shown in Figure 1, the EIA binding curves of the antibody alone and the conjugated antibody were essentially superimposable, indicating that the conjugation did not appear to alter the immunoreactivity of the antibody. Conjugation of T84.66 with DTPA and BzDTPA resulted in 0.4–0.9 and 1.1–1.4 molecules of chelater per molecule of antibody, respectively. A similar comparison of EIA binding curves showed no demonstrable alteration of immunoreactivity (data not shown).

The binding constant (K_{aff}) of ^{57}Co-VFC-T84.66 estimated using a solid phase EIA method (*23*), was found to be $2.7 \times 10^{10} M^{-1}$ compared to $2.6 \times 10^{10} M^{-1}$ for the T84.66 alone.

Serum stability, determined by integration of the FPLC elution profiles (described in Materials and Methods) showed that over 7 days, ^{57}Co-VFC-T84.66 retained 99.6% of the radioactivity present prior to incubation. Comparison with the two DTPA conjugates demonstrated that over the short-term, 24 hr, the fraction of radioactivity that remained associated with the antibody peak was similar for all three antibody preparations (VFC, 99.7%; BzDTPA, 96.7% and DTPA, 97.9%). After a longer incubation time of 168 hr, ^{111}In-BzDTPA-T84.66 retained 98% of the initial radioactivity, whereas ^{111}In-DTPA-T84.66 was less stable with 83% retention of the Mab-bound radioactivity. The average percent recovery of radioactivity in the FPLC analyses was 90%.

Attempts to label the DTPA-T84.66 conjugates with ^{57}Co in order to have a more direct comparison of chelates using the same Mab and radionuclide were not successful. The ^{57}Co labeling of the DTPA chelates resulted in a high degree of instability such that, during a 24 hr incubation in serum, only 13% of the label was retained by the DTPA-Mab. Cole et al. (*27*) also found that ^{57}Co-DTPA complexes were unstable in serum. ^{57}Co-BzDTPA complexes were not attempted, although there is some evidence that they may be somewhat more stable than the DTPA complexes (*27*). Indium-111, not being a transition metal, did not have the appropriate electronic configuration to form a stable complex with the VFC (*18–20*).

Pharmacokinetics of the Mab Conjugates

The pharmacokinetics of the three radioimmunoconjugates were examined in nude mice bearing LS174T tumors. The animals were injected intravenously with 20 μg of each Mab conjugate (VFC-Mab, 0.1 μCi/μg; DTPA-Mabs, 5 μCi/μg). Total body retention and tissue distribution of the radiolabel was followed for 7 days. Tumor burden was kept small and as similar as possible (0.5–1 g) to minimize the effects of tumor mass on antibody biodistribution (*28*).

Total body retention of the three radioimmunoconjugates is shown in Figure 2. Clearance followed a single exponential curve for all three conjugates with the clearance of VFC being more than twice as rapid ($t_{1/2}$ = 62 hr) as either DTPA ($t_{1/2}$ = 157 hr) or BzDTPA ($t_{1/2}$ = 167 hr). By 7 days, only 15% of the injected radioactivity remained in the animals receiving VFC radioimmunoconjugate,

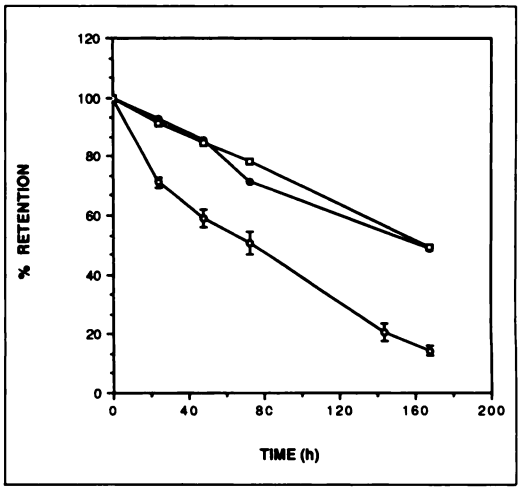

FIGURE 2. Whole-body retention curves of ^{57}Co-VFC-T84.66 (○), ^{111}In-DTPA-T84.66 (●) and ^{111}In-BzDTPA-T84.66 (□) radioimmunoconjugates in tumor-bearing nude mice. Each animal was injected with 20 μg of radioimmunoconjugate and the same animals were counted daily as described in Materials and Methods. Each point represents the mean ± s.e. There were five animals in each group.

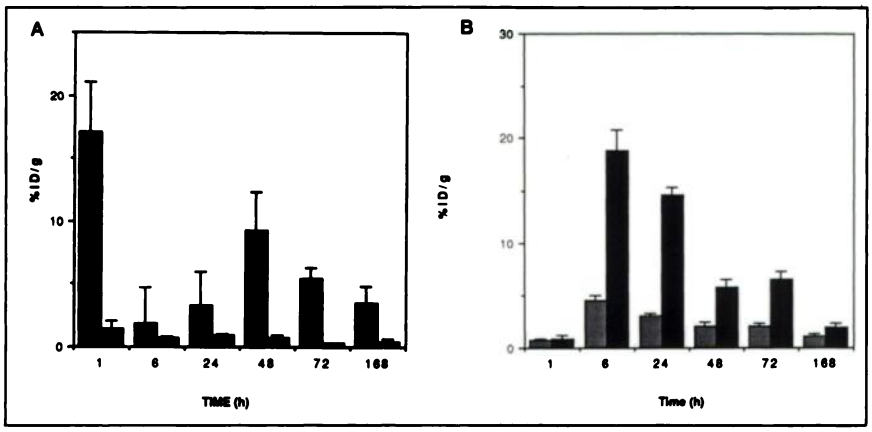

FIGURE 3. Excretion of radioactivity for ^{111}In-BzDTPA-Mab and ^{57}Co-VFC Mab administered to tumor-bearing nude mice. Radioactivity levels in urine (A) and feces (B) were expressed as %ID/g ± s.e. (n = 5). The BzDTPA radioimmunoconjugate (▩) was excreted predominantly via urine and the VFC radioimmunoconjugate (■) predominantly via feces.

whereas 50% of the radioactivity was retained in the animals given either of the DTPA radioimmunoconjugates.

Samples of both urine and feces were collected to determine the route of excretion of the various radioimmunoconjugates. Due to the difficulty in obtaining quantitative recovery of excreta, the results were expressed as %ID/g rather than total percent excreted. As seen in Figure 3A, very little of the radioactivity appeared in the urine of the animals injected with VFC. The feces, however, showed very high levels of radioactivity (18.8 %ID/g) which appeared to peak around 6 hr (Fig. 3B). Animals receiving the BzDTPA showed high levels of radiolabel (17.4 %ID/g) in the urine within the first hour and the amount of radiolabel present in the feces measured at each time point was <5 %ID/g. Radioactivity in the feces of animals receiving DTPA was not determined, however, the levels of radioactivity in urine were comparable to those seen with the BzDTPA.

Administration of the nonchelated $^{111}InCl_3$ and $^{57}CoCl_2$ to Balb/c (nontumor) mice resulted in the excretion of both radiometals in the urine. Radioactivity was barely detectable (<1 %ID/g) in the stool for either radiometal. On the other hand, administration of chelated cobalt (^{57}Co-VFC) resulted in the presence of substantial radioactivity in the feces, peaking around 3 hr (411.3 ± 45.2 %ID/g), slightly earlier than observed with ^{57}Co-VFC Mab. Chelated indium (^{111}In-BzDTPA) was excreted very rapidly (within the first hour) in the urine with <2 %ID/g remaining in the animals at 3 hr. Radioactivity in the stool was negligible (<2 %ID/g).

In contrast to the total body retention, blood clearance of the three radioimmunoconjugates was remarkably similar (Fig. 4A). The blood half-life for VFC-T84.66 was 19.6 hr, for DTPA-T84.66 was 24.2 hr and for BzDTPA-T84.66 was 23.3 hr. A single peak of radioactivity was seen for each of the radioimmunoconjugates on FPLC profiles of serum taken 24 hr postinjection. This single peak eluted in the same position as the intact radiolabeled Mab prior to injection (data not shown).

Biodistribution data for mice receiving ^{57}Co-VFC-T84.66 are shown in Table 1. Radioactivity in normal tissues such as liver, spleen and kidney decreased progressively across the 7-day time period to <1 %ID/g. Hepatic and splenic radioactivity decreased from 19.07 ± 0.57 and 6.56 ± 0.33 %ID/g respectively at 1 hr to 4.7 ± 0.2 and 2.61 ± 0.28 %ID/g respectively by 48 hr and was virtually gone by 168 hr (<1 %ID/g). Kidney uptake was low at all time points (<5 %ID/g) but showed a steady drop over 168 hr to

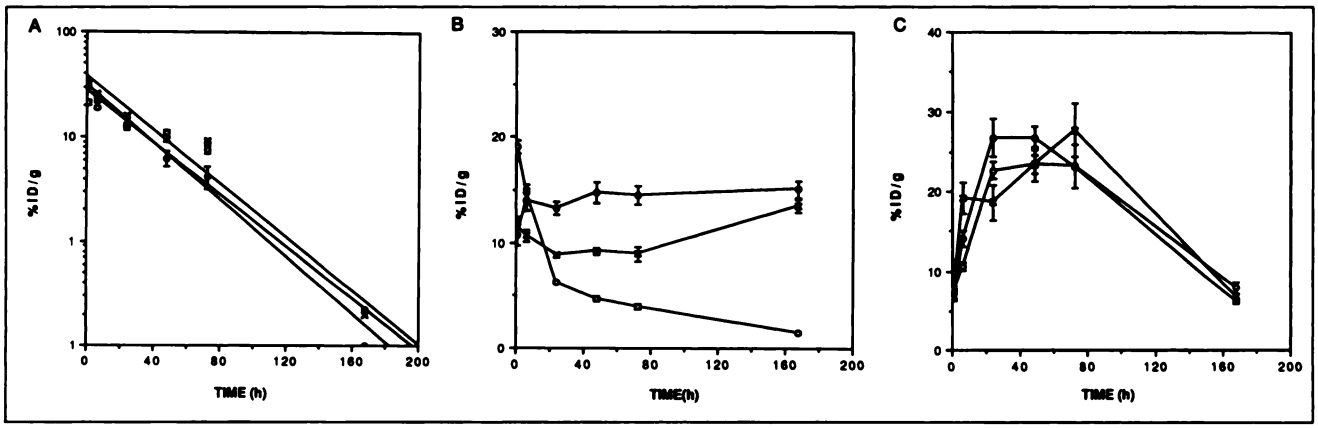

FIGURE 4. Pharmacokinetics of the three radioimmunoconjugates showing uptake and clearance in (A) blood, (B) liver and (C) tumor. Mice were injected with 20 μg of ^{57}Co-VFC-T84.66 (○), ^{111}In-DTPA-T84.66 (●) or ^{111}In-BzDTPA-T84.66 (x). Tissues were removed, weighed and counted on a gamma counter at each time point. Tissue radioactivity was expressed as %ID/g. Each time point represents the mean ± s.e. of five mice. Tumor size range was 0.5–1.0 g for all animals except those assessed at 168 hr. At 168 hr, the tumor burden was 1.0–1.8 g.

TABLE 1
Biodistribution of ^{57}Co-VFC-T84.66* in Nude Mice Bearing LS174T Xenografts at Various Time Intervals

	%ID/g (s.e.)					
	1 hr	6 hr	24 hr	48 hr	72 hr	168 hr
Blood	20.91	18.65	12.24	10.85	7.58	0.05
	(1.18)	(0.60)	(0.46)	(0.67)	(0.63)	(0.01)
Liver	19.07	14.70	6.29	4.65	3.91	0.88
	(0.57)	(0.75)	(0.09)	(0.24)	(0.22)	(0.07)
Spleen	6.56	6.23	3.08	2.61	2.42	0.69
	(0.33)	(0.32)	(0.30)	(0.28)	(0.17)	(0.12)
Kidney	5.21	5.05	3.30	2.70	2.00	0.23
	(0.09)	(0.18)	(0.18)	(0.20)	(0.09)	(0.02)
Tumor	7.33	10.66	22.69	23.42	23.26	4.71
	(0.87)	(0.51)	(1.00)	(2.13)	(0.09)	(0.50)
Urine	1.48	0.77	0.91	0.67	0.26	0.42
	(0.55)	(0.10)	(0.13)	(0.30)	(0.04)	(0.08)
Feces	0.92	18.80	14.57	5.75	6.50	2.01
	(0.36)	(1.99)	(0.83)	(0.79)	(0.77)	(0.28)
Tumor wt (g)	0.51	0.47	0.41	0.40	0.68	2.10
	(0.01)	(0.06)	(0.03)	(0.03)	(0.04)	(0.12)

*Dosage administered was 20 μg (2 μCi).
Five mice were in each group.

0.23 ± 0.02 %ID/g. Since the predominant route of excretion was via the feces, the radioactivity in the large and small bowel was examined. At both 1 hr and 24 hr time points, the amount of radiolabel present in these tissues was <3 %ID/g and by 48 hr <1 %ID/g. Thus, no selective bowel uptake of radioactivity was observed in animals receiving VFC radioimmunoconjugate.

Normal tissue radioactivity of mice given either of the ^{111}In-DTPA radioimmunoconjugates tended to remain higher. At 1 hr, the spleen showed 6.11 ± 0.34 %ID/g (DTPA) and 6.47 ± 0.41 %ID/g (BzDTPA). Very little change occurred by 168 hr with 5.74 ± 0.40 %ID/g and 6.16 ± 0.34 %ID/g, respectively, being retained. Kidney radioactivity at 168 hr (DTPA: 6.42 ± 0.4 %ID/g; BzDTPA: 4.89 ± 0.26 %ID/g) was about 50% of the 1-hr value.

The clearance of radiolabel from the liver was distinctly different for the ^{57}Co-VFC Mab compared to that for the two ^{111}In-DTPA radioimmunoconjugates (Fig. 4B). While the liver radioactivity remained relatively constant across the 7 days, for both DTPA radioimmunoconjugates (9–15 %ID/g), it decreased from 19 %ID/g at 1 hr to <1 %ID/g by 7 days for the VFC radioimmunoconjugate.

Kinetics of tumor targeting for the three radioimmunoconjugates are shown in Figure 4C, which demonstrates that tumor uptake was similar for each conjugate, reaching a maximum of 23–27 %ID/g over a 72-hr period. By 168 hr, the tumor mass had increased substantially and the %ID/g decreased to 4–7 %ID/g. For all three groups, tumor burden was 0.5–1.0 g at each time point except 168 hr, where the tumors ranged between 1.6–3.0 g. The larger tumor burden at 168 hr was partially responsible for the drop in %ID/g in these tumors.

Tumor-to-normal tissue ratios expressed as tumor-to-blood (T-to-B) and tumor-to-liver (T-to-L) were used as a measure of tumor specificity and are shown in Table 2. The T-to-B ratios for all three radioimmunoconjugates increased with time reflecting both the loss of labeled Mab from the blood as well as increased uptake by the tumor (Fig. 4). The T-to-B ratio of the VFC radioimmunoconjugate reached 2.2 ± 0.2 at 48 hr, 3.1 ± 0.2 at 72 hr and 109.4 ± 13.4 at 168 hr. Both BzDTPA and VFC radioimmunoconjugates gave lower T-to-B values at 48 hr (2.5 ± 0.1, BzDTPA; and 2.2 ± 0.2, VFC) and 72 hr (3.2 ± 0.2, BzDTPA; 3.13 ± 0.2, VFC) than the DTPA counterpart. However, at 168 hr (7 days) the situation was seen to reverse, with the T-to-B ratios for both DTPA (28.7 ± 2.3, $p < 0.0005$) and BzDTPA (40.9 ± 3.0, $p < 0.05$) radioimmunoconjugates being significantly lower than for the VFC radioimmunoconjugate (109.4 ± 13.4).

Tumor-to-liver ratios were also calculated for each radioimmunoconjugate to assess the usefulness of each conjugate in the imaging of tumors in or near this organ. Table 2 shows that the T-to-L ratios for the VFC and the DTPA radioimmunoconjugates were similar until 48 hr postinjection at which time the T-to-L ratio of the VFC radioimmunoconjugate was twice that of the other two radioimmunoconjugates. By 168 hr, the VFC T-to-L ratio was 5.3 ± 0.4, eight times that of the BzDTPA-Mab conjugate (0.62 ± 0.04). This was mainly due to the higher liver radioactivity in the animals receiving the DTPA radioimmunoconjugates. Overall, there was lower normal tissue radioactivity present in animals receiving the VFC radioimmunoconjugate compared to animals receiving either of the other two radioimmunoconjugates.

Scintiscans of animals injected with ^{57}Co-VFC T84.66 reflected the biodistribution data. Although the tumor was quite visible at 48 hr (Fig. 5B), the tumor image with the clearest background was obtained at 148 hr (Fig. 5C). At

TABLE 2
Tumor-to-Blood and Tumor-to-Liver Ratios for T84.66 Mab Conjugated with ^{111}In-DTPA, ^{111}In-BzDTPA or ^{57}Co-VFC in Nude Mice Bearing LS174T Xenografts Mean (s.e.)

	Tumor-to-blood			Tumor-to-liver		
Time	^{111}In-DTPA	^{111}In-BzDTPA	^{57}Co-VFC	^{111}In-DTPA	^{111}In-BzDTPA	^{57}Co-VFC
1 hr	0.65	0.22	0.36	0.22	0.67	0.39
	(0.08)	(0.01)	(0.06)	(0.02)	(0.03)	(0.05)
6 hr	0.63	0.75	0.57	1.01	2.02	0.74
	(0.04)	(0.04)	(0.03)	(0.34)	(0.30)	(0.06)
24 hr	2.04	1.17	1.86	2.01	2.12	3.62
	(0.17)	(0.07)	(0.09)	(0.13)	(0.12)	(0.20)
48 hr	4.64	2.47	2.19	1.87	2.53	5.05
	(0.60)	(0.09)	(0.21)	(0.20)	(0.09)	(0.43)
72 hr	6.56	3.19	3.13	1.60	3.44	5.98
	(0.94)	(0.16)	(0.16)	(0.18)	(0.23)	(0.29)
168 hr	28.73	40.88	109.43	0.41	0.62	5.34
	(2.32)	(2.97)	(13.37)	(0.01)	(0.04)	(0.40)

Five mice were in each group.

this time, only the tumor was visible with very little blood pool or organ background radioactivity observed, especially in the liver.

Although the specific activity of the VFC conjugates was 50-fold lower than that of the DTPA conjugates, tissue distribution (Table 1) and tumor imaging (Fig. 5) with the VFC radioimmunoconjugates was good. Tumor uptake (%ID/g) was comparable to that of the DTPA radioimmunoconjugates (Fig. 4C).

Effect of Mab Dose on Biodistribution

The effect of increasing the dose of unlabeled Mab on the liver uptake of the three immunoconjugates is shown in Figure 6. Mice receiving DTPA radioimmunoconjugates demonstrated a substantially higher liver radioactivity (DTPA: 22.2 %ID/g; BzDTPA: 17.9 %ID/g) than those injected with VFC radioimmunoconjugate (7.8 %ID/g) at the 10 μg dose. A Mab dose increase from 10 μg to 50 μg brought the level of radioactivity in the liver down to the 8–10 %ID/g range for both DTPA radioimmunoconjugates and to 5.5 %ID/g for VFC radioimmunoconjugate. Further increasing the dose to 100 μg or 200 μg did not alter the amount of radiolabel in the liver for any of the three Mab conjugates.

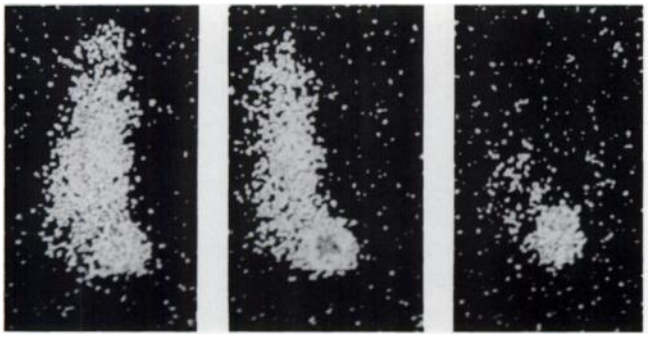

FIGURE 5. Scintiscans were performed on mice injected with 20 μg; 2 μCi of ^{57}Co-VFC-T84.66 at (A) 24 hr, (B) 48 hr, and (C) 148 hr following injection. At 148 hr, only the tumor was visible.

DISCUSSION

Concern regarding high uptake of radiometals conjugated to monoclonal antibodies by normal liver tissue has prompted considerable interest in the production of new, more stable bifunctional chelates. One of the possible reasons for the accumulation of radioactivity in the liver using the aminocarboxylate type chelates typified by DTPA and EDTA has been transchelation of the radiometal with serum proteins such as transferrin (*8,9,14,15*). A chelate which obviates this loss of radiometal to serum proteins should result in lower levels of radioactivity in normal liver and therefore be a more attractive radioimmunoconjugate. Modifications of DTPA and EDTA by addition of linker groups such as isothiocyanatobenzyl have demonstrated

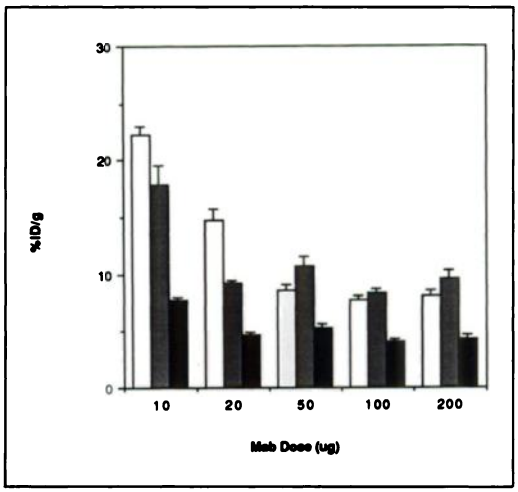

FIGURE 6. Effect of increasing the dose of antibody on liver radioactivity for ^{111}In-DTPA-T84.66 (□), ^{111}In-BzDTPA-T84.66 (▩) and ^{57}Co-VFC-T84.66 (■). Mice received 10 μg of radioimmunoconjugate plus 10, 40, 90 and 190 μg of unlabeled T84.66 Mab. The biodistributions were performed at 48 hr and liver radioactivity expressed as %ID/g ± s.e. The number of animals in each group ranged from four to nine.

less transchelation of the radiometal ^{111}In in serum (*27*) and some decrease in liver radioactivity in animal biodistributions (*8,29*). The synthesis of p-bromoacetamidobenzyl macrocyclic chelates, such as TETA (*30*) and DOTA (*31*), suitable for holding radiometals such as ^{67}Cu and ^{90}Y, respectively, has allowed the stable attachment of these radiometals (which normally form unstable bonds with DTPA) to monoclonal antibodies. However, animal biodistribution data showed T-to-L ratios of about 1 (0.6–1.3) over a 5-day study for ^{90}Y-DOTA Lym-1 radioimmunoconjugate (*29*) and T-to-L ratios of 0.7–2.7 by day 5 for ^{67}Cu-Lym-1 radioimmunoconjugate (*30*). Thus, normal liver uptake remained considerable for these two preparations.

The bifunctional carborane chelator VFC was designed to hold transition metals in a stable configuration utilizing enhanced pi-bonding between a transition metal and two covalently linked carborane cages (*18*). Chelates of this sort are not subject to the normal catabolic processes encountered in vivo because they are essentially inorganic and not subject to normal enzymatic attack. The radiotransition metal chosen to load the VFC in order to test the efficacy of this chelate for attaching radiotransition metals to an antibody for in vivo tumor targeting was ^{57}Co. This radiometal was determined appropriate for preclinical testing of the VFC chelate due to its accessibility, purity and appropriate photon energy (122 keV (89%) and 136 keV (11%) gamma emissions) for imaging studies, although its half-life (267 days) was not considered ideal for clinical use. Cobalt-57-VFC is highly hydrophobic and the requirement of loading the radiometal into the chelate prior to antibody conjugation is less desirable than labeling the already conjugated antibody. However, the in vivo behavior and the apparent lack of alteration to the antibody's immunological effectiveness make this chelate a strong candidate for attaching transition metals with therapeutic potential. Such transition metal nuclides include: ^{48}Cr (γ, $T_{1/2}$ = 21 hr), ^{66}Ni (β^-, $T_{1/2}$ = 55 hr), ^{67}Cu (γ, β^- $T_{1/2}$ = 62 hr), ^{105}Rh (γ, β^- $T_{1/2}$ = 35 hr), ^{186}Re (γ, β^- $T_{1/2}$ = 91 hr) and ^{188}Re (γ, β^- $T_{1/2}$ = 17 hr).

Previous work has demonstrated the synthesis and structural characterization of nickel and copper VFC chelates (*19*). Related bridged dicarbollide clusters which contain nickel and chromium (as well as iron and cobalt), prepared as prototypes for the second generation of VFC chelates (*20*), have been prepared and structurally characterized. Earlier work (*32*) has provided the synthesis of a ^{105}Rh *bis*-dicarbollide chelate which portends the usefulness of this nuclide in a VFC chelate structure. Pillai et al. (*33*) have demonstrated the conjugation of a ^{105}Rh chelate with human gamma globulin. As listed above, transition metal radionuclides that are potentially available have excellent properties for both diagnosis and therapy. They require a chemically appropriate chelation system, such as VFC, in order to maximize their stability and in vivo performance.

A comparison of the stabilities of the VFC radioimmunoconjugate and the two DTPA radioimmunoconjugates in serum both in vitro and in vivo showed the VFC radioimmunoconjugate to be very stable. The loss of ^{57}Co or the ^{57}Co-VFC from the Mab was <1% as identified by size exclusion HPLC analysis during the 7-day study. Thus, not only is the chelate exceptionally stable chemically (*18,21*) but it is also extremely stable biologically.

Pharmacokinetic studies in tumor-bearing nude mice indicated some interesting differences in the biodistribution of the T84.66 Mab conjugated with the VFC chelate as compared to the two DTPA chelates. On the one hand, blood clearance of the three radioimmunoconjugates was very similar with half the radiolabel removed by about 20 hr. This suggests that the rate of removal from the blood was determined by the antibody, which was the same for each preparation. On the other hand, total body clearance was more than twice as rapid for VFC radioimmunoconjugate than for the two DTPA radioimmunoconjugates. Since blood clearance was similar for all three radioimmunoconjugates, a more rapid clearance of VFC radioimmunoconjugate or its metabolites from tissues other than blood was indicated. This was observed in the hepatic clearance. As noted in Figure 4B, the liver demonstrated a higher initial uptake of radiolabel in animals receiving the VFC radioimmunoconjugate compared to those receiving the DTPA radioimmunoconjugates. This high level of hepatic radioactivity however, decreased exponentially to less than 1 %ID/g by day 7. Radioactivity in the livers of mice given either of the two T84.66-DTPA radioimmunoconjugates, although initially lower, remained at a relatively constant level across the same time period. Other normal tissues such as the spleen and kidneys also showed an overall lower retention of radiolabel in mice receiving the VFC radioimmunoconjugate.

Cobalt-57 administered in the ionic form ($^{57}CoCl_2$) was cleared very rapidly (<1 %ID/g in all tissues examined at 0.5 hr), did not appear in the liver (<0.5 %ID/g) and was excreted in the urine. Indium-111 administered in the ionic form ($^{111}InCl_3$) cleared more slowly, was retained in the liver and kidney (6–8 %ID/g; 21–27 %ID/g, respectively, out to 24 hr), and was excreted in the urine. The whole-body elimination of ionic cobalt was more rapid than that of ionic indium. However, both the high chemical and biological stability of the cobalt chelate, ^{57}Co-VFC, and the difference in excretion pattern for the chelated radiometal (feces) and the ionic form (urine) argue strongly against the suggestion that release and clearance of the cobalt ion may be responsible for the differences in tissue clearance observed with the VFC radioimmunoconjugate and the DTPA radioimmunoconjugates.

Furthermore, the pattern of liver uptake and clearance for ^{57}Co-VFC-Mab complex and ^{57}Co-VFC chelate was similar; both showed early transient high liver uptake with excretion being almost exclusively in the stool. The radiolabel in the stool was present predominantly in a hydrophobic form as demonstrated by the fact that aqueous (PBS) extraction removed <10% of the fecal radioactivity. FPLC analyses of the radioactive components in the aque-

ous extract was precluded by the low levels of radioactivity present. Preliminary radiochromatograms of diethyl ether extracts of stool from mice given either ^{57}Co-VFC or ^{57}Co-VFC Mab indicated the presence of two and three peaks, respectively, using reversed-phase HPLC (data not shown). Two peaks were common to both extracts and were the same peaks obtained with the ^{57}Co-VFC chelate prior to conjugation or administration to the animals. The VFC Mab catabolites showed an additional small peak of radioactivity. Further analysis of these catabolic products is in progress.

The prolonged retention of hepatic radioactivity observed with the ^{111}In-DTPA radioimmunoconjugates may be partially due to transchelation of ^{111}In with blood proteins such as transferrin. This would be determined by the conjugate stability. DTPA radioimmunoconjugate demonstrated a 7% loss and BzDTPA radioimmunoconjugate showed a 2%–3% loss of radiolabel over 7 days indicating that the contribution of transchelation to hepatic radioactivity during this time was small. Administration of the ^{111}In-DTPA chelate alone resulted in rapid blood and total body clearance with no uptake in the liver. This suggests that extracellular ^{111}In-DTPA is cleared rapidly. Intracellular chelate (catabolic product in liver cells), however, may behave differently. Previous work has demonstrated that for ^{111}In-DTPA radioimmunoconjugates, radioactivity present in the liver after 24 hr was seen predominantly as a low molecular weight species, not associated with intact antibody, as identified by HPLC analyses (*34,36,37*). These observations by ourselves and others indicate that indium may be retained intracellularly (hepatocytes) in the chelated form (*29,34–37*).

In contrast to the behavior of the ^{111}In-DTPA chelates, the results presented here show that there was no significant retention of the ^{57}Co-VFC in liver tissue. The advantage of the more efficient clearance mechanism for VFC radiometal conjugates in normal liver is reduced background radioactivity allowing improved visualization (and potential therapy) of tumors in the hepatic region. This is of great importance for colorectal disease in which hepatic metastases are common. Similarly, although excretion of the radiolabel is via the feces, no increased bowel tissue radioactivity was observed in biodistribution studies (<3 %ID/g) or in the scintiscans (Fig. 5).

We have previously demonstrated that the reason for the high liver uptake of anti-CEA Mabs in animals bearing CEA-producing tumors was the formation of CEA-Mab complexes that were cleared by the liver (*10,34*). Increasing the dose of unlabeled Mab with DTPA radioimmunoconjugates resulted in a decrease in the liver radioactivity without inhibiting the tumor uptake of radiolabel. The effective dose of unlabeled Mab was dependent on the size and level of CEA expression of the tumor, with higher doses required for larger, higher-expressing tumors. Increasing the dose of Mab with the VFC radioimmunoconjugate, however, did not alter the liver radioactivity to the same extent (Fig. 6). Because the Mab was the same for both types of conjugate and the tumor sizes were comparable, the formation of CEA-Mab complexes, and subsequent liver uptake, would be expected to be similar. This implies that lower levels of hepatic radioactivity, unaffected by Mab dose, observed with the VFC radioimmunoconjugates must be attributed to a faster rate of clearance of the radiolabeled catabolic products produced in the liver. Liver processing and excretion of the VFC radioimmunoconjugate appear to involve different mechanisms from those utilized for the DTPA radioimmunoconjugates. Further analysis of the liver processing of these two types of chelate is currently being pursued.

The use of high Mab doses to decrease liver background potentially increases the incidence of human anti-antibody (HAMA) formation. This suggests another advantage of VFC as a chelating agent for anti-CEA antibodies in that low doses of Mab may be administered while still maintaining low hepatic radioactivity.

In summary, the anti-CEA-Mab T84.66 conjugated to the VFC containing ^{57}Co retained its immunoreactivity, showed no loss of radiolabel in serum over a period of seven days, and demonstrated good targeting and imaging of the LS174T tumors in vivo. This prototype of a bifunctional chelate for transition metals showed a distinct improvement over the aminocarboxylate type chelates with respect to lower normal tissue levels of radioactivity, particularly in the liver, indicating its potential advantage for imaging and treating tumors in or near this organ.

ACKNOWLEDGMENTS

The authors thank Frances Greenspan-Stege and Susie Huthmacher for their excellent secretarial assistance. This work was supported in part by NIH Cancer Center Core Grant 33527 and Program Project CA 43904.

REFERENCES

1. Krejcarek GE, Tucker KL. Covalent attachment of chelating groups to macromolecules. *Biochem Biophy Res Commun* 1977;77:581–585.
2. Hnatowich DJ, Layne WW, Childs RL, Lanteigne D, Davis MA. Radioactive labeling of antibody: a simple and efficient method. *Science* 1983;220: 613–615.
3. Halpern SE, Hagan PL, Garver PR, et al. Stability, characterization, and kinetics of ^{111}In-labeled monoclonal antitumor antibodies in normal animals and nude mouse-human tumor models. *Cancer Res* 1983;43:5347–5355.
4. Paxton RJ, Jakowatz JG, Beatty JD, et al. High-specific-activity ^{111}In-labeled anticarcinoembryonic antigen monoclonal antibody: improved method for the synthesis of diethylenetriaminepentaacetic acid conjugates. *Cancer Res* 1985;45:5694–5699.
5. Esteban JM, Schlom J, Gansow OA, et al. New method for the chelation of indium-111 to monoclonal antibodies: biodistribution and imaging of athymic mice bearing human colon carcinoma xenografts. *J Nucl Med* 1987;28: 861–870.
6. Meares CF, McCall MJ, Reardon DT, Goodwin DA, Diamanti CI, McTigue M. Conjugation of antibodies with bifunctional chelating agents: isothiocyanate and bromoacetamide reagents, methods of analysis, and subsequent addition of metal ions. *Anal Biochem* 1984;142:68–78.
7. Brechbiel MW, Gansow OA, Atcher RW, et al. Synthesis of 1-(*p*-isothiocyanathobenzyl) derivatives of DTPA and EDTA. Antibody labeling and tumor imaging studies. *Inorg Chem* 1986;25:2772–2781.
8. Westerberg DA, Carney PL, Rogers PE, Kline SJ, Johnson DK. Synthesis of novel bifunctional chelators and their use in preparing monoclonal antibody conjugates for tumor targeting. *J Med Chem* 1989;32:236–243.
9. Sands H, Jones PL. Methods for the study of the metabolism of radiolabeled monoclonal antibodies by liver and tumor. *J Nucl Med* 1987;28:390–398.

10. Beatty BG, Beatty JD, O'Connor-Tressel M, Williams LE, Paxton RJ, Shively JE. Effect of specific antibody pretreatment on liver uptake of ^{111}In-labeled anti-CEA monoclonal antibody in nude mice bearing human colon cancer xenografts. *Cancer Res* 1989;49:1587–1594.
11. Jakowatz JG, Beatty BG, Vlahos WG, et al. High-specific-activity ^{111}In-labeled anticarcinoembryonic antigen monoclonal antibody: biodistribution and imaging in nude mice bearing human colon cancer xenografts. *Cancer Res* 1985;45:5700–5706.
12. Beatty JD, Williams LE, Yamauchi D, et al. Presurgical imaging with indium-labeled anti-CEA for colon cancer staging. *Cancer Res* 1990; 50(suppl):922s–926s.
13. Murray JL, Rosenblum MG, Sobol RE, et al. Radioimmunoimaging in malignant melanoma with ^{111}In-labeled monoclonal antibody 96.5. *Cancer Res* 1985;45:2376–2381.
14. Yokoyama K, Carrasquillo JA, Chang AE, et al. Differences in biodistribution of indium-111 and iodine-131-labeled B72.3 monoclonal antibodies in patients with colorectal cancer. *J Nucl Med* 1989;30:320–327.
15. Carney PL, Rogers PE, Johnson DK. Dual isotope study of iodine-125 and indium-111-labeled antibody in athymic mice. *J Nucl Med* 1989;30:374–384.
16. Paik CH, Yokoyama K, Reynolds JC, et al. Reduction of background activities by introduction of a diester linkage between antibody and a chelate in radioimmunodetection of tumor. *J Nucl Med* 1989;30:1693–1701.
17. Arano Y, Matsushima H, Tagawa M, et al. A novel bifunctional metabolizable linker for the conjugation of antibodies with radionuclides. *Bioconj Chem* 1991;2:71–76.
18. Hawthorne MF, Varadarajan A, Knobler CB, et al. Radiometallacarboranes as tumor imaging reagents. *J Am Chem Soc* 1990;112:5365–5366.
19. Varadarajan A, Johnson SE, Gomez FA, Chakrabarti S, Knobler CB, Hawthorne MF. Synthesis and structural characterization of pyrazole-bridged metalla-bis(dicarbollide) derivatives of cobalt, nickel, copper and iron: models for venus flytrap cluster reagents. *J Am Chem Soc* 1992;114: 9003–9011.
20. Gomez FA, Johnson SE, Knobler CB, Hawthorne MF. Synthesis and structural characterization of metallacarboranes containing bridged dicarbollide ligands. *Inorg Chem* 1992;31:3558–3567.
21. Paxton RG, Beatty BG, Hawthorne MF, et al. A novel transition metal cluster (venus flytrap cluster) for radioimmunodetection and radioimmunotherapy. *Proc Natl Acad Sci USA* 1991;88:3387–3391.
22. Wagener C, Clark BR, Rickard KJ, Shively JE. Monoclonal antibodies for carcinoembryonic antigen and related antigens as a model system: determination of affinities and specificities of monoclonal antibodies by using biotin-labeled antibodies and avidin as precipitating agent in a solution phase immunoassay. *J Immunol* 1983;130:2302–2307.
23. Hammarstrom S, Shively JE, Paxton RJ, Beatty BG, Larsson A. Antigenic sites in carcinoembryonic antigen. *Cancer Res* 1989;49:4852–4858.
24. Beatty JD, Beatty BG, Vlahos WG, Hill LR. Method of analysis of non-competitive enzyme immunoassays for antibody quantification. *J Immunol Methods* 1987;100:161–172.
25. Beatty JD, Beatty BG, Vlahos WG. Measurement of monoclonal antibody affinity by non-competitive enzyme immunoassay. *J Immunol Methods* 1987;100:173–179.
26. Baldwin HT, Rutzky LP, Jakstys MM, Oyasu R, Kaye CI, Kahan BD. Human colonic adenocarcinoma cells. I. Establishment and description of a new line. *In Vitro* 1976;12:180–191.
27. Cole WC, DeNardo SJ, Meares CF, et al. Comparative serum stability of radiochelates for antibody radiopharmaceuticals. *J Nucl Med* 1987;28:83–90.
28. Philben VJ, Jakowatz JG, Beatty BG, et al. The effect of tumor carcinoembryonic antigen (CEA) content and tumor size on tissue uptake of ^{111}Indium labeled anti-CEA monoclonal antibody. *Cancer* 1986;57:571–576.
29. Deshpande SV, Subramanian R, McCall MJ, DeNardo SJ, DeNardo GL, Meares CF. Metabolism of indium chelates attached to monoclonal antibody: minimal transchelation of indium from benzyl-EDTA chelate in vivo. *J Nucl Med* 1990;31:218–224.
30. Deshpande SV, DeNardo SJ, Meares CF, McCall MJ, et al. Copper-67-labeled monoclonal antibody lym-1, a potential radiopharmaceutical for cancer therapy: labeling and biodistribution in RAJI tumored mice. *J Nucl Med* 1988;29:217–225.
31. Deshpande SV, DeNardo SJ, Kukis DL, et al. Yttrium-90-labeled monoclonal antibody for therapy: labeling by a new macrocyclic bifunctional chelating agent. *J Nucl Med* 1990;31:473–479.
32. Francis JN. Reactions of some (3)-1,2-dicarbollyl cobalt complexes, PhD Thesis. University of California, Los Angeles; 1969.
33. Pillai MRA, John CS, Troutner DE. Labeling of human IgG with Rhodium-105 using a new pentadentate bifunctional ligand. *Bioconj Chem* 1990;1:191–197.
34. Beatty BG, O'Connor-Tressel M, Do T, Beatty JD. Mechanism of decreasing liver uptake of ^{111}In-labeled Mab by specific antibody pretreatment in tumor bearing mice. *Cancer Res* 1990;50(suppl):846s–851s.
35. Beatty JD, Beatty BG, O'Connor-Tressel M, Do T. Mechanisms of tissue uptake and metabolism of radiolabeled antibody. *Cancer Res* 1990; 50(suppl):840s–845s.
36. Jones PL, Brown BA, Sands H. Uptake and metabolism of ^{111}In-labeled monoclonal antibody B6.2 by the rat liver. *Cancer Res* 1990;50(suppl):852s–856s.
37. Motta-Hennessy C, Sharkey RM, Goldenberg DM. Metabolism of indium-111-labeled murine monoclonal antibody in tumor and normal tissue of the athymic mouse. *J Nucl Med* 1990;31:1510–1519.