Evaluation of Indium-111- and Yttrium-90-Labeled Linker-Immunoconjugates in Nude Mice and Dogs

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Rapid uptake and slow transit of radioactivity from normal organs are detrimental to any clinically utilized radioimmunoconjugate because they lower the target-to-nontarget ratio and deliver undesirable radiation to normal organs. To mitigate this problem, two labile chemical linkages (EGS and DST) were introduced between a monoclonal antiferritin antibody (QCI) and a chelating agent (DTPA). The biodistribution of labile-linker immunoconjugates (EGS and DST) and stable linker immunoconjugates (DSS and ITCB) were compared. In a nude mouse model, all of the four immunoconjugates labeled with ^{111}In targeted subcutaneously-implanted human tumor cells. Tumor-to-normal organ ratios were enhanced for the EGS linkage in comparison to the two stable linkages. Serial whole-body immunoscintigraphy confirmed the biodistribution study. The EGS and ITCB ^{90}Y-labeled immunoconjugates had biodistributions similar to their respective ^{111}In-labeled immunoconjugates. As the mouse model is not representative of the high uptake of monoclonal antibodies in the human liver, beagle dogs were used to further explore the retention of radiolabel in normal liver. The EGS-linked immunoconjugate significantly reduced the dog liver activity when compared to the ITCB immunoconjugate. The combination of the animal models (mouse and dog) appears to allow for a more complete and optimal preclinical analysis of chelated radiolabeled monoclonal antibodies for diagnosis or treatment and illustrates the potential clinical improvements possible with labile chemical linkages in radioimmunoconjugates.

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Radiolabeled immunoglobulin therapy (RIT) has induced complete and partial responses in patients with hepatoma (*1–3*), end stage Hodgkin lymphoma (*4–6*) or B-cell lymphoma (*7*). The results of experimental evaluation of radioimmunoconjugates in animal models have also been promising. High tumor uptake and rapid clearance from normal organs are essential for any radioimmunoconjugate to be considered as a clinically useful diagnostic and therapeutic agent. Several immunoconjugates with different chemical modifications have been tested to achieve this goal.

One approach is to use chelating agents with improved chelation stability for radiometals. This approach involves the conjugation of an antibody to a chelator with an appropriate denticity for stable complexation with ^{111}In or ^{90}Y (*8,9*). A second approach is a site-specific carbohydrate mediated conjugation of the chelating agent in the F_c region of the antibody to improve the tumor localization of immunoconjugates (*10*). A third approach investigated in this paper is the insertion of labile chemical linkages between antibody and chelating agent.

Ideally, a labile-linked radiolabeled chelate will be cleaved from the antibody by normal tissue metabolism. The chelated radiometal can be eliminated rapidly through the kidneys, thereby reducing the background activity. Metabolic cleavage of the radioimmunoconjugate should not occur in tumor tissues due to the absence of the appropriate enzymes. Quadri et al. (*11,12*); Haseman et al. (*13*); Deshpande et al. (*14*) and Paik et al. (*15,16*) have reported that the introduction of a diester linkage between the antibody and a radiometal chelate increases the clearance of the radioactivity from blood and substantially reduces radioactivity in normal organs such as bone marrow, kidney and liver.

In this paper, we report a comparison between two stable linkages and two chemically labile linkages in experimental animals. Using thiourea (ITCB) and hydrocarbon linkages (DSS) as relatively stable controls, diester (EGS) and tartaramide (DST), two chemically labile linkages, were introduced between a monoclonal antibody (Mab) and an aminobenzyl-DTPA derivative in order to evaluate pharmacokinetics in a nude mouse tumor model. The effect of labile-linker uptake in normal liver was also tested in beagle dogs.

MATERIALS AND METHODS

Aminobenzyl-DTPA was prepared by reacting methyl-p nitrophenylalaninate to ethylenediamine and followed by multistep reactions synthesis to achieve the bifunctional chelating agent

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(*8,17,18*). The intermediate compounds and final product were characterized by NMR, mass spectroscopy and elemental analysis. The ^1H NMR spectra were taken on a Varian XL-400 FT-NMR spectrometer. Mass spectra were run on a VG 70S spectrometer. The trimethylsilylester of pentacarboxylic acid was utilized in either the electron impact (70 eV, ion source temperature 200°C) or chemical ionization mode to provide the M^+ and (M + 1) molecular ions, respectively. HPLC purified aminobenzyl-DTPA was utilized for conjugation with cross-linkers. Most of the cross-linking agents used for the introduction of different chemical bonds are activated homobifunctional N-hydroxysuccinimide esters. The chelating agent, 1-(p-aminobenzyl)-diethylene triamine pentaacetic acid (ABDTPA) has a primary amino group available for conjugation with a bifunctional cross-linking agent. All reagents and chemicals were purchased from commercial suppliers with certificate of analysis and used as received, unless otherwise specified.

Monoclonal antiferritin antibody (QCI) is a murine IgG_1 that recognizes ferritin positive human hepatoblastoma (HepG2). Antibody was obtained from Hybritech, Inc., San Diego, CA. $^{111}InCl_3$ and $^{90}YCl_3$ isotopes were purchased from Du Pont New England Nuclear. Abbreviations used in text are defined as follows: Mab: monoclonal antibody; DTPA: diethylene triaminepentaacetic acid; ITCB-DTPA: 1-(p-isothiocyanatobenzyl)-DTPA; ABDTPA: aminobenzyl-DTPA; DMSO: dimethyl sulfoxide; EGS: ethylene glycol *bis*(succinimidylsuccinate); DST: disuccinimidyl tartarate; DSS: disuccinimidyl suberate; TLC: thin-layer chromatography; ITLC: instant thin-layer chromatography; HPLC: high-performance liquid chromatography.

Placement of Chemical Linkage

The primary amino group of ABDTPA was reacted with an activated carboxyl group of N-hydroxysuccinimide ester of disuccinimide tartarate (DST), disuccinimidyl suberate (DSS) or ethylene glycol *bis*(succinimidylsuccinate) (EGS); the remaining activated carboxyl group of the diester was then reacted with amino groups of the antibody to introduce the linkage between the chelator and the antibody.

Introduction of Labile Linkages (EGS, DST)

The method of Abdella et al. (*19*) was modified to conjugate Mab (IgG_1) with the labile linkers when DST and EGS were used as cross-linking agents. For the introduction of a linker, the cross-linking agent (0.04 mmole) was dissolved in 0.5 ml anhydrous DMSO and mixed with aminobenzyl-DTPA (0.04 mmole) in 0.5 ml DMSO solution in equimolar ratio. The solution was stirred gently at room temperature for 90 min. The reaction mixture was extracted twice with anhydrous ethyl acetate to remove the unreacted cross-linking agent. Fifteen milligrams of antibody (1×10^{-4} mmole) in 1 ml of 0.05 *M* phosphate buffer saline (PBS) pH 7.8 was added rapidly into 50 μl DMSO solution of chelate-crosslinker (2×10^{-3} mmole) mixture while stirring at 4°C. The coupling ratio of antibody and chelate-crosslinker was approximately 1:20. Under these optimized conditions, 1–2 chelates were conjugated per antibody molecule to minimize changes in immunoreactivity. The conjugation reaction was continued for 2 hr at room temperature. Before radiolabeling, immunoconjugates were separated from unconjugated chelate-crosslinker by dialysis against 0.05 *M* phosphate buffer pH 7.0.

Introduction of Hydrocarbon Linkages (DSS)

The reaction procedure for the introduction of the hydrocarbon chain was the same as that for the introduction of the labile linkage, except that disuccinimidyl suberate (DSS) was used as the cross-linking agent. Immunoconjugates were purified from unconjugated chelate moieties by centricon-30 filtration before radiolabeling with $^{111}InCl_3$ for biodistribution studies. The linker placement and chemical structure of immunoconjugates are described in Figure 1.

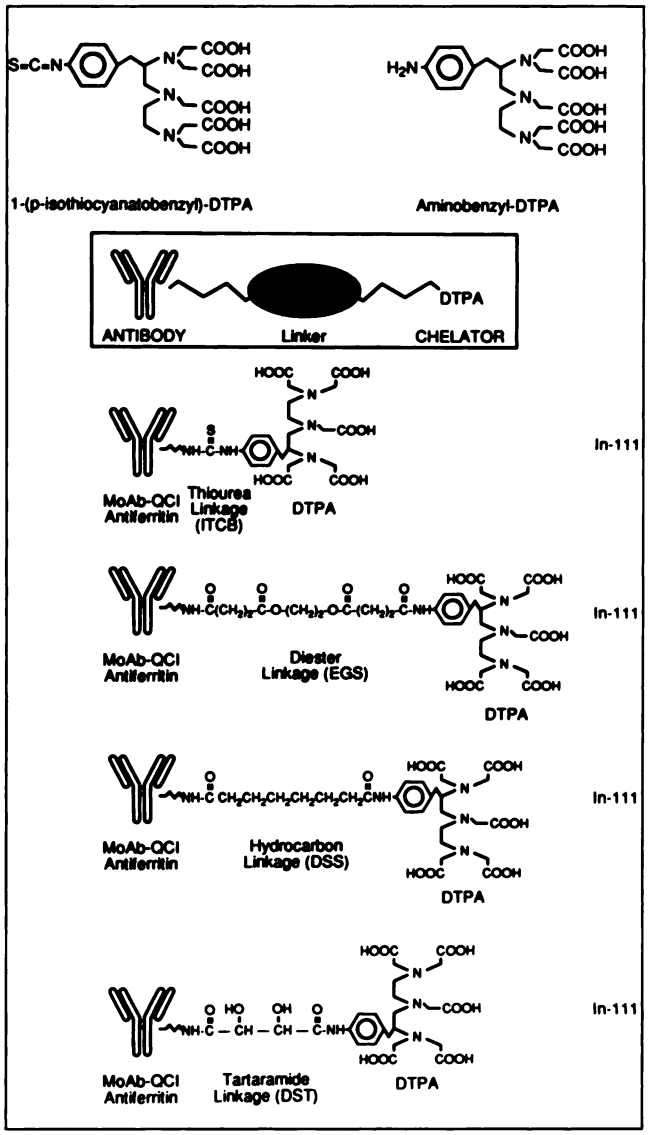

FIGURE 1. Chemical structures of chelating agents and antibody-linker-DTPA conjugates.

Introduction of Thiourea Linkages (ITCB)

Isothiocyanatobenzyl-DTPA (ITCB-DTPA) derivative was synthesized by reacting 50 mg of aminobenzyl-DTPA with 80% thiophosgene solution in 2 ml $CHCl_3$ (v/v)(*8,18*). The reaction mixture was stirred at room temperature for 5 hr and stopped when fluorescamine test was negative. The product was purified by column chromatography using florisil column and eluted with CH_3CN: H_2O (30:8). The solvent was lyophilized and dried ITCB-DTPA (45 mg, yield 80%) was stored at −20°C. The isothiocyanate functional group was characterized by FT-IR spectrophotometer (strong absorption at 2100 cm^{-1}). 1-(p-Isothiocyanatobenzyl)-DTPA was reacted with amino groups of antibody to conjugate through a thiourea bond. Monoclonal antibody (15 mg, 1×10^{-4} mmole) in 0.9 ml of 0.2 *M* HCO_3 buffer was reacted with

TABLE 1
Quality Control Analysis of Radioimmunoconjugates

Analysis	Ester linkage (EGS)	Tartaramide linkage DST	Thiourea linkage (ITCB)	Hydrocarbon linkage (DSS)
DTPA/IgG	1.8	1.8	2	1.5
Specific activity	2 mCi/mg	2 mCi/mg	2.5 mCi/mg	2 mCi/mg
Colloid formation	none	none	none	none
Serum stability*				
24 hr	80%–85%	92%–97%	99%	99%
48 hr	75%–80%	85%–90%	98%	97%
Cross-linking	<5%	<5%	none	<5%
Immunoreactivity	85%	85%	90%	90%
Dose injected	30–40 μCi/animal	30–40 μCi/animal	30–40 μCi/animal	30–40 μCi/animal

*Protein bound fraction after 24 and 48 hr incubation at 37°C in serum.

freshly prepared 100 μl aqueous solution of ITCB-DTPA (0.22 mg, 4×10^{-4} mmole) at a molar ratio of 1:4. The pH of the reaction mixture was adjusted to 8.0 by 1 *M* $NaHCO_3$ solution and mixture was incubated for 2 hr at room temperature. Prior to radiolabeling, Mab-thiourea-benzyl-DTPA was purified from unconjugated DTPA by centricon-30 filtration.

Radiolabeling and Determination of Chelates per IgG

An assay for the determination of the average number of chelates per IgG molecule was performed on a small aliquot of the coupling reaction mixture before separation of the unreacted chelating agent. This aliquot was diluted to 0.5 ml with a buffer mixture of 0.5 *M* sodium acetate and 0.05 *M* sodium citrate, pH 5.5, and 0.1 ml of $InCl_3$ solution (0.05 *M* HCl, pH ~ 2) of a known concentration spiked with a trace amount of $^{111}InCl_3$. The amount of indium metal added must be twice the amount of the ligand (coupled and uncoupled) in order to facilitate one indium per chelate complex. The presence of colloids or aggregates was analyzed during the labeling procedure by paper chromatography (Whatman No.1/saline). After 1 hr, the labeled antibody was separated from other ^{111}In complexes by TLC analysis and the number of moles of chelate per mole of antibody was calculated. Radiolabeling of immunoconjugate was determined by ITLC using saline as developing solvent. In this system the labeled immunoconjugate stayed at the origin while labeled DTPA moved with the solvent front. Silica-gel TLC strip (1 × 12 cm) was spotted with radiolabeled conjugate and developed by using methanol: 10% NH_4OAc (1:1) as a mobile phase. In this TLC analysis, ^{111}In-chelated IgG appeared at $R_f = 0$, ^{111}In-DTPA derivative at $R_f = 0.55$ and ^{111}In-citrate at $R_f = 1.0$. TLC strips were monitored by a radiochromatograph scanner (Vista-100, Packard Instruments). The percent activity bound to the antibody peak was also determined by HPLC analysis and correlated with TLC results. An average of 2 DTPA chelates were conjugated to antibody molecules in antibody-linker-chelate conjugates.

An aliquot (10 μl) of pure $^{111}InCl_3$ (2.0 mCi) in 0.1 *M* HCl was equilibrated with 50 μl of 0.5 *M* acetate buffer pH 5.3 and 50 μl of 0.05 *M* citrate buffer pH 5.5. Fifty microliters of antibody-linker-chelate conjugate (1.0 mg) solution in PBS was added into buffered indium, mixed well and incubated at room temperature for 30 min. The labeled immunoconjugate was separated from low molecular weight compounds by Sephadex G50 gel column (1.5 × 20 cm) chromatography using 0.05 *M* PBS as an eluant. The labeled immunoconjugate was collected and assayed in a dose calibrator, and the labeling efficiency was determined by TLC and HPLC analysis. Yttrium-90 radiolabeling was achieved by using ^{90}Y-acetate buffer solution at pH 6.0. The buffered ^{90}Y-acetate was incubated with immunoconjugate for 2 hr at room temperature. The yttrium-labeled immunoconjugates were purified on Sephadex G100 column (1.5 × 25 cm) chromatography by eluting with 0.05 *M* PBS.

Quality Control Analysis

HPLC Analysis. Size exclusion HPLC analysis with a Bio-Sil SEC 250 column (300 × 7.8 mm) was used to determine the molecular weight and purity of immunoconjugates. The column was eluted with buffer (containing 50 m*M* NaH_2PO_4, 50 m*M* Na_2HPO_4, 150 m*M* NaCl and 10 m*M* NaN_3, pH 6.8) at a flow rate of 1 ml/min.

Immunoreactivity. Affinity column chromatography was employed to determine the immunoreactivity of immunoconjugates. Affinity gel was prepared by conjugating 2.5 mg of ferritin protein to 2 ml of CNBr-activated Sepharose-4B gel. The maximum binding capacity of affinity gel was standardized by monoclonal anti-ferritin antibody and found to be 1 mg antibody per ml affinity gel. Purified radiolabeled linker-immunoconjugate (100 μg; 100 μCi) in 300 μl PBS buffer was loaded on a small affinity gel column (1 × 5 cm) containing 2 ml bed volume of affinity gel. After 2 hr incubation at room temperature, the column was eluted with 20 ml 0.05 *M* PBS at pH 7.6 to separate unbound immunoconjugates from the bound immunoreactive conjugates. The immunoreactive fraction was then dissociated from ferritin antigen with 3 *M* ammonium thiocyanate. The percent radioactivity in bound and unbound fractions were assayed to determine the affinity of linker immunoconjugates. Percent radioactivity recovered in bound fraction ranged between 85% and 90% (Table 1). Control incubation with a labeled nonspecific IgG of the same subclass showed less than 5% of the bound radioactivity to the affinity gel.

Serum Stability. In vitro serum stability study of ^{111}In-labeled linker-immunoconjugates was carried out using analytical chromatography procedures. After radiolabeling, the product was purified on Sephadex G-50 (1.5 × 20 cm) to remove the free isotope or other small contaminates. The purified labeled antibody (10 μg/0.1 ml of 0.05 *M* PBS, pH 7.2) was added to 1 ml aliquot of fresh human serum in triplicates. This mixture was incubated at 37°C for 24 and 48 hr. The serum samples were analyzed on size-exclusion HPLC columns (TSK G3000SW, 7.8 × 300 mm and Bio-Sil TSK 250, 7.8 × 300 mm) connected in tandem. Traces of radioactivity in antibody and nonantibody peaks were monitored by counting the fractions in a gamma counter. TLC and

TABLE 2
Liver Uptake of Indium-111-Labeled Immunoconjugates in Dogs (n = 2)

Analysis	Linker-chelate	Specificity	Dose injected	% Injected activity in liver
Poly (rabbit) (antidog ferritin)	Thiourea (ITCB-DTPA)	Dog ferritin	2.0 mCi	16
Mab QCI (mouse) (antihuman ferritin)	Thiourea (ITCB-DTPA)	Human ferritin	1.0 mCi	64
Mab QCI (mouse) (antihuman ferritin)	Diester (EGS-ABDTPA)	Human ferritin	1.35 mCi	33

ITLC were also employed to analyze the activity associated with antibody and small molecular species. As described in the previous section, labeled antibody stayed at the origin while small radiolabeled moieties moved along the solvent front.

Biodistribution Studies

Injectates were filtered through a sterile 0.2 micron acrodisc before biodistribution studies. The biodistribution of these linker-immunoconjugates was evaluated in nude mice implanted with human hepatoma xenografts (HepG2). Human tumor (HepG2) was xenografted into the left hind leg of nude mice by a subcutaneous injection of tumor cells (5×10^6 cells). When the size of the tumor was approximately 0.5 to 1 cm in diameter, the mice were injected with purified ^{111}In- or ^{90}Y-labeled immunoconjugates (40 μCi, specific activity 2 mCi/mg) via the tail vein. Animals were killed at 1, 2, 4 and 6 days after the injection of the radioimmunoconjugates. Organs were excised, weighed promptly and counted in a gamma counter.

Whole-Body Clearance

The whole-body clearance of radioactivity was monitored for labile and stable linker-chelate conjugates labeled with ^{111}In. The nude mice bearing human hepatoma HepG2 xenografts were injected via tail vein with 100 μCi of ^{111}In-labeled immunoconjugates. The retention of radioactivity in the whole body was measured by holding the mouse inside a 50-ml plastic centrifuge tube in a radioisotope dose calibrator (Capintec CRC-7, Capintec, Inc., Ramsey, NJ). The clearance of radioactivity was monitored from Day 0 to Day 6 for the labile and stable conjugates.

Imaging Studies

Athymic nude mice bearing xenografted human hepatoma HepG2 tumor were injected with 65 μCi of QCI-EGS-aminobenzyl-DTPA-^{111}In (EGS) and QCI-thiourea-benzyl-DTPA-^{111}In (ITCB) for immunoscintigraphic analysis. Static gamma camera images of nude mice were acquired in the dual-energy mode with 10% windows, centered on the 172-keV and 247-keV photopeaks of ^{111}In and stored in 128 × 128 matrices. Mice were placed on a parallel-hole collimator, protected by absorbent padding, and serial images were acquired at 4, 12 and 48 hr postinjection to visualize the differences in the biodistributions of ITCB and EGS radioimmunoconjugates.

Dog Studies

Female beagle dogs were used to analyze the retention of radiolabel in the normal liver (Table 2). After ^{111}In-labeled immunoconjugate injections, serial planar gamma camera images and SPECT scans were performed followed by autopsies 7 days postinjection. Serial blood and urine samples were taken for radioactivity determinations. Activity quantification obtained from SPECT scans was confirmed by direct counting of tissue samples that were excised at autopsy in a gamma counter (*19*).

RESULTS

Linker-Chelate Conjugation

The interposition of chemical linkages between the antibody and the chelating agent is shown schematically in Figure 1. First, the chelator, aminobenzyl-DTPA, is coupled covalently to one of the activated arms of the cross-linker. Then, the remaining activated carboxyl group is reacted with the amino group of lysine residues of the antibody in order to establish linkage between the linker-chelator and the antibody. The linker-chelate conjugation yield was 10%–20% under optimal conditions (2 DTPA per antibody molecule). For ITCB conjugates, the antibody was directly conjugated to 1-p-ITCB-DTPA in order to obtain a stable thiourea linkage between chelator and antibody. The chelate conjugation yield was 50% (2 DTPA per antibody).

Quality Control

Stability studies, characterization of immunoconjugates, and quality control analyses were performed. Results are summarized in Table 1. The immunoconjugates, containing two DTPA molecules per antibody, were radiolabeled with ^{111}In or ^{90}Y, purified by Sephadex G50 (for ^{111}In) or G100 (for ^{90}Y) column chromatography, and tested for immunoreactivity. Colloids or aggregates were not detected during the radiolabeling procedure. HPLC analysis of the linker-immunoconjugates showed one major peak (containing 95% of total activity) with a retention time identical to that of the native antibody. A minor peak (5% of activity) represented the dimer. All labeled immunoconjugate preparations were immunoreactive and radiochemically pure so that any differences in their biodistributions resulted primarily from the differences in chemical linkage. For all in vivo and in vitro experiments, the radiolabeled reagents used were shown to have more than 95% radioactivity bound to immunoconjugates.

Biodistribution

A comparison of the pharmacokinetics and biodistribution between the labile (EGS, DST) and stable (DSS, ITCB) linker immunoconjugates labeled with ^{111}In is shown in Figure 2. Excellent tumor localization is achieved

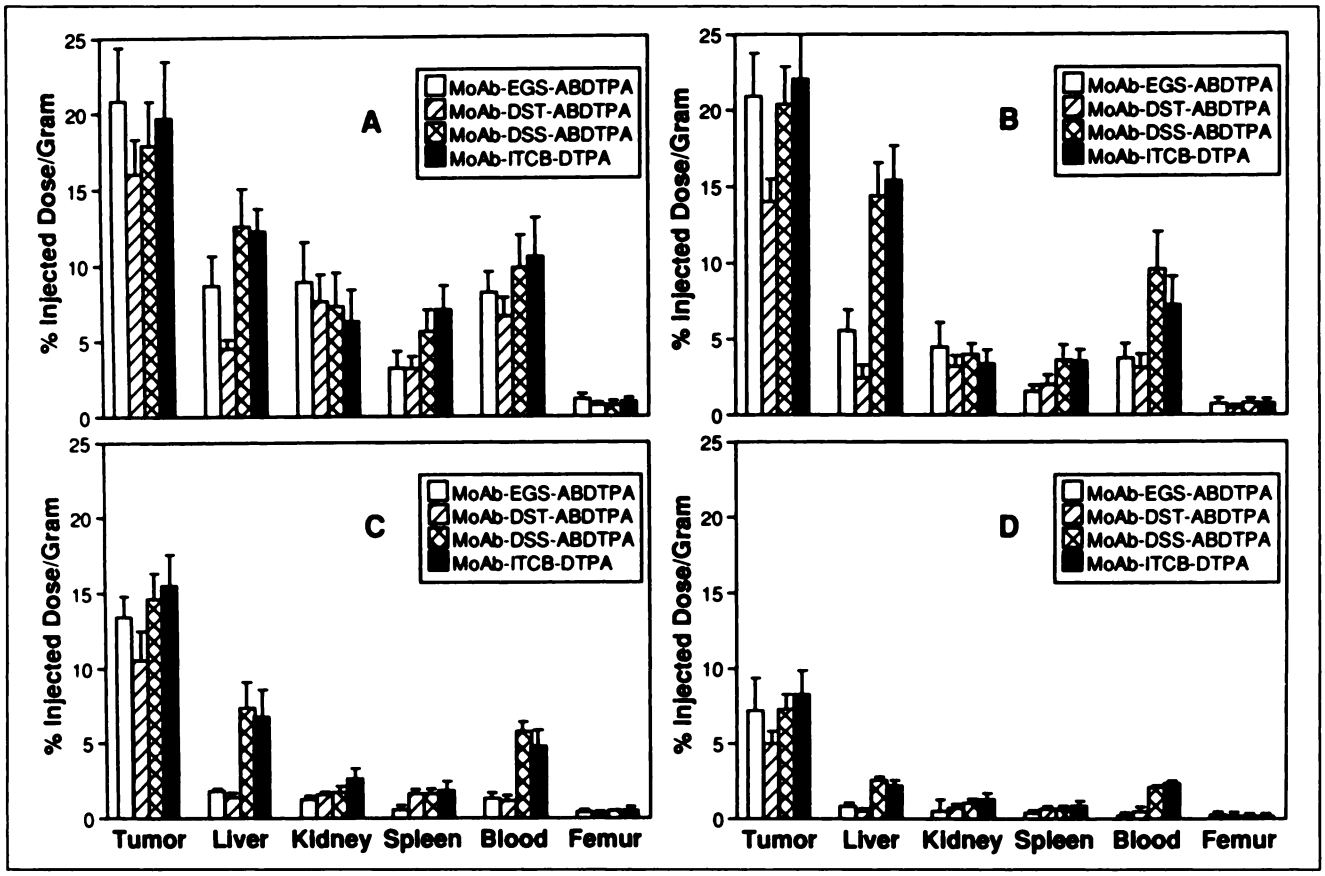

FIGURE 2. Biodistribution of ^{111}In-labeled QCI-linker-chelate conjugates in nude mice (n = 6) xenografted with human hepatoma (HepG2) at different time intervals. (A) Day 1; (B) Day 2; (C) Day 4; (D) Day 6.

on Day 2 following injection. The DST linker immunoconjugate did not target as well as the ones containing EGS or stable linkers. DST and EGS immunoconjugates are cleared more rapidly from blood than DSS- or ITCB-containing immunoconjugates. After 48 hr, the liver uptake of labile linkers is three- to fourfold lower than the stable ITCB and DSS linker conjugates. Localization of DSS-linked conjugate in normal organs such as blood, liver, kidney and spleen is very similar to the biodistribution patterns for the thiourea-linked conjugate (Fig. 2). Bone marrow uptake of indium-labeled immunoconjugates is not observed in any of the mice in this study. Similar biodistribution patterns are obtained when ^{90}Y-radiolabeled EGS and ITCB-linked immunoconjugates are compared in the nude mice tumor model as illustrated in Figure 3A–D. Bone uptake of ^{90}Y remains less than 2% injected activity per gram of bone throughout the biodistribution studies. Significant amplification of the target-to-nontarget ratios is achieved by both indium- and yttrium-labeled EGS linker conjugates as shown in Table 3. The biodistribution data were analyzed by Student's t-test and Wilcoxon rank sum test. All blood and liver activity levels are significantly different ($p < 0.01$) on Day 2 and Day 4 between EGS and ITCB immunoconjugates for both labels (^{111}In or ^{90}Y). The radioactivity levels in tumors are slightly lower for EGS than for ITCB conjugate. This is of marginal significance on Day 4 ($0.02 < p < 0.05$ for yttrium; $0.05 < p < 0.1$ for indium).

Whole-Body Retention of Radioactivity

The whole-body retention of radioactivity in nude mice was measured over a period of six days, and the data are plotted in Figure 4. The labile EGS linker-immunoconjugate clears three times faster from the whole body than the stable immunoconjugates (ITCB, DSS) with a biological half-life of 40 and 120 hr, respectively. The other labile linker (DST) was excreted twice as fast as stable control conjugates (Fig. 4). Whole-body retention and blood clearance half-life showed a strong positive correlation for all radioimmunoconjugates. The radioactive metabolic products excreted in urine are found to be associated with chelated indium complexes.

Whole-Body Immunoscintigraphy

The distribution of ^{111}In-labeled linker-immunoconjugates shows preferential localization of the radiolabel in tumor and excellent visualization within 48 hr of injection. Tumor-to-nontarget ratios and image quality improves over time as demonstrated in Figure 5A–C. A significant amplification of the target-to-nontarget ratios is discernible for the EGS linker conjugate. The differences seen between the EGS and ITCB immunoscintigraphs reflect the tumor-to-organ ratios obtained in the biodistribution stud-

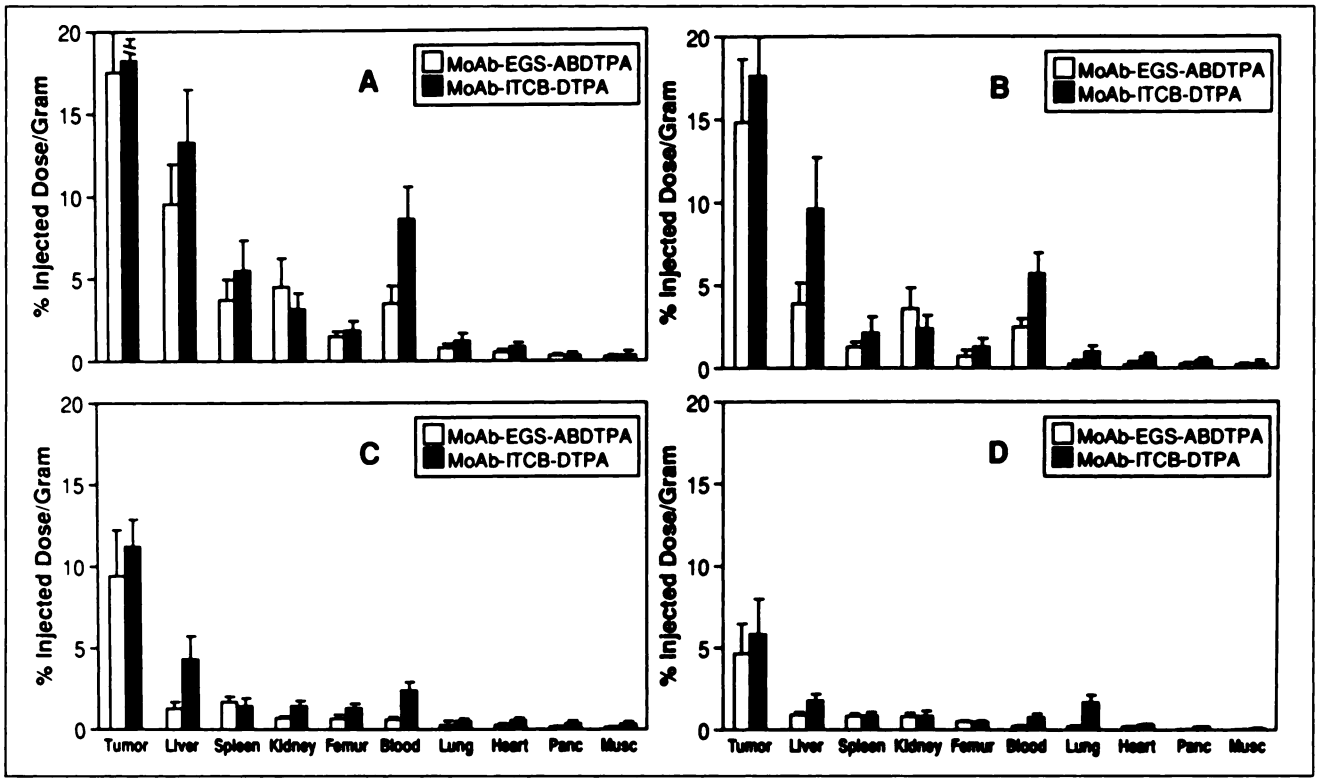

FIGURE 3. Biodistribution of ^{90}Y-labeled QCI-linker-chelate conjugates in nude mice (n = 6) xenografted with human hepatoma (HepG2) at different time intervals. (A) Day 1; (B) Day 2; (C) Day 4; (D) Day 6.

ies. Liver activity is apparent in early scans, but for EGS only declines rapidly thereafter, indicating clearance of the isotope, most likely due to hydrolysis of the diester bond in liver and blood (Fig. 5A–C).

Indium-111-Labeled Immunoconjugates in Dog Model

The introduction of the EGS linker in the immunoconjugate significantly reduced (−50%) the dog liver activity when compared to the thiourea-linked immunoconjugate. Rabbit polyclonal antidog ferritin showed a four-fold reduction in liver uptake when compared to relatively stable noncleavable linked QCI. The results are summarized in Table 2. Previously, we have shown in dogs that the liver volume and activity predictions based on SPECT scans were in accordance (±5%) with autopsy data (*20*). In the case of the EGS linker, 15% of injected ^{111}In activity was excreted in the dog's urine within 20 hr compared to 3% excretion of thiourea linkage. The half-life in the blood was biphasic for all immunoconjugates. The rapid and slow component had a half-life of ~10 and ~33 hr, respectively. The ratio between the rapid and slow component was ~1.55 for EGS immunoconjugates and ~1.25 for ITCB immunoconjugates. The two major radioactive compounds found in serum, analyzed by HPLC, are representative of intact immunoconjugate and a low molecular weight species similar to the ^{111}In-DTPA complex. The excreted activity in the urine is composed of one major component similar to if not identical to the ^{111}In-DTPA complex.

DISCUSSION

The objective of this study was to develop and evaluate linker-chelates for improved radioimmunoimaging and radioimmunotherapy. An optimal therapeutic radio-labeled immunoconjugate would achieve maximum tumor deposition and retention while rapidly clearing the isotope from normal tissues. Hypothetically, labile linker-immunoconjugates would attach radionuclides to the antibody in a

TABLE 3
Tumor to Normal Organ Ratios of Mab-Linker DTPA Conjugates in Nude Mice Xenografted with Human Hepatoma HepG2

	Blood				Liver				Kidney				Femur			
	^{111}In		^{90}Y		^{111}In		^{90}Y		^{111}In		^{90}Y		^{111}In		^{90}Y	
Linkages	2d	4d	2d	4d	2d	4d	2d	4d	2d	4d	2d	4d	2d	4d	2d	4d
Mab-EGS-ABDTPA	5.1	10.6	5.9	15.4	3.6	8.1	3.9	7.2	4.7	11.1	4.1	13.8	22.3	23.9	19.5	10.8
Mab-ITCB-DTPA	2.9	3.2	3.1	4.7	1.4	2.3	1.8	2.6	6.0	5.7	7.3	8.6	20.4	20.9	12.9	5.75
Mab-DST-ABDTPA	4.2	7.5	—	—	5.5	5.6	—	—	4.4	5.1	—	—	33.8	35.0	—	—
Mab-DSS-ABDTPA	2.1	2.5	—	—	1.4	2.0	—	—	5.0	6.7	—	—	18.6	19.7	—	—

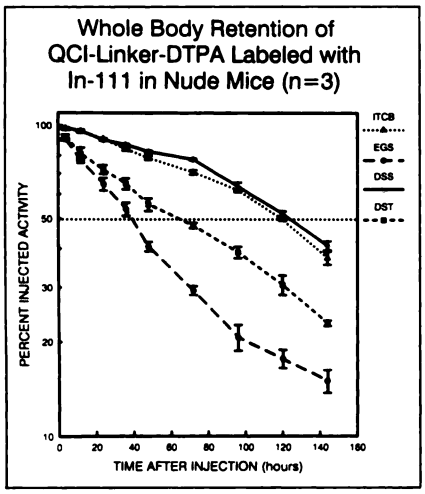

FIGURE 4. The whole-body retention of linker-immunoconjugates labeled with ^{111}In in a nude mouse model (n = 3).

manner that delivers and retains radiation in the tumor without dissociating the antibody-chelate-isotope complex, minimizing normal tissue uptake and retention. This report validates the labile linker-immunoconjugate concept in two experimental animal models.

Antibody immunoreactivity greatly influences the localizing efficacy for any immunoconjugate. Coupling conditions were, therefore, optimized so that the immunoglobulin was modified as little as possible. The affinity chromatography data (Table 1) for the immunoconjugates used in this study demonstrate only minimal changes in affinity compared to unconjugated antibody. The aminobenzyl-DTPA derivative was selected as a chelator over the more conventional DTPA-p-aminoethyl-anilide derivative reported previously (*16*) because the former better stabilizes the ^{111}In or ^{90}Y complex in vivo due to the availability of an additional carboxylate ligand for complexation. Moreover, the steric blockage of the benzyl group at the carbon backbone can also contribute to the improved stability of the metal chelate complex. The results obtained in the nude mouse system show that labile linker-chelates clear the blood circulation more rapidly than the stable ITCB-DTPA or DSS immunoconjugates. Additionally, both radioactivity in other normal tissues is lower and the effective half-life is shorter for the labile linkers than for either the noncleavable DSS linker or ITCB-DTPA. Slightly lower activity in the tumor with the EGS immunoconjugate than with the ITCB immunoconjugate can be explained by the lower activity in the blood pool due to the rapid clearance of labile EGS linkage in immunoconjugates. This does not decrease the potential advantage of the EGS linker, since it has a higher targeting ratio and a tumor half-life similar to stable linker immunoconjugates.

Whole-body immunoscintigraphy confirmed all three significant findings of the biodistribution studies: (1) good tumor targeting by both EGS and ITCB immunoconjugates; (2) clearance of EGS from the liver and (3) rapid excretion of the EGS immunoconjugate from normal tissues into the urine. The biodistribution data and imaging studies indicate a prominent difference in the EGS cleavage between tumor site and normal organs in nude mice with hepatoma xenografts. The 40-hr whole-body biological half-life of the diester (EGS) was consistent with previously reported results (*16*) for antimelanoma antibody in the nude mouse.

With the exception of the femur, comparable tumor-to-normal ratios were obtained with ^{111}In- and ^{90}Y-labeled EGS-immunoconjugates (Table 3). Lower tumor-to-femur ratios of ^{90}Y-labeled EGS and ITCB are explained by lower tumor uptake and relatively higher femur activity compared to ^{111}In-labeled EGS and ITCB conjugates. For the great majority of results in Table 3, the difference between the ratios for indium and yttrium is less than two-fold. The current precision of the best dosimetric es-

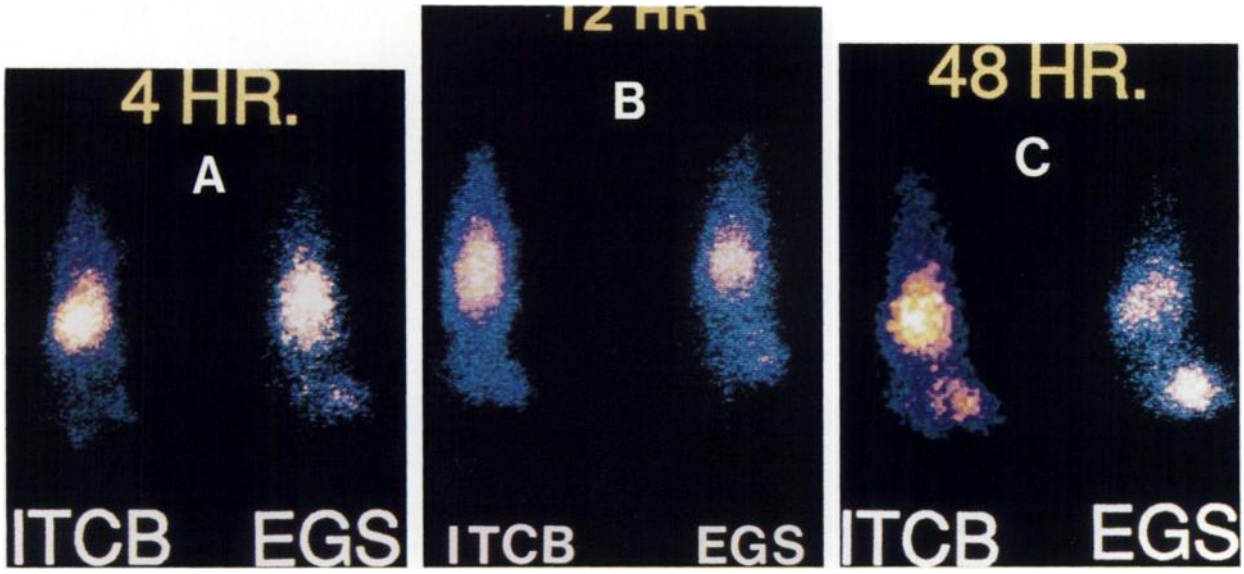

FIGURE 5. Immunoscintigraph of EGS and ITCB after injection of radiolabeled immunoconjugates into nude mice bearing human xenografted (HepG2 hepatoblastoma) in the right rear flank of the leg. Radioisotope concentration shown in upper light area is the abdominal region. Tumor is located in the lower right section of the light area. (A) Four hour images; (B) 12-hr images; (C) 48-hr images.

timates is considered 50% (± a factor of 2) (*21*). This indicates that the results for indium and yttrium are sufficiently similar to allow for state of the art dosimetric efforts. In the future with improved dosimetric accuracy, greater similarity between indium and yttrium results will become necessary.

The excessive accumulation of radioactivity in nontarget organs, particularly the liver, is one of the major clinical problems associated with monoclonal immunoconjugates labeled with ^{111}In or ^{90}Y radionuclides. Monoclonal antibodies radiolabeled with ^{111}In or ^{90}Y have targeted human tumors in nude mice well and have not shown significant liver uptake in mice or rats, contrary to the experience in clinical studies, which have demonstrated high liver uptake in patients. Binding of the murine F_c fragment to human liver cells and slow metabolism of radioimmunoconjugates in the liver would be responsible for the release of indium to transferrin and could cause prolonged entrapment of the radiometal in the human liver. Our preliminary results for beagle dogs indicate that the "human" high liver uptake is reproduced in this model. Liver uptake is dependent on the species in which the antibody is produced as well as on linker-chelate chemistry (Table 2). The labile linker approach could allow for the selective removal of the radioactive chelate from the liver while the cold antibody remains attached to the F_c receptors.

The combination of the two models (mouse and dog) used in this study improve the preclinical evaluation of radiolabeled monoclonal antibodies for the treatment and diagnosis of cancer. Tumor targeting can be evaluated in the murine model, whereas normal tissue toxicity (liver, bone marrow) is better analyzed in the canine model (*22*). Our study in two complementary animal models has shown that labile linker-chelates may provide an important improvement in tumor-to-normal tissue ratios. Application of these radioimmunoconjugates in human patients may, in turn, lead to improvements in clinical radioimmunodiagnosis and radioimmunotherapy.

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