Pharmacokinetics of Chimeric L6 Conjugated to Indium-111- and Yttrium-90-DOTA-Peptide in Tumor-Bearing Mice

Sally J. DeNardo, Gao-Ren Zhong, Qansy Salako, Min Li, Gerald L. DeNardo and Claude F. Meares

Departments of Internal Medicine and Chemistry, University of California, Davis, California

A bifunctional chelating agent, DOTA-Gly_3-L-(p-isothiocyanato)-phenylalanine amide (DOTA-peptide-NCS), was studied in nude mice bearing human breast cancer xenographs (HBT 3477) to determine its potential for radioimmunoconjugate therapy. **Methods:** Indium-111 and yttrium-90 were attached to an anti-adenocarcinoma chimeric L6 (ChL6) monoclonal antibody (MAb) after pre-chelation to the DOTA-peptide-NCS and the desired neutral radiochelates were obtained by purification. The unique characteristic of the DOTA-peptide-NCS to form neutral complexes with trivalent metals was utilized to separate the resulting ^{111}In and ^{90}Y radiochelates from excess chelating agent and other anionic by-products resulting from metal impurities. The purified radiochelates were then conjugated to ChL6. The pharmacokinetics of ^{111}In- and ^{90}Y-DOTA-peptide-ChL6 were obtained for 5 days after injection in nude mice bearing HBT 3477 xenographs. The results were compared with the pharmacokinetics of ^{125}I-ChL6 obtained in the same mouse model. **Results:** The whole-body clearance of ^{125}I-ChL6, ^{90}Y- and ^{111}In-DOTA-peptide-ChL6 was monoexponential with biologic half-times of 92, 104 and 160 hr, respectively. Blood clearances of the three radiopharmaceuticals were biphasic. The radiometal immunoconjugates had greater tumor uptake and slower clearances. **Conclusion:** Indium-111- and ^{90}Y-DOTA-peptide-ChL6 can be produced at high specific activity with fewer than one chelate per MAb by using a pre-labeling method that permits radiochelate purification by charge selection. Studies in mouse xenografts indicate that tumor uptake is enhanced and a favorable therapeutic index is achieved using these agents.

Key Words: bifunctional chelating agents; monoclonal antibody; indium-111; yttrium-90; pharmacokinetics; breast carcinoma; radioimmunoconjugate therapy

J Nucl Med 1995; 36:829–836

Yttrium-90 is an appealing radionuclide for treating solid cancers because of its energetic beta emission (*1–3*). Indium-111 has usually been used to trace the pharmacokinetics and estimate the radiation dosimetry of equivalent ^{90}Y-immunoconjugates. Since the chemistry of indium is similar to that of yttrium, it has been assumed that chelating agents designed for indium would also control ^{90}Y. It is now established that ^{90}Y escapes from many chelates to the skeleton resulting in increased hematopoietic toxicity (*4–7*). We have reported enhanced stability in vitro and in vivo for ^{90}Y immunoconjugates when ^{90}Y was chelated to the macrocycle 1,4,7,10-tetraazocyclododecane-N,N′,N″,N‴-tetraacetic acid (DOTA). In these studies, a bifunctional derivative of DOTA, 2-[p-(bromoacetamido)benzyl]-DOTA (BAD) was conjugated to monoclonal antibodies (MAbs) via 2-iminothiolane (2IT) (*8,9*). Whole-body autoradiography of mice injected with ^{90}Y-2IT-BAD immunoconjugates has revealed no significant uptake of ^{90}Y in the skeleton and a substantial increase in the LD_{50} when compared to that of comparable ^{90}Y-MXDTPA immunoconjugates (*10*). Yttrium-90 clearance from the liver and body of these mice, however, was appreciably slower than that of equivalent ^{125}I-labeled MAbs.

Since hepatic accumulation of radioactivity has proven to be a problem for radiometal immunoconjugates, several approaches to produce a cleavable linkage between the chelate and MAb have been studied. Radiometal immunoconjugates have been designed to be metabolized in intracellular locations, such as the hepatocyte, by enzyme-rich lysosomes in the hope that this would result in release of the radiometal chelate to the blood for rapid excretion by the kidney (*11–13*). The peptide linkage chosen by us was based on evidence that intrahepatocyte cathepsins (endopeptidases) are responsible for most of the protein metabolism in hepatic lysosomes, so that a peptide linkage susceptible to endopeptidase activity would allow hepatocyte cleavage and excretion of the radiochelate (*14*).

In the preparation of ^{90}Y-DOTA immunoconjugates, other metal ions present in $^{90}YCl_3$ supplies compete with yttrium because formation of yttrium-DOTA is relatively slow. Suboptimal radiolabeling yields can frequently occur depending on the amounts of these competing metals present in the $^{90}YCl_3$ supply. Immunoconjugate preparations containing more chelates per antibody can be used to

Received Sept. 16, 1994; revision accepted Dec. 22, 1994.

For correspondence or reprints contact: Sally J. DeNardo, MD, Section of Radiodiagnosis and Therapy, Molecular Cancer Institute, 1508 Alhambra Blvd., Suite 214, Sacramento, CA 95816.

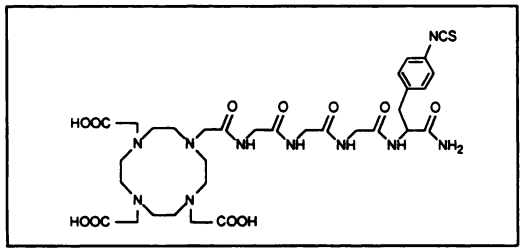

FIGURE 1. Structure of the DOTA-peptide-NCS chelate (DOTA-Gly_3-L-(p-isothiocyanato)-phenylalanine amide).

overcome this problem, but preparations with excess macrocycle over that needed to carry the yttrium may increase the immunogenicity of the pharmaceutical (*15*). In order to increase the specific activity of ^{90}Y immunoconjugates while at the same time limiting DOTA chelate to no more than one per MAb molecule, a novel DOTA peptide bifunctional chelating agent, DOTA-Gly_3-L-(p-isothiocyanato)-phenylalanine amide (DOTA-peptide-NCS), has been developed (Fig. 1) (*16,17*). This bifunctional chelating agent has been designed concurrently with an alternative method of radiolabeling (prelabeling) in order to take advantage of the neutral-charged chelate created when a trivalent (i.e., ^{90}Y, ^{111}In) metal is present on the DOTA peptide. Because the desired ^{111}In and ^{90}Y chelates can be separated from the competing divalent metal chelates, the major contaminants of the ^{90}Y supply, and from excess DOTA, ^{90}Y or ^{111}In-immunoconjugates, can be prepared at high specific activity with minimal DOTA chelates (Fig. 2). This paper describes the prelabeling, purification and conjugation of ^{111}In- and ^{90}Y-DOTA-peptide-ChL6 radiopharmaceuticals and their behavior in mice bearing tumor xenografts. The characteristics of these radiopharmaceuticals are compared to those of the radioiodinated ChL6 analog previously studied (*18*).

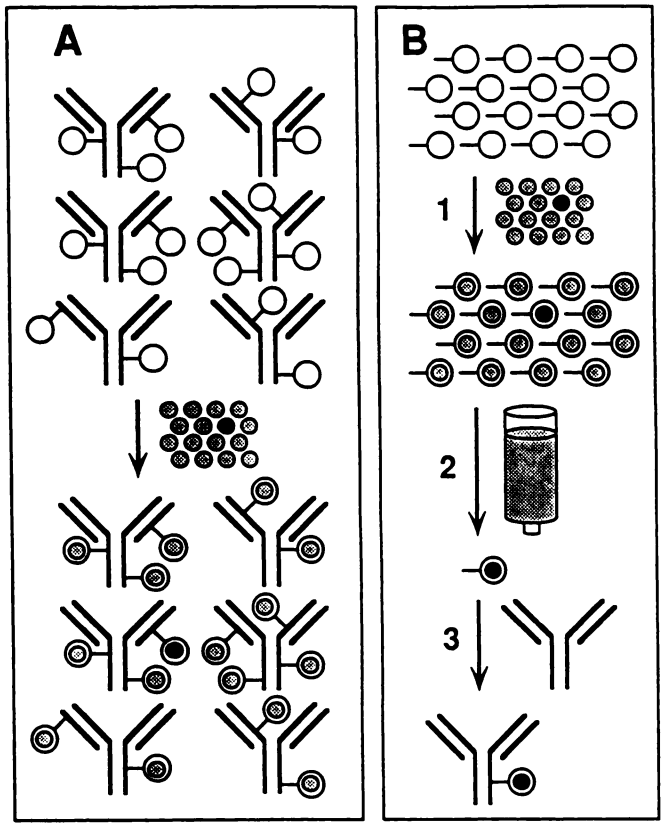

FIGURE 2. Schematic of (A) radiolabeling of DOTA-peptide-NCS after conjugation to MAb and (B) radiolabeling of DOTA-peptide-NCS before conjugation to MAb. Because ^{90}Y and ^{111}In (●), and contaminant metal ions (○), are all complexed by DOTA (—○), excess immunoconjugate is required to efficiently capture ^{90}Y or ^{111}In radiolabeling by either method. In method A, this results in varied and complex molecular species, low specific activity and large amounts of DOTA in the final product. In method B, excess bifunctional chelating agent is added to the radioisotope (1), then removed by charge on anion exchange chromatography (2) to provide relatively pure, uncharged ^{90}Y-DOTA-NCS or ^{111}In-DOTA-NCS chelates (—⊙) without excess chelate or chelated divalent metal contaminants (—⊙). The purified ^{90}Y or ^{111}In chelate is conjugated to antibody (3) to prepare radioimmunoconjugates with minimum chelates and high specific activity.

MATERIALS AND METHODS

Chelating Agents

The bifunctional chelating agent, DOTA-peptide-NCS, was prepared according to the method previously described (*16*). This lot was purified by C18 reverse-phase, high-performance liquid chromatography (HPLC). The concentration was measured by UV spectroscopy and purified DOTA-peptide-NCS was stored in 0.1 *M* tetramethylammonium acetate, pH 5.0, in 25- or 50-μl aliquots at −70°C. Aliquots were thawed immediately before use and re-examined by C18 reverse-phase HPLC to assure storage stability. The MAb used was chimeric L6 (ChL6), an anti-adenocarcinoma MAb in which the constant domains C-G2a and C-kappa of the mouse MAb have been replaced with human IgG1 equivalent domains (*19,20*). ChL6 was supplied at 5 mg/ml in normal saline by Bristol-Myers Squibb Pharmaceutical Research Institute (Seattle, WA). Prior to use, the ChL6 was transferred into 0.1 *M* tetramethylammonium phosphate, pH 8.0, concentrated to 56 mg/ml using a centricon concentrator (Amicon, Inc., Beverly, MA), and stored at −70°C.

All radioisotopes were obtained commercially and designated as carrier-free. Indium-111-Cl_3 in 0.04 *M* HCl was obtained from Medi-Physics-Amersham (Arlington Heights, IL) and $^{90}YCl_3$ in 0.04 *M* HCl from Mallinckrodt (St. Louis, MO), NEN-DuPont (Billerica, MA) and Westinghouse Hanford Company (Richland, WA). DEAE cellulose anion exchange resin was purchased in the chloride form (Sigma Chemical Co., St. Louis, MO) but was converted to acetate before it was used to purify radiochelate mixtures. The acetate form of the resin was packed in a 1-ml syringe barrel at a ratio of 25 μl resin per 10 μl crude radiochelate solution at the time of separation.

MAb Radioiodination

ChL6 was labeled with ^{125}I using a chloramine-T method. The molar ratio of ^{125}I-to-MAb and the mass ratio of chloramine-T-to-MAb were 1:10. Free ^{125}I was separated from MAb bound ^{125}I by using a Sephadex G-50-80 column (Sigma Chemical Co., St. Louis, MO).

Analytical Methods

HPLC was performed using a Beckman model 332 system (Beckman Instruments Inc., Palo Alto, CA) consisting of a ultra-

spherogel SEC 3000 SW (30 cm × 7.5 mm id.) size exclusion column or Dynamax C18 column (250 cm × 4.6 mm id., 300 Å), a model 170 radioisotope detector and model 166 UV detector. Radiolabeled MAb was eluted on the SEC 3000 column with 0.1 *M* sodium phosphate pH 7 (containing 0.025% NaN_3 (w/v)), at a flow rate of 1 ml/min, and detected by UV at 280 nm. Radiometal chelates were eluted on the C18 column using solvents A (0.1% trifluoroacetic acid (w/v) in water) and B (methanol) over a 30-min linear gradient from 15%A, 85%B to 100%B, at a flow rate of 0.7 ml/min and detected at 254 nm.

Cellulose acetate electrophoresis (CAE) was performed using a Gelman Sciences electrophoresis unit (Ann Arbor, MI). The CAE strips were run in 0.05 *M* sodium barbital buffer, pH 8.6 (Sigma Chemical Co., St. Louis, MO), at 5mA/strip for 11 and 45 min for each sample. The radioactive species were determined using an AMBIS radioisotope gel scanner (Ambis Systems, San Diego, CA). Under these conditions, the free radiometal ions travel ahead of the radiolabeled MAb toward the anode and can be quantitated after an 11-min electrophoresis, while after 45 min, monomeric radiolabeled MAb can be distinguished from slower moving aggregate species.

Thin layer chromatography (TLC) was performed on plastic-backed silica-coated plates (EM Science, Cherry Hill, NJ) and developed in a 1:1 (v/v) mixture of 10% (w/v) aqueous ammonium acetate and methanol as mobile phase. The solvent front was allowed to run for 5 cm per strip and the plates were scanned for radioactivity on the AMBIS scanner. In this solvent system, unchelated radiometal has an R_f of 0, radiolabeled DOTA-peptide-ChL6, an R_f of 0.2, and radiolabeled DTPA an R_f of 0.6 (*13,14*).

Live cell assay was used to evaluate immunoreactivity of the radiolabeled antibodies (*21*). Radiolabeled antibody (5 ng) was added to 3 × 10^6 HBT 3477 breast cancer cells or to Raji cells (negative control cells) in triplicate and incubated at room temperature for 30 min. Each tube was counted for total radioactivity in a gamma well counter, then centrifuged at 800 g for 5 min and washed three times with 0.15 *M* phosphate-buffered saline (PBS), pH 7. The radioactivity of the resulting pellets in each tube was counted and divided by the initial radioactivity to obtain the percent of binding to the pellet. Radiometal immunoconjugate preparations were run in parallel with and their immunoreactivity compared to that of ^{125}I ChL6 reference standard preparations that behaved like unlabeled ChL6 by Scatchard analysis.

Titration of Radiometals Against DOTA-Peptide-NCS

Because ^{111}In and ^{90}Y supplies contained varying amounts of other metal contaminants, a titration with varying amounts of DOTA-peptide-NCS was conducted on each shipment to determine the molar ratio of DOTA-peptide-NCS-to-^{90}Y, or ^{111}In needed to chelate 80%–90% of the radiometal. The radiometal solution was dried at 60°–80°C under a gentle stream of nitrogen gas, and reconstituted in 100 μl 0.1 *M* tetramethylammonium acetate, pH 5. A fixed volume of 10 μl of this reconstituted radioactive solution was added to each 10 μl of 0.2 μ*M*, 2 μ*M*, 20 μ*M*, 200 μ*M*, 2 m*M*, 20 m*M* and 100 μl of 20 m*M*, DOTA-peptide-NCS solution in 0.1 *M* tetramethylammonium acetate, pH 5. The solution from each mixture was incubated at 37°C for 30 min, then analyzed by TLC. The result of TLC for each titration was plotted as percent ^{90}Y (or ^{111}In) chelated versus the molar ratio of DOTA-peptide-NCS-to-radiometal (Fig. 3). Preliminary experiments showed that the pure, trace radiolabeled yttrium ion was quantitatively complexed under all titration conditions described.

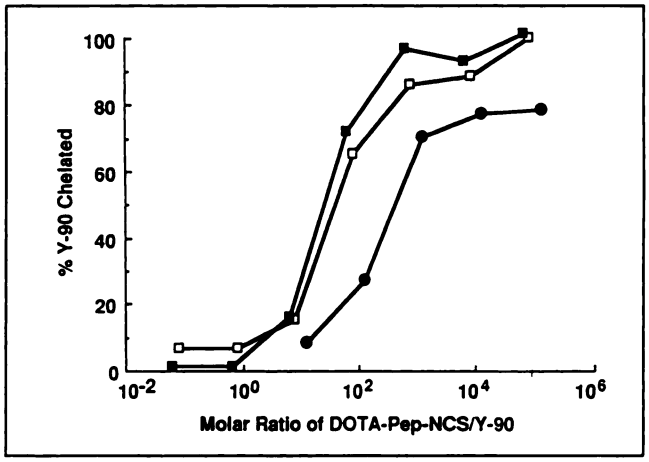

FIGURE 3. Titration curves of $^{90}YCl_3$ supplies illustrating the moles of DOTA-peptide-NCS needed to chelate the ^{90}Y. Molar ratios of DOTA-peptide-NCS needed to bind 80% of the ^{90}Y in an aliquot of $^{90}YCl_3$ from each shipment varied from 6 × 10^2–6 × 10^4. Titration curves for $^{111}InCl_3$ shipments were similar.

Pre-chelation of DOTA-Peptide-NCS with ^{90}Y or ^{111}In

The molar ratio of DOTA-peptide-to-mCi of ^{90}Y or ^{111}In that had yielded ≥ 80% chelation in the titration procedure (Fig. 3), was used to radiolabel DOTA-peptide-NCS for animal studies. The DOTA-peptide-NCS radiometal mixture was incubated at 37°C for 30 min, and 50 m*M* DTPA in 0.1 *M* ammonium acetate, pH 6, was added to the mixture to a final DTPA concentration of 10 m*M* to scavenge any remaining unchelated metal ions. The solution was further incubated for 15 min at room temperature, then assayed by TLC and C18 HPLC. The ^{111}In- or ^{90}Y-DOTA-peptide-NCS was purified from excess chelate and other anionic by-products using a DEAE cellulose anion-exchange resin column (Fig. 2). The column was spun for 2 min at approximately 2,000 g and washed with 4 × 20 μl water, following similar centrifugation conditions. The radioactive fractions were pooled and measured on a dose calibrator for total radioactivity content, then concentrated to the desired volume on a vacuum concentrator (Savant Instruments, Farmingdale, NY). An aliquot was taken and assayed by TLC and C18 HPLC (Fig. 4).

Conjugation of ChL6 with ^{90}Y or ^{111}In-DOTA-Peptide-NCS

The antibody was added to the purified DOTA-peptide-NCS radiometal chelate, and the pH of the mixture adjusted to 9.5 with 0.1 *M* aqueous triethylamine solution before incubating at 37°C for 60 min. The mixture was purified in PBS by Penefsky's gel-filtration chromatography method (*22*) using Sephadex G50/80 (Sigma Chemical Co., St. Louis, MO). The product was then evaluated by TLC, CAE, SEC and C18 HPLC and HBT 3477 live cell assay for immunoreactivity. In order to confirm the absence of unchelated radiometal ions in the final product, a 2-μl aliquot was further challenged in 10 m*M* DTPA for 15 min, and a pre- and postchallenge aliquot were examined by CAE for evidence of any increase in non-MAb bound radiometal in the postchallenge aliquot.

Preparation of ^{90}Y-DOTA-Peptide-ChL6 at Therapeutic Dose Levels

Three lots of ^{111}In-DOTA-peptide-ChL6 and two lots of ^{90}Y-DOTA-peptide-ChL6 were prepared for animal studies using 2.1–

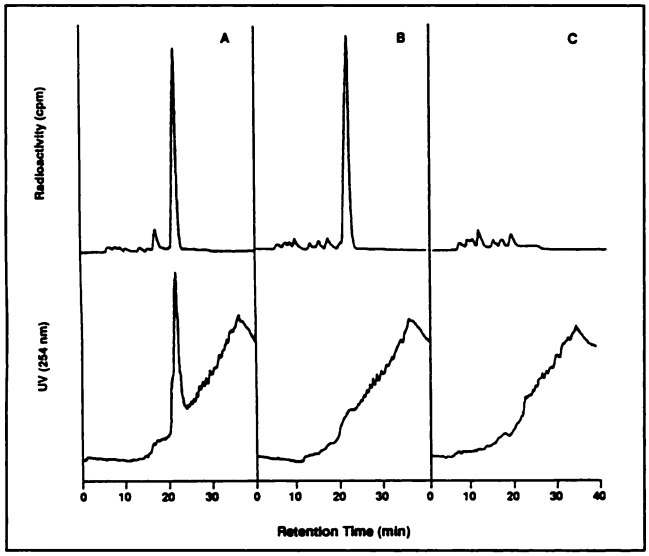

FIGURE 4. Elution from HPLC C18 chromatography of (A) ^{90}Y-DOTA-peptide-NCS chelate reaction mixture containing 10 m*M* DOTA-peptide-NCS and 2–4 mCi ^{90}Y in YCl_3 before DEAE anion exchange purification; (B), after anion exchange purification; and, (C) after conjugation to ChL6 and before purification. Disappearance of the major UV absorption peak and retention of the major radioactive peak between A and B demonstrates that divalent metal chelates and excess DOTA-peptide chelates were removed by the DEAE resin. Disappearance of the DOTA-peptide-NCS radioactive peak in C (with only residual minor, unreactive ^{90}Y species in the eluent) indicates retention of ^{90}Y-DOTA-peptide-ChL6 by the C18 column and completeness of ^{90}Y-DOTA-peptide-NCS conjugation to ChL6.

6.2 mCi of radiometal. To assure the feasibility of scale-up of this method to quantities of the radiopharmaceutical needed for human doses and to verify that the procedure could give higher product yield and specific activity when performed on a larger (mCi) scale, 80 mCi of ^{90}Y was used to prepare a third lot of ^{90}Y-DOTA-peptide-ChL6 (Table 1).

Animal Studies

Pharmacokinetic studies were performed in female, athymic nude mice (nu/nu Balb/c) obtained from Harlan Sprague Dawley (Indianapolis, IN). The mice were maintained on normal diet ad libitum and pathogen-free conditions and were used at approximately 4 wk of age.

HBT 3477 cells were harvested in the log phase from plastic culture flasks using RPMI 1640 medium, and 2.5–5.0 × 10^6 cells were injected subcutaneously into both ventral flanks of each mouse. When the tumors were 50–300 mg (about 3 wk), 100 μl of radiolabeled ChL6 were injected intravenously into the tail vein of each mouse. Radioimmunoconjugate injections contained 15–30 μCi of ^{90}Y or 30–40 μCi of ^{111}In and unconjugated ChL6 was added to a total of 100 μg of MAb protein. The radioactive dose injected into each mouse was measured with a Capintec CRC-12 radionuclide dose calibrator (Capintec, Inc., Pittsburg, PA) and confirmed by counting the mouse immediately after injection using two opposed sodium iodide detectors (Picker Nuclear, White Plains, NY) calibrated against appropriate standards for radionuclide, volume and geometry.

In order to determine the whole-body clearance of ^{111}In- or ^{90}Y-DOTA-peptide-ChL6, each mouse was counted at times zero, 1 hr and 1,2,3 and 5 days postinjection using the dual-probe system. The counts were decay-corrected and expressed as percent injected dose (%ID). The blood clearance was evaluated by collecting 10-μl blood samples from the tail vein at 1 hr and 1, 3 and 5 days postinjection and counting them in a sodium iodide gamma well counter (Pharmacia LKB Nuclear, Inc., Gaithersburg, MD). Decay-corrected radioactivity in the blood was expressed as %ID/ml of blood. Biodistributions were obtained by killing the mice 1, 3 and 5 days postinjection, removing and weighing relevant organs and counting them in the same gamma well counter. The uptake in each organ was calculated and expressed as %ID/g. By using standards of comparable volume and geometry for blood and organs, all ^{90}Y sample counts were corrected for attenuation in addition to decay (^{90}Y window = 320–1500 keV; ^{111}In window = 150–320 keV). The mean (%ID/g) blood and whole-body clearances for the three radiopharmaceuticals at each time point were compared utilizing the Student t-test for two independent groups.

Whole-Body Autoradiography

Whole-body autoradiography was performed as previously reported (*10,23*). Briefly, each mouse was injected with approximately 70 μCi of either ^{90}Y or ^{111}In-radioimmunoconjugate. Two mice were killed at 3 and 5 days postinjection by anesthetizing with pentobarbital and flash freezing in a hexane-dry ice bath. The mice were embedded in carboxymethyl-cellulose, and sagittal sections were taken using a PMV 2250 cryomicrotome at −17°C. Sections of 50 μm thickness were taken every 250 μm. The sections then were desiccated, and autoradiograms were prepared by exposing the sections to microvision mammography film (E.I. DuPont De Nemours & Company, Inc., Wilmington, DE) for various time intervals. The film was developed using standard procedures.

TABLE 1
DOTA Peptide Radiochelate and Radioimmunoconjugate Yields and Immunoreactivity for ^{111}In and ^{90}Y DOTA-Peptide-ChL6

Radionuclide	Preparation	Initial radioactivity (mCi)	Purified radiochelate (mCi)	Radioimmunoconjugate product (mCi)	Specific activity (mCi/mg)	Overall yield (%)	HBT binding (%)
^{111}In	1	4.9	3.7	2.3	2.3	47	80
	2	6.1	3.6	2.4	2.4	40	82
	3	6.2	4.6	2.4	2.4	40	89
^{90}Y	4	2.1	1.6	0.5	0.5	24	73
	5	3.9	3.4	1.3	1.3	33	78
	6	80	63.6	38.4	3.8	48	74

TABLE 2
Biodistribution of ^{90}Y and ^{111}In-DOTA-Peptide-ChL6 in Mice (%ID/g)*

	^{90}Y-DOTA-peptide-ChL6			^{111}In-DOTA-peptide-ChL6			^{125}I-ChL6†		
	Day 1	Day 3	Day 5	Day 1	Day 3	Day 5	Day 1	Day 3	Day 5
Tumor	17.5 ± 8.0	18.0 ± 8.0	13.8 ± 5.2	13.3 ± 1.8	12.0 ± 1.9	10.4 ± 3.0	10.7 ± 2.4	8.9 ± 5.4	7.4 ± 1.8
Liver	6.3 ± 1.4	5.5 ± 1.4	5.8 ± 0.9	6.2 ± 1.0	7.2 ± 1.3	7.0 ± 1.1	3.7 ± 0.7	2.4 ± 0.7	1.9 ± 0.2
Lung	8.4 ± 2.2	5.9 ± 1.0	4.9 ± 1.0	7.3 ± 1.1	4.8 ± 0.8	5.0 ± 1.0	7.4 ± 0.7	3.6 ± 0.8	4.9 ± 0.7
Muscle	1.2 ± 0.2	0.9 ± 0.2	0.7 ± 0.2	1.2 ± 0.2	0.9 ± 0.1	0.7 ± 0.1	1.4 ± 0.3	0.8 ± 0.2	0.8 ± 0.1
Intestine	2.5 ± 0.5	1.9 ± 0.6	1.6 ± 0.7	3.3 ± 0.6	2.7 ± 0.6	2.2 ± 0.7			
Kidneys	5.6 ± 0.3	4.6 ± 0.7	3.6 ± 0.9	8.9 ± 0.6	7.9 ± 1.0	6.4 ± 1.8	4.3 ± 0.6	2.0 ± 0.6	2.4 ± 0.5
Brain	1.2 ± 0.3	1.0 ± 0.2	0.7 ± 0.2	0.9 ± 0.3	0.8 ± 0.3	0.6 ± 0.3			
Spleen	6.1 ± 1.8	5.9 ± 1.9	5.7 ± 1.9	4.7 ± 1.0	5.2 ± 1.8	5.1 ± 1.3	3.0 ± 1.4	2.1 ± 0.5	2.2 ± 0.5
Heart	4.5 ± 0.9	3.2 ± 0.6	2.4 ± 0.4	3.7 ± 0.5	2.9 ± 0.4	2.6 ± 0.6			
Stomach	2.3 ± 0.3	1.9 ± 0.3	1.5 ± 0.2	2.4 ± 0.4	2.0 ± 0.3	1.7 ± 0.3			
Bone‡	2.0 ± 0.3	1.9 ± 0.6	1.8 ± 0.3	2.1 ± 0.6	2.0 ± 0.4	1.8 ± 0.4	1.7 ± 0.2	0.7 ± 0.6	0.7 ± 0.2
St. Cont.	0.4 ± 0.7	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.2	0.2 ± 0.2	0.3 ± 0.3			
Int. Cont.	0.3 ± 0.2	0.2 ± 0.1	0.2 ± 0.1	0.5 ± 0.3	0.6 ± 0.5	0.3 ± 0.1			
n =	12	12	12	15	15	14	3	4	5

*Mean ± s.d.
†Reference GP Adams et al., 1992 (*18*).
‡Represents bone with marrow removed.

RESULTS

Radiochemistry

Three ^{111}In-DOTA-peptide-ChL6 and three ^{90}Y-DOTA-peptide-ChL6 preparations were produced for these studies with specific activities of 2.3–2.4 and 0.5–3.8 mCi/mg of ChL6, for ^{111}In and ^{90}Y, respectively (Table 1). Each preparation contained less than one DOTA chelate per antibody molecule. Titration of aliquots from each $^{111}InCl_3$ and $^{90}YCl_3$ shipment indicated that greater than 10^3 molar excess of DOTA-peptide was needed to chelate ≥ 80% of the radiometal in most shipments of $^{111}InCl_3$ or $^{90}YCl_3$ (Fig. 3). C18 HPLC assays of aliquots from the prelabeling and conjugation steps demonstrated that excess DOTA-peptide (Fig. 4A) was no longer present after anion purification (Fig. 4B). Conjugation of almost all ^{111}In- or ^{90}Y-DOTA-peptide-NCS to ChL6 radiochelates occurred in most reactions leaving no residual radioactivity in the election volume of DOTA-peptide-NCS after the conjugation reaction (Fig. 4C).

Radiolabeled DOTA-peptide-ChL6 eluted from the SEC HPLC as a monomeric peak corresponding to 150 kD in molecular mass (retention time of 8.5 min). For the six radiolabelings, this monomeric peak represented 97% ± 3% of the final radioactivity. Less than 2% (0%–2%) total radioactivity was associated with ChL6 preconjugation aggregates and the remaining radioactivity (0%–4%) was ^{111}In or ^{90}Y-DTPA. No other radiochemical species were observed. These results were confirmed by TLC and CAE, which also did not detect any difference in radioimmunoconjugate aliquots obtained before and after DTPA challenge.

Live cell assays showed that ^{111}In and ^{90}Y-labeled DOTA-peptide-ChL6 had greater than 70% binding to HBT 3477 cells. This binding represents 90%–100% immunoreactivity relative to unmodified ChL6.

Animal Studies

The results of mouse biodistribution studies for ^{111}In and ^{90}Y-DOTA-peptide-ChL6 and previously reported ^{125}I-ChL6 data (*18*) are summarized in Table 2. The whole-body and blood clearances are shown in Figures 5 and 6, respectively. Whole-body clearances were monoexponential with biological half-lives of 92, 104 and 160 hr for ^{125}I-ChL6, ^{90}Y-DOTA-peptide-ChL6 and ^{111}In-DOTA-peptide-ChL6, respectively. Whole-body clearances of ^{90}Y-DOTA-peptide-ChL6 and ^{125}I-ChL6 were not statistically different. The blood clearances of all three radiopharmaceuticals were biexponential with a beta phase $T_{1/2}$ of

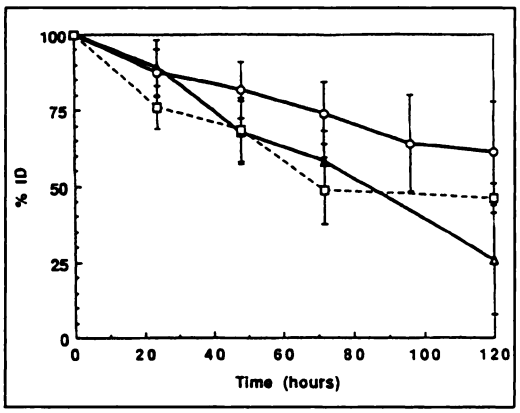

FIGURE 5. Whole-body clearance curves for ^{90}Y (△) and ^{111}In-DOTA-peptide-ChL6 (○) and ^{125}I-ChL6 (□) in HBT tumor-bearing nude mice. Data represent 5–15 mice for each time point and are expressed as percent injected dose (%ID). Error bars represent 1 standard deviation.

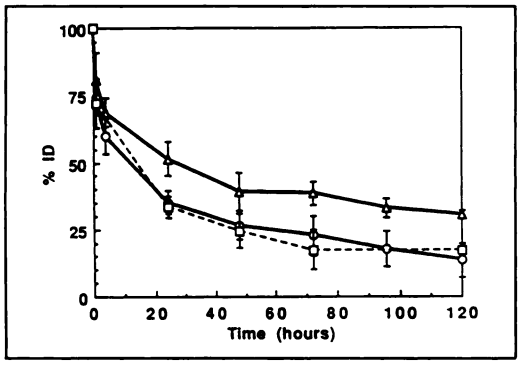

FIGURE 6. Blood clearance curves for ^{90}Y (△) and ^{111}In-DOTA-peptide-ChL6 (○) and ^{125}I-ChL6 (□) in HBT tumor-bearing nude mice. Data represent 5–15 mice for each time point and are expressed as percent injected dose (%ID). Error bars represent 1 standard deviation.

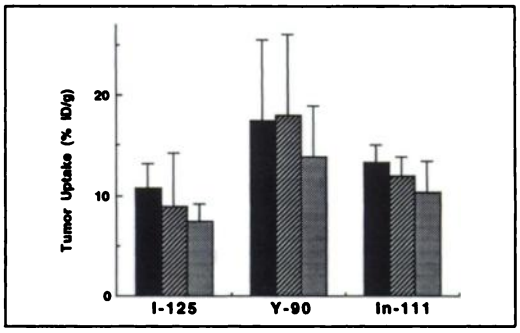

FIGURE 7. Tumor uptake results for ^{111}In- and ^{90}Y-DOTA-peptide-ChL6, and ^{125}I-ChL6 on Days 1 (■), 3 (▨) and 5 (■) post-injection. The ^{90}Y-DOTA-peptide-ChL6 uptake is greater than that of ^{125}I-ChL6. Error bars represent 1 standard deviation.

104, 104 and 148 hr for ^{125}I-ChL6, ^{90}Y-DOTA-peptide-ChL6 and ^{111}In-DOTA-peptide-ChL6, respectively. Tumor uptakes of radioimmunoconjugates at 1, 3 and 5 days postinjection and an autoradiograph from a mouse killed 3 days after injection of ^{90}Y-DOTA-peptide-ChL6 are shown in Figures 7 and 8, respectively. Concentration of ^{90}Y in the tumor and minimal bone accumulation of ^{90}Y are demonstrated by the data in Table 2 and Figure 7 and are also visually apparent in the autoradiograph in Figure 8.

DISCUSSION

In spite of greater tumor uptake and radiation dose per unit of radioactivity, therapy with ^{90}Y-labeled MAb has been limited by a lower maximum tolerated dose (MTD) and a therapeutic index than equivalent ^{131}I-immunoconjugates (*5,6*). Therapy utilizing ^{90}Y-immunoconjugates has been hindered because of the instability of the chelated yttrium resulting in deposition of ^{90}Y in bone sufficient to increase the radiation dose to the bone marrow, thereby decreasing the MTD and therapeutic index (*1,2*). Macrocyclic chelating agents make it possible to prepare more stable ^{90}Y-chelated immunoconjugates for medical use (*8–10*). Furthermore, DOTA chelates of ^{111}In behave like ^{90}Y-DOTA chelates in vitro (*8,16*), and ^{111}In- and ^{90}Y-DOTA-peptide-ChL6 had similar pharmacokinetics in the present study. Whether ^{111}In-DOTA MAbs can be used to extrapolate human dosimetry for ^{90}Y-DOTA MAbs awaits careful evaluation in patients.

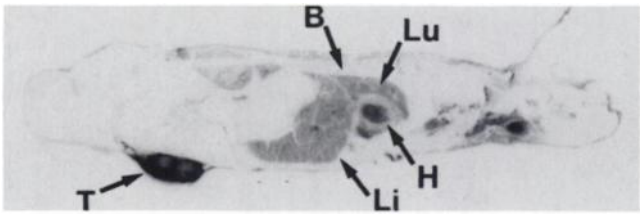

FIGURE 8. Whole-body autoradiograph of a mouse killed 3 days after injection of 70 μCi of ^{90}Y-DOTA-peptide-ChL6. Tumor uptake is uniform and greater than liver radioactivity, and lung and bone radioactivity are minimal. The autoradiograph visually suggests a good therapeutic index.

The stability of ^{90}Y-DOTA radioimmunoconjugates has been previously demonstrated by whole-body autoradiography studies in mice that showed significantly less ^{90}Y accumulation in bone when compared to an equivalent ^{90}Y-MXDTPA radioimmunoconjugate (*10*). When compared to radioiodinated analogs, however, ^{90}Y-DOTA MAb was retained in the liver and whole body of the mice. Several approaches have been used to decrease radiometal accumulation in the liver after radioimmunoconjugate administration. Rosenblum et al. (*24*) modified the Fc portion of antibodies to impede their intracellular access. Pretargeting approaches with bispecific antibodies are subjects of ongoing studies because they lead to rapid excretion of nontargeted radioactivity and a very high therapeutic index (*25–28*). A number of different linkers between the chelate and MAb have been designed to enhance the cleavage of the radiochelate from the immunoconjugate when internalized in cells, so that the radiochelate can return to the blood and be excreted by the kidneys. The novel linkage described herein was chosen as a likely substrate for intrahepatocyte cathepsin enzymes. The effectiveness of this cleavable linker in fulfilling its purpose is reflected in the data showing that whole-body clearance of ^{90}Y-DOTA-peptide-ChL6 was not significantly different from that of ^{125}I-ChL6 ($p < 0.01$) (Fig. 5) and that normal tissue clearances were similar, whereas tumor uptake and retention were increased (Table 2). The clearance of ^{111}In-DOTA-peptide-ChL6 through 72 hr was also not significantly different from that of the other two labeled MAbs, but later time points suggested some retention of ^{111}In ($p < 0.05$). Although the results indicate that ^{90}Y-DOTA-peptide is cleaved from ChL6 and excreted in a manner analogous to ^{125}I released from ^{125}I-ChL6, the data also suggest that very small amounts of ^{111}In may escape from the DOTA chelate so that the total excretion is somewhat less (%ID) than that of ^{90}Y and ^{125}I.

Importantly, tumor uptake and retention of ^{90}Y-DOTA-peptide-ChL6 were much greater than that of ^{125}I-ChL6 throughout the five study days, suggesting that there was

little if any enzymatic digestion in the tumor tissue where the radioimmunoconjugate is presumably located on the cell surface membrane (*29*).

Despite the shorter half-life of ^{90}Y, greater tumor uptake retention of ^{90}Y-DOTA-peptide-ChL6 resulted in a cumulative activity (16.6 μCi hr/μCi) equivalent to that of ^{131}I-ChL6 (17.2 μCi hr/μCi), assuming that ^{131}I-ChL6 behaves like ^{125}I-ChL6. Because energy deposition per disintegration of ^{90}Y is much greater than that of ^{131}I, equivalent cumulative activities of these radionuclides are associated with four to five more radiation doses from the ^{90}Y. This potential advantage only proves useful for therapy if the radiation dose to normal tissues is tolerable. When toxicity is displaced from bone marrow by the use of growth factors and/or bone marrow reconstitution to the liver and/or lungs, toxicity remains dose-limiting to ^{90}Y-radioimmunoconjugate treatment. Thus, the therapeutic success of ^{90}Y immunoconjugates in cancer patients depends on the therapeutic index which in the latter circumstances is determined by hepatic and/or pulmonary toxicity, and in turn, can be favorably influenced by the DOTA-peptide linker described herein.

In order to prepare therapeutic doses of expensive radiometals like ^{90}Y economically, it is necessary to maximize the product yield. Because the amount of excess chelate needed for the DOTA-peptide chelation step depends on the amount of metal ion contaminants present in the ^{90}Y or ^{111}In supply, a rapid titration method was used to measure the total amount of these competing metals in each shipment. Based on the results from the titration curve, the amount of DOTA-peptide necessary to chelate of $\geq$ 80% of the ^{90}Y or ^{111}In in the shipment was determined. DOTA-peptide-NCS complexed by nontrivalent contaminant metal ions and excess DOTA-peptide-NCS were removed before MAb conjugation using anion exchange column chromatography. During the conjugation step, virtually all of the reactive DOTA-peptide-NCS was attached to the antibody leaving only nonreactive, putatively hydrolyzed, chelate (Fig. 4C). Because these nonreactive species compete with reactive DOTA-peptide for ^{90}Y and ^{111}In, repurification of DOTA-peptide-NCS with C18 chromatography before use when stored for several months improves labeling yields.

CONCLUSION

The potential immunogenicity of radioimmunoconjugates must be minimized in order to decrease the possibility that HAMA will interrupt therapy prematurely. The radiochemical methods that we describe in this article limit the amount of DOTA macrocycles in the radiopharmaceutical to a few nanograms, ensure that each MAb molecule has no more than one DOTA macrocycle and ensure that most DOTA macrocycles carry a radiometal. Therefore, the final immunoconjugate radiopharmaceutical contains the least amount of MAb and DOTA required for optimal product yields. By using as much as a 50:1 molar excess of ChL6-to-^{111}In- or ^{90}Y-DOTA-peptide for the conjugation reaction to ensure not more than one DOTA macrocycle on each MAb molecule, specific activities of 2 mCi ^{111}In or 4 mCi ^{90}Y per mg of MAb have currently been obtained. Despite large and variable amounts of metal contaminants in many shipments, purification of the ^{111}In- or ^{90}Y-DOTA-peptide-NCS and postpurification MAb conjugation has led to high specific activity ^{111}In- and ^{90}Y-DOTA-peptide-ChL6 preparations with minimal chelate and no cross-conjugated antibody. Thus, ^{111}In and ^{90}Y radiopharmaceuticals prepared by these methods should have excellent immunologic, biochemical and pharmaceutical properties for radioimmunoimaging and radioimmunotherapy.

ACKNOWLEDGMENTS

Supported by U.S. Department of Energy grant DE-FG03-84ER60233, National Cancer Institute grant CA47829 and U.S. Public Health Service grant CA16861.

REFERENCES

1. Sharkey R, Kaltovich FA, Shih LB, et al. Radioimmunotherapy of human colonic cancer xenografts with yttrium-90-labeled monoclonal antibodies to carcinoembryonic antigen. *Cancer Res* 1988;48:3270–3275.
2. Hnatowich DJ. Antibody radiolabeling, problems and promises. *Nucl Med Biol* 1990;17:49–55.
3. Wessels BW, Rogus RD. Radionuclide selection and model absorbed dose calculations for radiolabeled tumor associated antibodies. *Med Phys* 1984; 11:638–645.
4. Hnatowich DJ, Virzi F, Doherty PW. DTPA-coupled antibodies labeled with yttrium-90. *J Nucl Med* 1985;26:503–509.
5. Stewart JS, Hird V, Snook D, et al. Intraperitoneal ^{131}I and ^{90}Y labeled monoclonal antibodies for ovarian cancer: pharmacokinetics and normal tissue dosimetry. *Int J Cancer Suppl* 1988;3:71–76.
6. Stewart JSW, Hird V, Snook D, et al. Intraperitoneal yttrium-90-labeled monoclonal antibody in ovarian cancer. *J Clin Oncol* 1990;8:1941–1950.
7. Order SE. Presidential address: systemic radiotherapy—the new frontier. *Int J Radiat Oncol Biol Phys* 1990;18:981–992.
8. Meares CF, Moi MK, Diril H, et al. Macrocyclic chelates of radiometals for diagnosis and therapy. *Br J Cancer Suppl* 1990;10:21–26.
9. 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.
10. DeNardo GL, Kroger LA, DeNardo SJ, et al. Comparative toxicity studies of yttrium-90 MX-DTPA and 2-IT-BAD conjugated monoclonal antibody (BrE-3). *Cancer* 1994;73(suppl):1012–1022.
11. Paik CH, Quadri SM, Reba RC. In vivo comparison of antibody DTPA conjugates with different linkages [Abstract]. *J Nucl Med* 1987;28:572.
12. Yokoyama K, Paik CH, Quadri SM, et al. Amplification of antibody tumor targeting by chemical modification with ester bond [Abstract]. *J Nucl Med* 1987;28:572.
13. Deshpande SV, DeNardo SJ, Meares CF, et al. Effect of different linkages between chelates and monoclonal antibodies on levels of radioactivity in the liver. *Nucl Med Biol* 1989;16:587–597.
14. Studer M, Kroger LA, DeNardo SJ, Kukis DL, Meares CF. Influence of a peptide linker on biodistribution and metabolism of antibody-conjugated benzyl-EDTA: comparison of enzymatic digestion in vitro and in vivo. *Bioconj Chem* 1992;3:424–429.
15. Kosmas C, Snook D, Gooden CS, et al. Development of humoral immune responses against a macrocyclic chelating agent (DOTA) in cancer patients receiving radioimmunoconjugates for imaging and therapy. *Cancer Res* 1992;52:904–911.
16. Li M, Meares CF. Synthesis, metal chelate stability studies, and enzyme digestion of a peptide-linked DOTA derivative and its corresponding radiolabeled immunoconjugates. *Bioconj Chem* 1993;4:275–283.
17. Li M, Meares CF, Zhong G-R, et al. Labeling monoclonal antibodies with ^{90}Y- and ^{111}In-DOTA chelates: a simple and efficient method. *Bioconj Chem* 1994;5:101–104.
18. Adams GP, DeNardo SJ, Amin A, et al. Comparison of the pharmacokinetics in mice and the biological activity of murine L6 and human-mouse

chimeric Ch-L6 antibody. *Antibod Immunoconjug Radiopharm* 1992;5:81–95.
19. Hellstrom I, Horn D, Linsley P, et al. Monoclonal antibodies raised against human lung carcinomas. *Cancer Res* 1986;46:3917–3923.
20. Liu AY, Robinson RR, Hellstrom KE, et al. Chimeric mouse-human IgG1 antibody that can mediate lysis of cancer cells. *Proc Natl Acad Science USA* 1987;84:3439–3443.
21. Beaumier PL, Neuzil D, Yang H-M, et al. Immunoreactivity assay for labeled anti-melanoma monoclonal antibodies. *J Nucl Med* 1986;27:824–828.
22. Penefsky HS. A centrifuged-column procedure for the measurement of ligand binding by beef heart F_1. *Methods Enzymol* 1979;56:Part G,527–530.
23. Fand I, McNally WP. The technique of whole-body autoradiography. In: Johnson JE Jr, ed. *Current trends in morphological techniques*, vol 2. Boca Raton, FL: CRC Press, 1981:1–28.
24. Rosenblum MG, Murray JL, Haynie TP, et al. Pharmacokinetics of ^{111}In-labeled anti-p97 monoclonal antibody in patients with metastatic malignant melanoma. *Cancer Res* 1985;45:2382–2386.
25. Goodwin DA, Meares CF, McCall MJ, et al. Pre-targeting immunoscintigraphy of murine tumors with indium-111-labeled bifunctional haptens. *J Nucl Med* 1988;29:226–234.
26. Paganelli G, Riva P, Deleide G, et al. In vivo labeling of biotinylated monoclonal antibodies by radioactive avidin: a strategy to increase tumor radiolocalization. *Int J Cancer Suppl* 1988;2:121–125.
27. Hnatovich D, Virzi F, Ruskovski M. Investigations of avidin and biotin for imaging applications. *J Nucl Med* 1987;28:1294–1302.
28. Paganelli G, Belloni C, Magnani P, et al. Two-step tumor targeting in ovarian cancer patients using biotinylated monoclonal antibodies and radioactive streptavidin. *Eur J Nucl Med* 1992;19:322–329.
29. Shih LB, Thorpe SR, Griffiths GL, et al. The processing and fate of antibodies and their radiolabels bound to the surface of tumor cells in vitro: a comparison of nine radiolabels. *J Nucl Med* 1994;35:899–908.