Copper-67-Labeled Monoclonal Antibody Lym-1, A Potential Radiopharmaceutical for Cancer Therapy: Labeling and Biodistribution in RAJI Tumored Mice

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Copper-67 (^{67}Cu) is one of the most promising radiometals for radioimmunotherapy because of its 61.5 hr physical half-life, abundant beta particles, and gamma emissions suitable for imaging. However, ^{67}Cu is readily transferred from the usual chelates of EDTA or DTPA to albumen. We developed a new macrocycle (6-p-nitrobenzyl-TETA) to chelate copper. Bifunctional chelating agent p-bromoacetamidobenzyl-TETA was conjugated to Lym-1, a monoclonal antibody against human B cell lymphoma, without significantly altering its immunoreactivity. This conjugate was stably labeled with ^{67}Cu under conditions chosen to optimize the yield of a high specific activity radiopharmaceutical. The biodistribution in RAJI tumor bearing mice demonstrated significant tumor uptake (14.7% ID per gram) and extended residence time (120 hr) in contrast to normal organs. After 24 hr, radioactivity was continuously cleared from all tissues except the tumor. This study suggests ^{67}Cu labeled Lym-1 to be a promising radiopharmaceutical for potential use for radioimmunotherapy of B cell lymphoma.

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The development of radiopharmaceuticals from tumor targeting monoclonal antibodies has been primarily focused on their potential for radioimmunoimaging. We have suggested that the target-to-nontarget relationship required for radioimmunotherapy is no greater than that for radioimmunoimaging if there is a prolonged residence time of the radiolabeled antibody on the tumor cell (*1,2*). Except in isolated patients (*3–5*), however, the few reported preliminary trials using iodine-131(^{131}I) on first or second generation monoclonal antibodies have demonstrated only modest therapeutic success (*6–8*). Many factors may have had a role in limiting these results, such as nonspecific antigenic targets, low affinity of the radiolabeled antibody, and the use of ^{131}I as the therapeutic radionuclide. Animal studies have demonstrated a lower and less prolonged tumor content of radioactivity when radioiodinated antibodies were administered as compared to when radiometal labeled antibodies were administered (*9–11*).

Copper-67 (^{67}Cu) is one of the most promising radiometals for radioimmunotherapy. It has a 61.5 hr physical half life which is well matched to the residence time of a typical antibody on the tumor; copper-67 releases abundant beta particles as well as a moderate abundance of 93 and 184 keV gamma emissions (Table 1). These gamma emissions permit radiation dosimetry to be predicted from the information obtained from quantitative imaging studies on pretherapy doses of ^{67}Cu. This pretherapy imaging and kinetics, followed by similar pharmacokinetics of the actual therapy doses, allows treatment planning and on-going verification of that planning with actual dosimetric information on the radiation delivered.

In order to use ^{67}Cu for radioimmunotherapy it must be stably chelated to the monoclonal antibodies. Serum stability studies of copper chelates of EDTA and DTPA have indicated that copper is readily transferred from these chelates to serum albumen (*12*). We recently reported the synthesis and characterization of a new

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TABLE 1
Properties of Copper-67

Half-life: 62 hr
Decay mode: beta minus
Energy (Emax), keV, particle: 577 (20%), 486 (35%), 395 (45%)
Gamma: 91 (7%), 93 (17%), 184 (47%)
Production method: ^{68}Zn (P, 2P) ^{67}Cu
Decay product: ^{67}Zn

macrocycle ([6-p-nitrobenzyl-1,4,8,11-tetraazacyclotetradecane-N,N′,N″,N‴ tetraacetate (TETA)] (*13*) which forms a copper chelate that remains stable in serum for several days (*12*).

In this paper, we describe a method for the conjugation of this macrocycle to the anti-lymphoma monoclonal antibody, Lym-1, subsequent labeling of the antibody chelator conjugates with ^{67}Cu, and the biodistribution of the radiolabeled antibody chelate conjugate in tumor-bearing nude mice.

MATERIALS AND METHODS

p-Nitrobenzyl-TETA was prepared according to the method of Moi et al. (*13*). 2-Iminothiolane (Traut's reagent), (Pierce Chemical Co.) carrier-free cobalt-57 (^{57}Co) chloride, (ICN Chemical Radioisotope Div.) and ^{67}Cu were used. The specific activity of the ^{67}Cu was 10 mCi/μg and was used without any dilution with cold copper. Lym-1, an IgG2a monoclonal antibody, was produced in murine ascites and purified by protein A affinity column chromatography (*14*). Nude mice on a BALB/c background were purchased from Harlen-Sprague-Dawley and transplanted with RAJI tumor (*15*) by subcutaneous injection of 10^6 cells. Metal-free conditions were maintained during the synthetic chemistry by using metal-free glassware and plastic ware (*16*).

Analytical Techniques

Thin layer chromatography (TLC) was performed on the samples using 0.2 mm silica gel as stationary phase on plastic backing and a 1:1 mixture of 10% ammonium acetate and methanol as the mobile phase. Cellulose acetate electrophoresis (CAE) of samples was carried out by using CAE apparatus (Gelman Electrophoresis Chamber) with barbital buffer (0.05 *M*, pH 8.6) at 350 V. High performance liquid chromatography (HPLC) was performed on the monoclonal antibody, antibody chelator conjugates, and labeled protein by using a Beckman HPLC system (Model 110, control model 410) on a spherogel TSK-3000 molecular sieving column. Sodium phosphate buffer (0.1*M*, pH 7) containing on a spherogel TSK-3000 molecular sieving column. Sodium phosphate buffer (0.1*M*, pH 7) containing 0.025% sodium azide was used to elute the column at a flow rate of 1 ml per min (pressure 1,000 psi). Radioactive fractions were detected with a continuous flow-through radioisotope detector (Beckman Model 170), and protein was detected by a flow-through ultraviolet detector at 280 nm. These profiles were recorded by a strip chart recorder. The metal binding capacity of bifunctional chelators and the number of chelators bound to the antibody was assayed by titration with ^{57}Co-labeled standard cobalt chloride solutions due to the ready availability of ^{57}Co in the carrier free form and its 271 day half-life.

Sephadex Gel Filtration (Centrifuged Column Method)

A centrifuged Sephadex G-50-80 column method first described by Penefsky and modified by Meares et al., was used to separate proteins from lower molecular weight compounds (*17*).

Preparation of p-Aminobenzyl-TETA

p-Nitrobenzyl-TETA (55.1 mg, 97.1 μmol) was dissolved in 20 ml of distilled water and the pH was adjusted to 11.5 using 2*M* NaOH. The solution was cooled in an ice bath, and 1 mg palladium catalyst (10% palladium on powdered charcoal) was added. The mixture was placed under hydrogen (1 atmosphere) and temperature was maintained at 0°C. The appearance of a faint greenish color was noted during the course of the reaction. After 6 hr, the reaction mixture was neutralized and filtered through a nylon 66-Millipore filter, having a pore size of 0.45 μ. The filtrate was analyzed for the presence of aromatic amines by use of the fluorescamine test (*18*). Lyophilization of the filtrate left 42.5 mg of residue

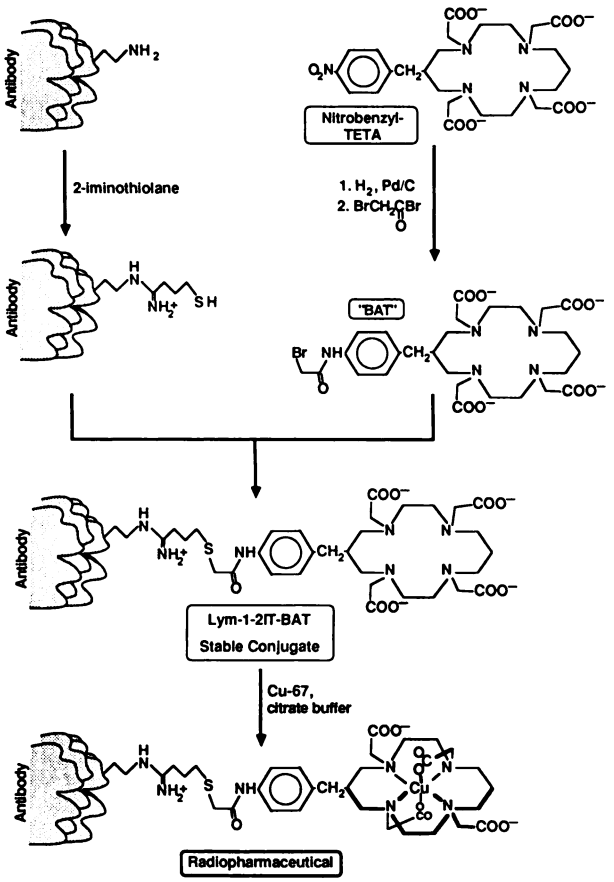

FIGURE 1
Conjugation of macrocyclic ligand TETA to the monoclonal antibody. The nitro group of p-nitrobenzyl TETA is reduced to p-aminobenzyl TETA and then alkylated to give p-bromoacetamidobenzyl TETA (BAT). 2-Iminothiolane treatment modifies lysine amino groups on Lym-1, producing free sulfhydryl groups. These may be alkylated by BAT to yield the protein-chelator conjugate Lym-1-2IT-BAT. Addition of ^{67}Cu (see Fig. 2) produces the radiopharmaceutical.

containing the p-aminobenzyl-TETA. An aliquot of the residue was subjected to a ^{57}Co metal binding assay, which showed that the residue was 86.7% pure p-aminobenzyl-TETA by weight. This material could be used in the following synthesis without further purification.

Preparation of p-Bromoacetamidobenzyl-TETA (BAT)

The p-aminobenzyl-TETA residue (39.2 mg, 73 μmol p-aminobenzyl-TETA) was dissolved in 2 ml of distilled water and the pH was adjusted to 6.5 using 2*M* HCL. Fifty microliters of bromoacteyl bromide was added in 10-μl aliquots. Completion of the reaction was determined by a negative fluorescamine test (showing the absence of aromatic amine groups) and a positive 4-(p-Nitrobenzyl)pyridine (NBP) test, showing the presence of alkyl bromide (*19*). The final reaction mixture was extracted with ether (4 × 1.4 ml) and lyophilized. The lyophylization residue weighed 45.8 mg and was shown by ^{57}Co metal binding assay to be 81.8% pure p-bromoacetamidobenzyl-TETA (BAT) by weight. This material could be used in subsequent conjugation reactions without further purification.

Synthesis of Antibody Chelator Conjugate

Twenty millimolars 2-iminothiolane (2IT) solution was prepared by dissolving 2.7 mg in 1 ml of 50 mm triethanolamine at pH 8.6. To 1 mg Lym-1 in 65 μl 100 m*M* sodium phosphate buffer at pH 8, 32 μl of freshly prepared 2-iminothiolane solution and 3.2 μl 2-mercaptoethanol was added. The resulting solution was vortex-mixed for 30 sec and incubated at 4°C for 30 min. The centrifuged column procedure (G-50-80, 0.1*M* sodium phosphate buffer, pH 8) was used to remove small molecules, including unreacted 2-iminothiolane and 2-mercaptoethanol. The effluent containing the protein was directly collected in a metal-free microcentrifuge tube containing 25 μl of 11 m*M* BAT in 0.1*M* sodium phosphate buffer at pH 8 (Fig. 1). Argon gas was gently blown on the solution, and the cap of the tube was closed to maintain an inert atmosphere. The reaction mixture was incubated at 4°C overnight.

Iodoacetamide (1 mg) was added at room temperature to alkylate unreacted sulfhydryls on the protein. After 30 min, the protein chelator conjugate (Lym-1-2IT-BAT) was separated from lower molecular weight compounds by the centrifuged column method, which also changed the buffer to 0.1*M* citrate, pH 8. The protein concentration was determined mined by measuring the ultraviolent absorbance at 280 nm.

Quantitating the Number of Chelating Groups Bound

A nonradioactive sample of pure cobalt metal was weighed to four significant figures, dissolved in 6*M* HCl at 60°C and then diluted with a known amount of deionized water in a volumetric flask. A measured volume of this solution was again diluted in a volumetric flask using 0.06*M* HCl to obtain 500 μ*M* in Co (exact value accurately calculated). A 500-μl amount of this solution was added to ~0.01 mCi of dry carrier-free ^{57}Co to yield a solution with radioactivity concentration >5,000 cpm per μl and an accurately known concentration of cobalt.

Assay

Ten microliters of protein conjugate solution was mixed with 10 μl of 0.1*M* sodium phosphate buffer at pH 8.5 and 10 μl of standardized $CoCl_2$ solution. After 20 min, 10 μl of 10 m*M* EDTA and 60 μl 0.1*M* sodium phosphate buffer at

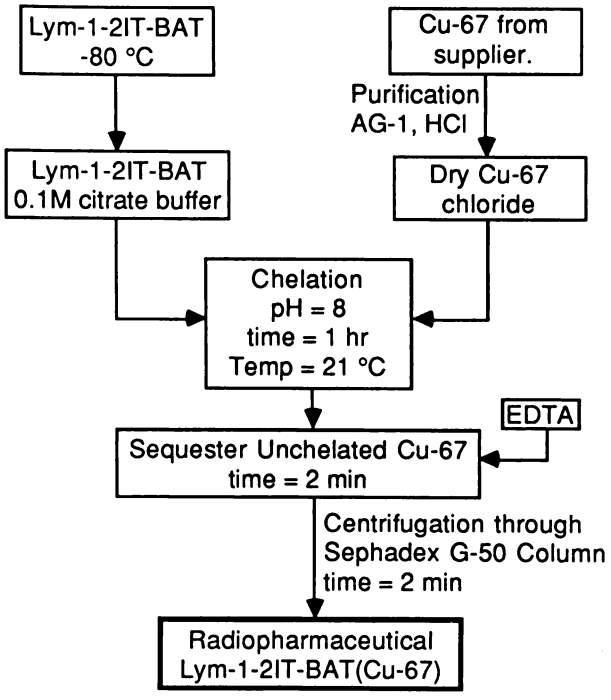

FIGURE 2
Flow chart for radiolabeling antibody chelator conjugate Lym-1-21T-BAT with ^{67}Cu and isolating the product. Molecular structures are given in Figure 1.

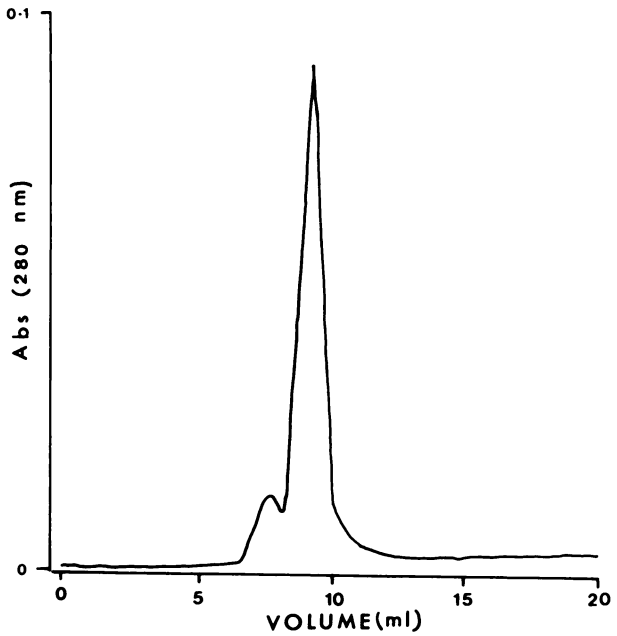

FIGURE 3
HPLC of antibody-chelator conjugate using molecular sieving column. The major peak eluted at 8.4 ml represents the conjugate Lym-1-21T-BAT having a molecular weight of 150,000 D. The small peak at 6.9 ml presumably represents cross-linked protein and has an apparent molecular weight of 300,000 D.

pH 8.5 were added. Cobalt-57-labeled protein was separated from the lower molecular weight species by the centrifuged column method. The column and the effluent were separately counted in a gamma well counter. The concentration of protein bound chelators available for metal binding was calculated as described by Moi et al. (*13*).

Labeling Antibody-Chelator Conjuate (Lym-1-2IT-BAT) with Copper-67

7.2 mCi of ^{67}Cu solution in 2*M* HCl, purified by AG-1 anion exchange chromatography (*20*), was evaporated to dryness in a metal free plastic test tube using a heat lamp and a gentle flow of nitrogen gas. Seventy-nine microliters of protein conjugate solution (600 μg protein) in 0.1*M* citrate buffer at pH 8 was then added and incubated at room temperature for 1 hr with occasional vortex mixing. Ten microliters of 10 m*M* EDTA solution and 11 μl citrate buffer (0.1*M*, pH 8) was added to the reaction mixture to chelate ^{67}Cu that had not been bound to chelating groups on the antibody. After 2 min labeled protein was separated from lower molecular weight compounds by the centrifuged column procedure into 0.1*M* sodium phosphate buffer at pH 7.4 (Fig. 2). Reaction yield was estimated from the TLC of the reaction mixture after addition of EDTA. The overall radiolabeling yield was calculated as follows:

$$\% \text{ Yield} = \frac{\text{Radioactivity on protein after purification}}{\text{Radioactivity initially added}} \times 100.$$

To check for nonspecifically bound ^{67}Cu on the protein conjugate, 1 μl of radiopharmaceutical was mixed with 2 μl of 1 m*M* EDTA, incubated at room temperature for 10 min and a TLC was performed.

Preparation of Control: p-Nitrobenzyl Copper-67 TETA

To 500 μCi of dry ^{67}Cu, 20 μl of 11 m*M* aqueous solution of p-nitrobenzyl-TETA at pH 7 was added and incubated for 30 min at room temperature. The solution was passed through Chelex ion exchange resin (bed volume 1 ml) to remove unchelated ^{67}Cu. Thin layer chromatography of this effluent was performed to analyze for bound and free ^{67}Cu.

The immunoreactivity of ^{67}Cu-labeled protein chelator conjugate was determined by solid phase radioimmunoassay as previously described (*14*).

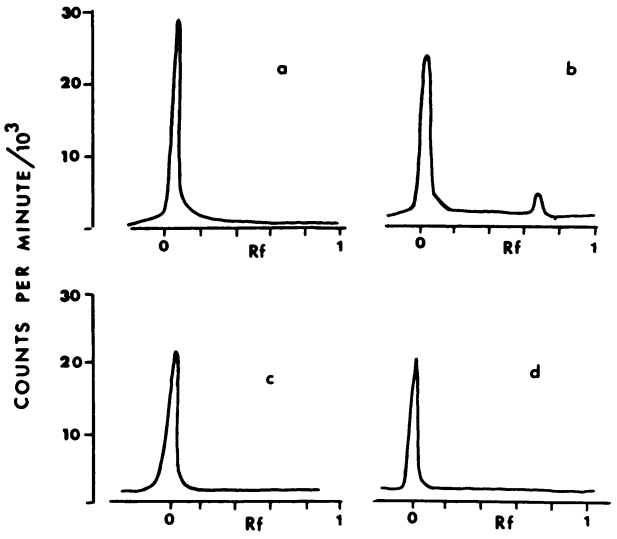

FIGURE 4
Analysis by thin layer chromatography. (a) Lym-1-21T-BAT in 0.1*M* citrate buffer at pH 8 which was added to dry ^{67}Cu chloride and evaluated by TLC. Since neither ^{67}Cu citrate nor ^{67}Cu-Lym-1-21T-BAT move on TLC, all the radioactivity was found at the origin. (b) EDTA was added to the previous mixture, ^{67}Cu-EDTA was seen on TLC at an Rf value of 0.7. ^{67}Cu-Lym-1-21T-BAT remained at the origin. (c) Copper-67 labeled protein after removing [^{67}Cu]EDTA by gel filtration was evaluated by TLC. All the ^{67}Cu was bound to the protein and remained at the origin. No mobile radioactivity was detected. (d) An aliquot of radiopharmaceutical (from Step c) was challenged with 1 m*M* EDTA to evaluate nonspecific binding of ^{67}Cu to Lym-1. The absence of any peak at an Rf of 0.7 suggested the absence of nonspecific binding of ^{67}Cu.

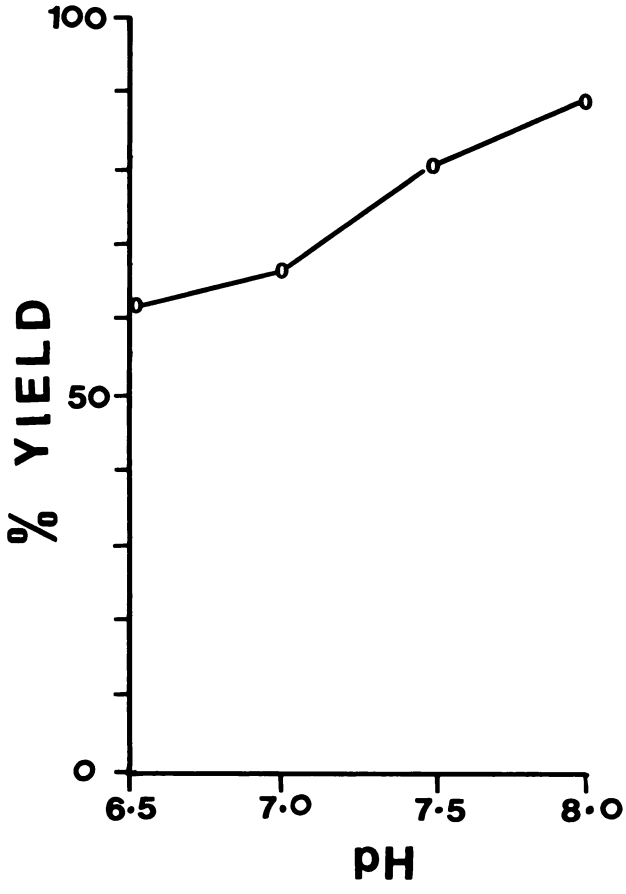

FIGURE 5
Variation of ^{67}Cu labeling yields with pH. Lym-1-2IT-BAT was added to ^{67}Cu (ratio of concentration of BAT to copper ions was 1) at different pH in 0.1*M* citrate buffer. After 1 hr incubation, the unchelated ^{67}Cu was chelated by EDTA and [^{67}Cu]EDTA complex was separated by the centrifuged column method. The labeling yield was calculated as the percent radioactivity on the protein after gel filtration. Note that the yield increased as the pH increased from 6.5 to 8.

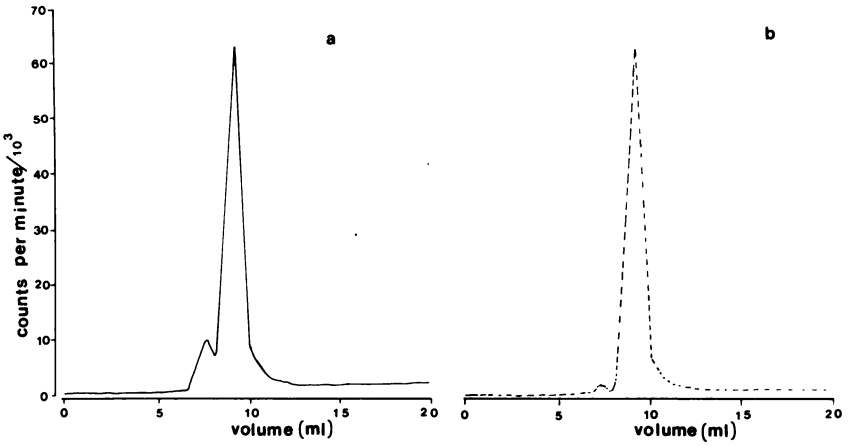

FIGURE 6
Gel filtration HPLC of ^{67}Cu-Lym-1-2IT-BAT radiopharmaceutical (a). The major peak at 8.4 ml was ^{67}Cu labeled Lym-1 corresponding to a molecular weight of 150,000 D. The radiopharmaceutical also contained a small amount of labeled, cross-linked protein with a molecular weight of 300,000 D (peak at 6.9 ml). Fractions (0.5 ml each) were collected and rechromatography of the antibody peak (8.0–8.5 ml fraction) removed most of the 300,000-D molecules (b).

Mouse Study Method

The experiments were performed with adult, female, athymic nude mice with RAJI tumors. The mice were first treated with 400 rad whole-body irradiation prior to implantation of 10^6 RAJI cells in suspension by subcutaneous injection (*15*). After ~3 wk, solid tumors were present (0.1–0.4 g), and the mice were given an injection of 20 μg ^{67}Cu-Lym-1 (155–165 μCi) into a tail vein. The whole body retention of radioactivity at different time points after injection was measured by a balanced and standardized dual sodium iodide crystal probe system (*21*). The blood clearance was obtained by taking measured (10–30 μl) aliquots of blood from the tail vein and counting them in a automated gamma well counter with a 50–250 keV window setting. The animals were killed and tissues were removed and counted in the same manner. The uptake of radioactivity by tissues was calculated as percent injected dose per gram and percent dose per organ. All the results were decay corrected to day zero. The mean and s.d. was calculated for each time point by using the data from at least six mice.

A control mouse biodistribution study was performed in the same manner using 40 μCi ^{67}Cu-labeled p-nitrobenzyl-TETA in each mouse.

Imaging

Mice injected with the radiopharmaceutical were anesthetized with chloroform and imaged on Day 1 and Day 2 after injection with a Siemens LFOV gamma camera using a 20% window centered at 190 keV. A LEAP collimator was used and 90,000 counts were collected.

RESULTS

Conjugation Chemistry

Conjugation of macrocyclic bifunctional chelating agent BAT with Lym-1 typically gave a protein conjugate with an average of 2.7 cheolates per antibody. HPLC analysis demonstrated that more than 90% of the conjugate had a molecular weight of 150,000 D, although a higher molecular weight component was present (9%) (300,000 D) (Fig. 3). This was presumably a cross-linked antibody dimer.

Labeling and Quality Control

The reaction mixture demonstrated 80% radiolabeling yield (Figs. 4 and 5). The ratio of total copper (cold and hot) to the total chelators available for metal binding was 1:1, so 80% of the available chelators were filled with copper in this labeling experiment. Presumably, the remaining 20% of the chelators bound other metal ions present as impurities in the solution. The final recovery of radiolabeled antibody was ~72%. This is based on the original amount of ^{67}Cu and the amount of ^{67}Cu in the final product after the labeling and purification processes. Control experiments showed that 6% of the labeled protein was lost during gel filtration by the centrifuged column method. The specific activity of final product was 9.4 mCi of ^{67}Cu per mg protein. EDTA challenge demonstrated no nonspecific binding of ^{67}Cu to the protein. HPLC of the purified product showed more than 90% to be present as radiolabeled antibody monomer and 9% as labeled molecules of 300,000 D (Fig. 6). The immunoreactivity of the radiopharmaceutical was 70–75% by solid phase assay (*14*).

Biodistribution

The biodistribution of ^{67}Cu-Lym-1 was evaluated in athymic nude mice with RAJI tumors. The radioactivity in the whole body decreased in a continuous manner with biologic half-life of three days (Figure 7). Radiolabeled protein was cleared from the blood in two phases with half-lives of 5 hr and 3.6 days (Figure 8).

The tissue distribution of ^{67}Cu-Lym-1 is given in Table 2. Tumor uptake increased to 14.7% injected dose (ID) per gram on Day 3 and remained near this level through Day 5 (Fig. 9 and Table 2). The radioactivity in all normal tissues decreased steadily from Day 1.

Biodistribution of ^{67}Cu-p-nitrobenzyl-TETA control showed an extremely rapid renal excretion of radioactivity. The organs had very little uptake of radioactivity (Table 2).

Imaging Studies

An image from a mouse that received the ^{67}Cu-Lym-1 is shown in Figure 10. Tumor localization of radiopharmaceutical was obvious on Day 1 after injection.

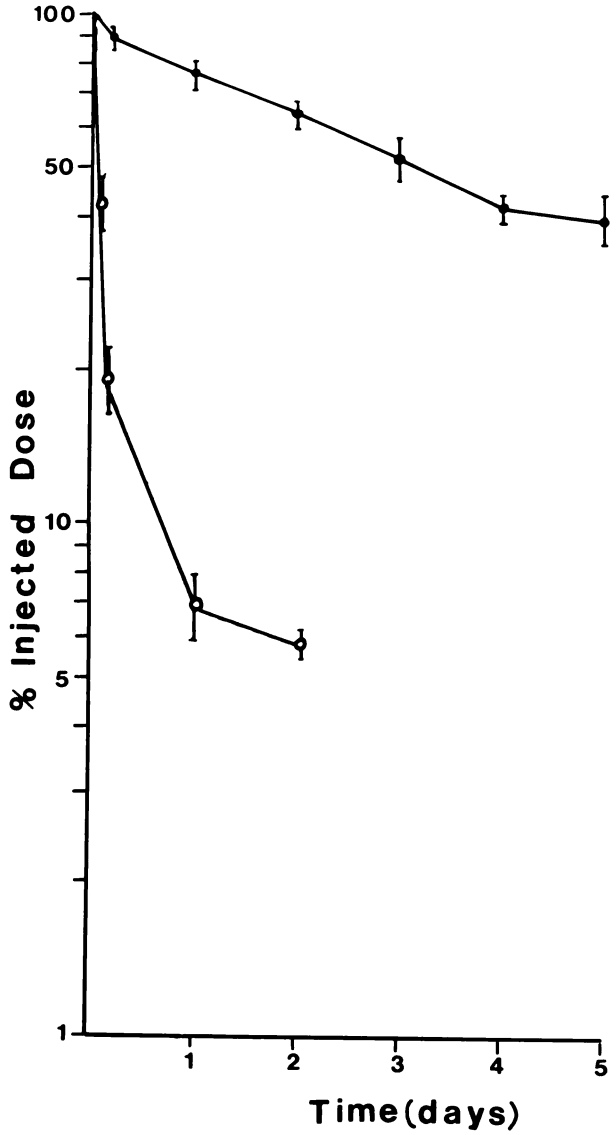

FIGURE 7
Whole body retention curves obtained in tumor bearing nude mice injected with ^{67}Cu-Lym-1-2IT-BAT (●——●) or p-nitrobenzyl [^{67}Cu]TETA (○——○). The control radiopharmaceutical p-nitrobenzyl [^{67}Cu]TETA was excreted very quickly in the urine.

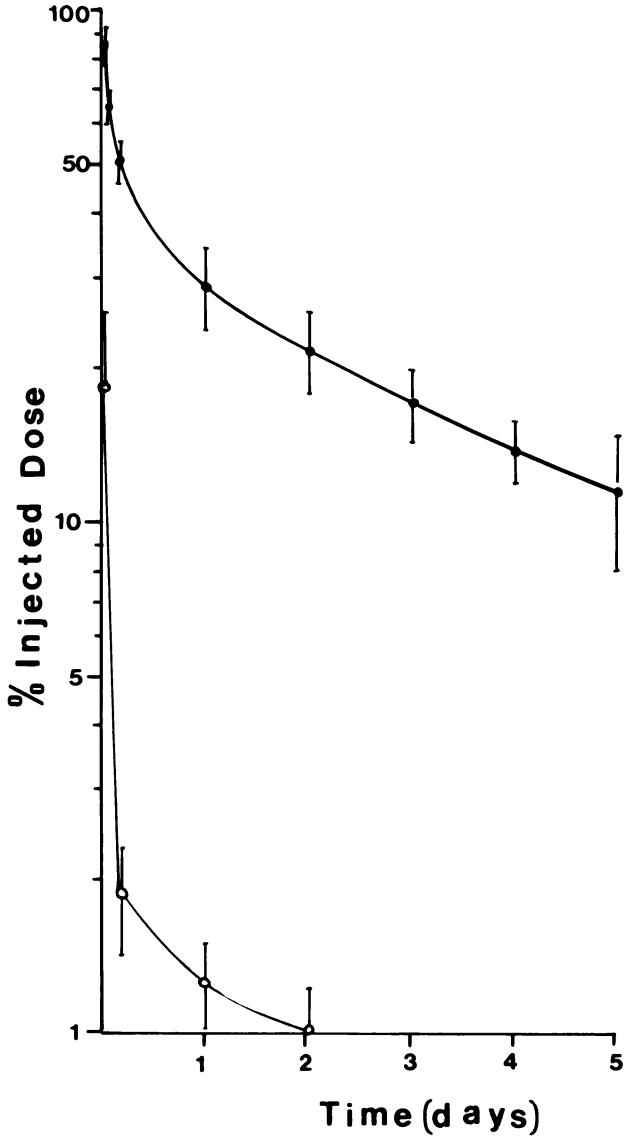

FIGURE 8
Blood clearance curves obtained in tumor bearing nude mice for ^{67}Cu-Lym-1-2IT-BAT (●——●) or p-nitrobenzyl TETA-Cu-67 (○——○). Blood clearance of ^{67}Cu-Lym-1-2IT-BAT had an initial $T_{½}$ of 5 hr followed by slower clearance. The p-nitrobenzyl [^{67}Cu]TETA was cleared very rapidly from the blood and from the mouse.

DISCUSSION

Copper-67 possesses abundant beta particles useful to treat solid tumors using tumor-targeting monoclonal antibodies and its less abundant gamma emissions are suitable for imaging and kinetic evaluation in vivo. Its properties as a radiometal provide the opportunity to utilize bifunctional chelates that cannot be easily metabolized by extracellular enzymes at the tumor sites. The kinetic lability of many copper chelates was at first a challenge problem; copper was observed to be lost quickly from commonly used chelators but not from the macrocycle used here (*12,22*).

The quality assessment of Lym-1-2IT-BAT by HPLC gel filtration and immunoassay consistently demonstrated a chelation yield of 80% of the ^{67}Cu by antibody conjugate containing 1–3 chelators per antibody, a final recovery of 70–75%, 91% of the ^{67}Cu associated with a molecular weight of 150,000 D and an immunoreactivity of 70–75%. The biodistribution studies in nude mice with human lymphoma demonstrated that this copper attachment to the antibody was stable, providing good tumor uptake, and had an extended residence time of the copper on a tumor (greater than 5 days). Tumor uptake was comparable to that found in other studies of radiometal labeled antibodies and tumor residence was similar to the longest seen in other studies (*11,23–25*). It is important to observe that the radioactivity in the normal organs, such as the liver, was cleared during

TABLE 2
Organ Distribution Data for Copper-67-Labeled Lym-1 (^{67}Cu-Lym-1-2IT-BAT) and Control p-Nitrobenzyl-TETA-^{67}Cu in Tumor-Bearing BALB/c Athymic Nude Mice

	% ID/gram tissue					% ID				
	^{67}Cu-Lym-1-2IT-BAT			p-Nitrobenzyl TETA-^{67}Cu		^{67}Cu-Lym-1-2IT-BAT			p-Nitrobenzyl-TETA-^{67}Cu	
	Day 1	Day 3	Day 5	Day 1	Day 2	Day 1	Day 3	Day 5	Day 1	Day 2
Blood	13.8 ± 2.5	8.7 ± 1.2	5.8 ± 1.2	0.73 ± 0.24	0.40 ± 0.14	28.0 ± 5.0	17.8 ± 2.4	11.8 ± 2.4	1.50 ± 0.24	0.80 ± 0.28
Tumor	8.2 ± 1.8	14.7 ± 4.5	13.2 ± 1.9	0.17 ± 0.04	0.11 ± 0.04	1.4 ± 0.3	2.4 ± 0.7	2.2 ± 0.3	0.03 ± 0.01	0.02 ± 0.01
Liver	10.9 ± 0.6	9.4 ± 2.3	4.8 ± 2.3	0.98 ± 0.18	0.68 ± 0.12	10.9 ± 0.6	9.4 ± 2.3	4.7 ± 2.3	0.97 ± 0.18	0.68 ± 0.12
Lung	5.4 ± 0.5	4.8 ± 1.6	2.9 ± 0.7	0.50 ± 0.07	0.06 ± 0.02	0.8 ± 0.1	0.7 ± 0.2	0.4 ± 0.1	0.07 ± 0.01	0.01 ± 0.00
Muscle	1.0 ± 0.5	0.8 ± 0.4	0.6 ± 0.3	0.06 ± 0.02	0.06 ± 0.03					
Intestines	4.7 ± 0.5	3.8 ± 1.0	1.7 ± 0.5	0.49 ± 0.07	0.26 ± 0.08	4.9 ± 0.5	4.0 ± 1.1	1.8 ± 0.5	0.51 ± 0.07	0.28 ± 0.08
Kidneys	5.1 ± 0.4	4.9 ± 1.8	3.2 ± 0.5	0.49 ± 0.05	0.42 ± 0.14	1.5 ± 0.1	1.5 ± 0.5	0.9 ± 0.1	0.15 ± 0.01	0.12 ± 0.04
Brain	1.2 ± 0.6	0.4 ± 0.2	0.3 ± 0.1	0.04 ± 0.01	0.03 ± 0.01	0.4 ± 0.2	0.2 ± 0.1	0.1 ± 0.0	0.01 ± 0.00	0.01 ± 0.00
Spleen	9.1 ± 1.8	8.5 ± 3.0	3.2 ± 1.2	0.43 ± 0.08	0.18 ± 0.07	0.9 ± 0.2	0.9 ± 0.3	0.3 ± 0.1	0.50 ± 0.01	0.02 ± 0.01
Heart	2.8 ± 1.3	2.7 ± 0.7	2.0 ± 0.4	0.32 ± 0.02	0.25 ± 0.06	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.0	0.03 ± 0.00	0.02 ± 0.00
Stomach	2.7 ± 0.6	2.4 ± 0.5	1.6 ± 0.4	0.26 ± 0.15	0.73 ± 0.66	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.04 ± 0.02	0.11 ± 0.09

Mean ± s.d.

a 5-day interval in parallel with clearance from the blood and from the entire mouse. This suggests that metabolism of the copper TETA antibody proceeds in the liver with the copper still held in the chelate macrocycle form. Otherwise, the copper would be recycled into proteins particularly ceruloplasmin appearing in the blood (*26*). One of the key steps in designing the radiopharmaceutical, ^{67}Cu-Lym-1-2IT-BAT, for radioimmunotherapy is the synthesis of the conjugate Lym-1-2IT-BAT. Understanding the chemistry of conjugation and setting the right conditions for the synthesis of the conjugate are critical for successful results.

When 2-iminothiolane (*27*) is mixed with Lym-1 in the presence of 2-mercaptoethanol, it reacts rapidly with epsilon amino groups of lysine residues to form amidines, in the process creating free sulfhydryl groups on the antibody. These sulfhydryl groups are susceptible to oxidation when 2-mercaptoethanol is removed. An inert atmosphere is maintained to avoid oxidation of the sulfhydryl groups which would form disulfide cross-linkages. These sulfhydryl groups react with the bromoacetemide group of BAT to yield the conjugate. Generally, conjugation reactions follow second order kinetics and, therefore, concentrations of protein and chelator must be kept as high as possible in order to obtain good conjugation yields.

When the conjugate is prepared, it is necessary to know the concentration of chelator bound to the antibody. Generally, for labeling with ^{67}Cu the concentration of chelators on protein available for chelation should be more than 50 μM to obtain good labeling yields. In the present work, the concentration was 135 μM.

Labeling of antibody chelator conjugate with ^{67}Cu involves several competitive reactions. The pH at which labeling is carried out may hydrolyse copper to give

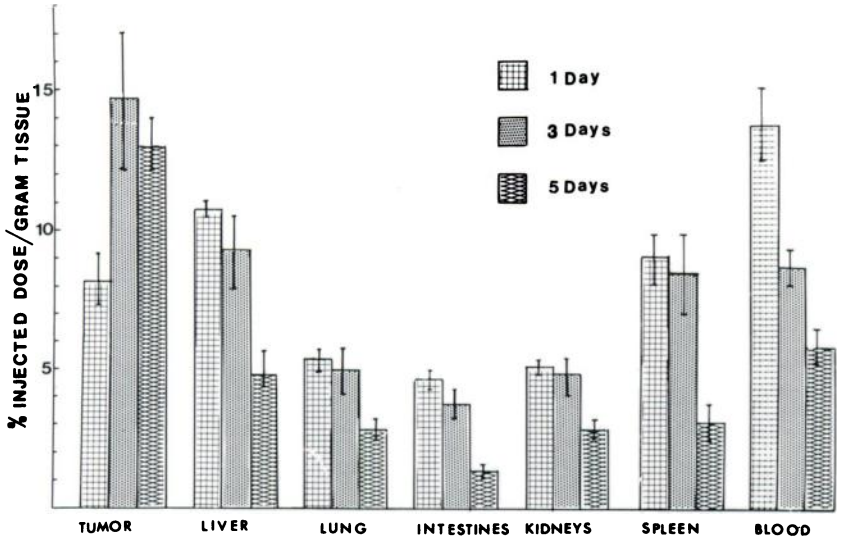

FIGURE 9
Tissue concentrations of ^{67}Cu. Tumor concentration of ^{67}Cu reached maximum on Day 3 (14.7% ID per gram) and did not decrease significantly by Day 5 in spite of rapidly decreasing blood activity. Radioactivity was continuously cleared from all other tissues.

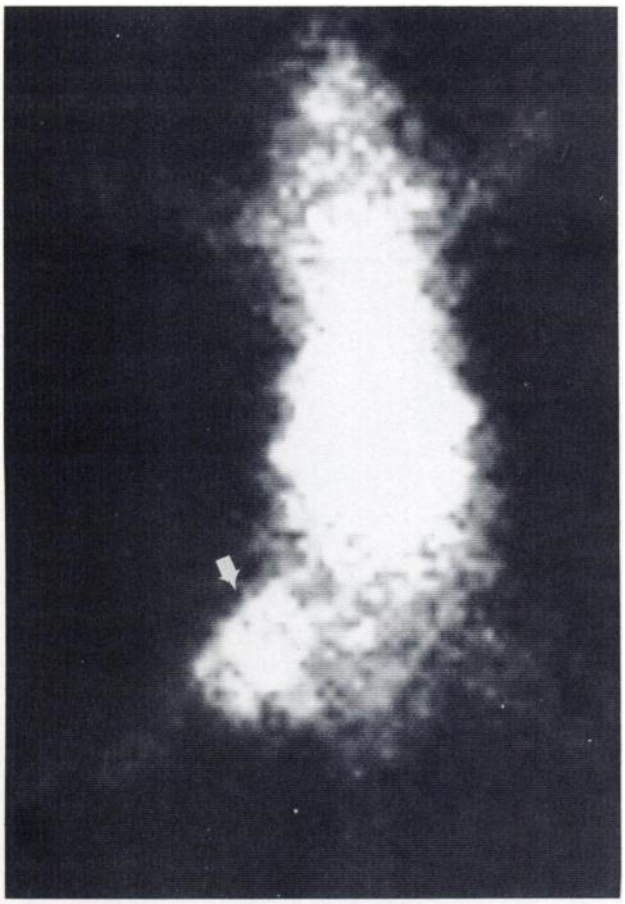

FIGURE 10
Anterior gamma camera image of tumor bearing nude mouse injected (i.v.) with ^{67}Cu-Lym-1-2IT-BAT. The tumor (Arrow) was readily apparent on Day 1 (illustrated) and Day 2 postinjection. Activity was also present in the abdominal organs but was less on Day 2.

insoluble copper hydroxide or the reaction buffer may complex copper strongly. Side chains of amino acid residues of Lym-1 may bind copper thus making it unavailable for complexation with the Lym-1 bound macrocycle. These side reactions need to be suppressed for optimum labeling conditions. The buffer system should form a moderately stable complex with ^{67}Cu in order to keep it in solution and deliver it quickly to the antibody bound macrocycle.

It is desirable to ensure that there is no nonspecifically bound ^{67}Cu in the radiopharmaceutical. The presence of nonspecifically bound ^{67}Cu can be easily determined by mixing an aliquot of radopharmaceutical (see Materials and Methods) with 1 m*M* EDTA. When nonspecifically bound ^{67}Cu is present, it is complexed by EDTA which can be demonstrated by TLC or gel filtration. Control experiments with unmodified antibody verify the efficacy of this EDTA treatment for removing nonspecifically bound metals.

The HPLC evaluation of the ^{67}Cu monoclonal antibody demonstrated a small shoulder preceding the major peak. The molecular weight of this material was ~300,000 D and probably represented monoclonal antibody cross-linked by disulfide bond formation. Removal of these cross-linked molecules in future preparations of the conjugate could improve the immunoreactivity and decrease the reticuloendothelial organ uptake.

The purity of ^{67}Cu affects labeling yields. Copper-67 is produced by bombardment of stable zinc-68 (^{68}Zn) targets and its decay product is stable ^{67}Zn. Copper-67 therefore contains trace amounts of zinc (Zn^{2+}) as a metallic impurity. To obtain good labeling yields, we routinely purified ^{67}Cu by ion exchange column chromatography before labeling (*20*).

More extensive metabolic studies in animals and humans will be necessary to corroborate these in vitro and in vivo findings. However, the specific activity of the ^{67}Cu labeled Lym-1 monoclonal antibody (9.4 mCi ^{67}Cu per mg protein) is already satisfactory for pretreatment dosimetric imaging studies as well as for therapy in cancer patients. In patients, 19 mg of unlabeled and 1 mg of labeled monoclonal antibody has been reported to provide effective radiometal imaging in many monoclonal antibody studies (*28*). Furthermore, we presently treat patients with lymphoma using multiple doses of 60 mCi of ^{131}I-Lym-1 (specific activity 10 mCi per mg of protein) (*29*). Having developed and tested ^{67}Cu-Lym-1 as described in this manuscript, we intend to proceed with imaging to determine dosimetry in patients. If this is encouraging, we intend to pursue a similar therapeutic approach using this radiopharmaceutical.

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