^{90}Y-DOTA-hLL2: An Agent for Radioimmunotherapy of Non-Hodgkin's Lymphoma

Gary L. Griffiths, PhD[1]; Serengulam V. Govindan, PhD[1]; Robert M. Sharkey, PhD[2]; Darrell R. Fisher, PhD[3]; and David M. Goldenberg, ScD, MD[2]

[1]*Immunomedics, Incorporated, Morris Plains, New Jersey;* [2]*Garden State Cancer Center, Belleville, New Jersey; and* [3]*Pacific Northwest National Laboratory, Richland, Washington*

The goal of this work was to determine an optimal radioimmunotherapy agent for further development against non-Hodgkin's lymphoma. We sought to establish the stability profile of ^{90}Y-labeled humanized LL2 (hLL2) monoclonal antibody (mAb) when prepared with different chelating agents and, from these data, to estimate the dosimetric improvement to be expected from use of the most stable ^{90}Y-chelate-hLL2 complex. **Methods:** The complementarity-determining region–grafted (humanized) anti-CD22 mAb, hLL2 (epratuzumab), was conjugated to 3 different chelating agents, 2 of which were derivatives of diethylenetriaminepentaacetic acid (DTPA) and 1 of which was the macrocyclic chelate 1,4,7,10-tetraazacyclododecane-*N,N′,N″,N‴*-tetraacetic acid (DOTA). The 3 hLL2 conjugates were radiolabeled with ^{90}Y and tested for stability in vitro against a 10,000-fold molar excess of free DTPA over 9 d. They were also tested against normal human serum at 37°C over 12 d. Each conjugate was radiolabeled with the γ-emitting radionuclide, ^{88}Y, and compared for biodistribution in normal and lymphoma xenograft–bearing athymic mice. In vivo data were analyzed for statistical differences in the uptake of yttrium in bone and washed bone when either the DOTA or the Mx-DTPA chelates were used, and dosimetry calculations were made for each complex. **Results:** ^{90}Y-DOTA complex of the hLL2 mAb was completely stable to either DTPA or serum challenge for the duration of either experiment (equivalent to 3.3–4.5 half-lives of ^{90}Y radionuclide or >90% of possible ^{90}Y decays from any initial starting activity). Complexes of hLL2 that had been prepared using the DTPA-type chelates lost 3%–4% of initially bound ^{90}Y over the first few days and about 10%–15% over the duration of the challenges. In vivo, these stability differences manifested as significantly lower yttrium uptake in bone and cortical bone over a 10-d period when DOTA was used as the yttrium chelating agent. Absorbed doses per 37 MBq (1 mCi) of ^{90}Y-mAb were 3,555 and 5,405 cGy for bone and 2,664 and 4,524 cGy for washed bone for ^{90}Y-DOTA-hLL2 and ^{90}Y-MxDTPA-hLL2, respectively, amounting to 52.0% and 69.8% increases in absorbed radiation doses for bone and washed bone, respectively, when a DOTA chelate was switched to a Mx-DTPA chelate. **Conclusion:** ^{90}Y-hLL2 prepared with the DOTA chelate represents an improved agent for radioimmunotherapy of non-Hodgkin's lymphoma, with an in vivo model demonstrating a large reduction in bone-deposited yttrium, compared with ^{90}Y-hLL2 agents prepared with open-chain DTPA-type chelating agents. Dosimetry suggests that this benefit will result in a substantial toxicologic advantage for a DOTA-based hLL2 conjugate.

Key Words: CD22; dosimetry; DOTA; humanized LL2; ^{90}Y

J Nucl Med 2003; 44:77–84

Radioimmunotherapy for hematologic malignancies has now reached a stage of useful clinical fruition, after many years of dedicated research and development (*1*). At the time of writing, one agent has been approved by the Food and Drug Administration for treatment of non-Hodgkin's lymphoma (ibritumomab tiuxetan, or Zevalin; IDEC Pharmaceuticals Corp., San Diego, CA) (*2*), and another (tositumomab and ^{131}I tositumomab, or Bexxar; Corixa Corp., Seattle, WA) (*3*) remains under review by the Food and Drug Administration. The latter agent uses a radioiodinated (^{131}I) monoclonal antibody (mAb) and the former a ^{90}Y-labeled mAb, with both antibodies directed against the CD20 antigen on B cells. Both antibodies are also murine in origin, although ibritumomab tiuxetan will also involve administration of the chimeric anti-CD20 antibody, rituximab (Rituxan; IDEC Pharmaceuticals Corp.).

We developed an antibody, humanized LL2 (hLL2), directed against a different B-cell antigen, CD22 (*4*), and for clinical development prepared a complementarity-determining region–grafted, or humanized, version of this mAb (*5*). Naked hLL2 antibody (Immunomedics, Inc., Morris Plains, NJ) was shown to be a potentially effective therapeutic agent, as were ^{131}I- and ^{90}Y-versions of the mAb (*1*,*6*–*8*). Because an anti-CD22 agent has the advantage of targeting a different antigen from that targeted by the above earlier-developed agents, it could be used ultimately more easily, in some form (radiolabeled or not) in combination with either of the above anti-CD20 agents (*9*). Also, because it is a

Received Mar. 7, 2002; revision accepted Aug. 21, 2002.

For correspondence or reprints contact: Gary L. Griffiths, PhD, Immunomedics, Inc., 300 American Rd., Morris Plains, NJ 07950.

E-mail: ggriffiths@immunomedics.com

humanized version of the original murine mAb, it may prove more effective on repeated use, with less concern for human antimouse antibody responses in patients treated. These projected advantages should hold true whether nonradiolabeled, radiolabeled, or combinations of radiolabeled and nonradiolabeled versions of the hLL2 mAb are given in series, in repeated courses, or even after prior imaging with ^{99m}Tc (*10*) or ^{111}In versions of the same mAb.

Having been concerned with proceeding to clinical trials with a humanized and, therefore, in our view, fully optimized mAb, we also desired to develop and test a fully optimized radiolabeling method. The choice between ^{131}I and ^{90}Y radionuclide could be made by comparison of the properties of the 2 radionuclides taken in combination with the properties of the anti-CD22 mAb itself. Unlike the anti-CD20 mAbs, hLL2 internalizes after antigen binding (*11*). This meant that radioiodinated hLL2 prepared by standard iodination methods, using direct attachment to protein tyrosine residues, resulted in a product that was readily internalized and then metabolized by target B cells. Radioiodine in low-molecular-weight form was then excreted from the cells, meaning that much of the targeted ^{131}I-hLL2 did not result in an iodine decay event at the targeted B cells (*11*). To overcome this deficiency of a "nonresidualizing" radioiodinated mAb, we have developed new methods for preparing iodinated rapidly internalizing mAbs, and their use has resulted in sustained residualization of targeted radioiodine at tumor cells. The therapeutic advantages to these agents over standard iodination methodologies have been demonstrated for an internalizing mAb (*12*).

Unlike internalizing mAb conjugates prepared using standard methods of iodination, ^{90}Y radiolabeling results in mAb radioimmunotherapy agents in which ^{90}Y is retained to a much greater extent inside cells even after the antibody has been catabolized (*13*). Added to this observation were other desirable properties of ^{90}Y versus ^{131}I. Most notably, the lack of γ-emissions with the former nuclide suggested a greater likelihood, in both the United States and Europe, that a procedure can be performed on an outpatient basis. In addition, the β-particle from ^{90}Y has a much deeper tissue penetration than that from ^{131}I, because of the much higher average energy of the respective β-particle emissions (*14*). It seemed likely to us that such a property could only prove even more favorable toward ^{90}Y when one considered clinical situations involving patients with sites of disease sized in the 0.5- to 5-cm range, as is most often seen in lymphoma patients. What remained was to decide on the most appropriate method of coupling ^{90}Y to the hLL2 mAb, bearing in mind that one of the most negative aspects of ^{90}Y is its bone-seeking, and therefore toxic, potential when present in vivo as a free radionuclide (*15*).

Several methods have been described for the coupling of yttrium to proteins using bifunctional chelating agents (Fig. 1). Early work successfully enabled coupling of yttrium to antibodies using the diethylenetriaminepentaacetic acid

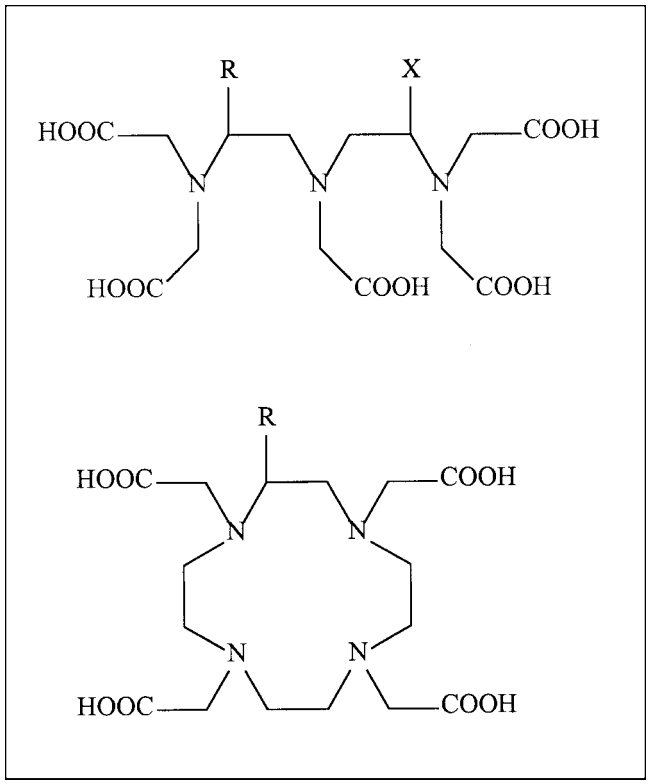

FIGURE 1. Chemical structures of chelates discussed in article (top: DTPA type; bottom: DOTA type). When R and X both are hydrogen, either chelate is coupled to mAbs through one of their pendant carboxylic acid groups, leading to unstable yttrium complexes in the case of DTPA-type chelates but stable yttrium complexes in the case of DOTA-type chelates. When R is a backbone-substituted linking group, typically a benzyl isothiocyanate, all carboxyl groups remain free for yttrium binding after mAb conjugation, in turn leading to stable complexes for both chelate types, albeit more stable complexes for DOTA type. X is typically hydrogen (Bz-DTPA) or methyl (Mx-DTPA), with yttrium complexes displaying similar stability profiles when used as hLL2 mAb conjugates.

(DTPA) chelate (*16*), but it quickly became evident that these agents were not stable enough for in vivo application. Use of backbone-modified DTPA analogs, wherein all 5 carboxyl groups remained free for metal binding after conjugation of a separate functional group to antibody, dramatically improved the in vivo stability profiles of yttrium-labeled antibodies (*17,18*). Separately, cyclic chelators were investigated for yttrium binding (*19,20*), with the 12-membered ring macrocycle 1,4,7,10-tetraazacyclododecane-N,N′,N″,N‴-tetraacetic acid (DOTA) found most suitable. Initially, DOTA agents were designed such that a separate functional group appended to the cyclic backbone was used for antibody coupling, leaving all 4 DOTA carboxyl groups free for binding, in a manner that paralleled development of the backbone-modified DTPA derivatives.

Backbone-substituted DOTA and DTPA agents are difficult to make, with expensive, multistep organic syntheses required. We previously described the agent, DOTA-hLL2

(*21*), which is not attached through backbone substitution but uses one of the DOTA carboxyl groups as a mAb attachment site. Such DOTA-mAb conjugates, perhaps surprisingly, show a good yttrium complex stability profile, especially when contrasted with the initially made carboxyl-linked DTPA species described above. In the current work, we have compared hLL2 labeled with yttrium using DOTA, coupled through a carboxyl moiety, and hLL2 labeled using the popular open-ring, backbone-substituted DTPA analogs Bz-DTPA and Mx-DTPA. We also describe the pertinent properties of ^{90}Y-DOTA-hLL2, the agent we have selected for further clinical trials.

MATERIALS AND METHODS

hLL2 was manufactured at Immunomedics, Inc., using an amplified cell line grown in serum-free culture, as previously described (*22*). The rat anti-idiotypic antibody to hLL2, WN, has been described previously (*23*) and was also prepared at Immunomedics. The macrocyclic chelate, DOTA, was obtained from Macrocyclics, Inc. (Dallas, TX). The bifunctional chelate 2-(4-isothiocyanatobenzyl)DTPA (designated Bz-DTPA) was synthesized following a published method (*24*). The amino group–reactive bifunctional chelate 2-(4-isothiocyanatobenzyl)-6-methyl-DTPA (designated Mx-DTPA) was a kind gift of Dr. Martin Brechbiel of the National Cancer Institute. DOTA-hLL2 was prepared as described previously (*21*) and contained an average of 2.8 DOTA molecules per hLL2 mAb, as determined by an ^{111}In-binding assay (*24*). Both Bz-DTPA-hLL2 and Mx-DTPA-hLL2 conjugates were produced using the published procedure (*25*) and contained, respectively, an average substitution ratio of 1.1 Bz-DTPA and 1.4 Mx-DTPA per unit of hLL2, by the ^{111}In-binding assay. ^{90}Y, as yttrium chloride, in 0.05N hydrochloric acid was purchased from Perkin-Elmer Life Sciences (Boston, MA). ^{88}Y, as the chloride salt in 0.1 mol/L hydrochloric acid, was purchased from Los Alamos National Laboratory (Los Alamos, NM). DTPA was obtained from Sigma Chemical (St. Louis, MO), human serum albumin (HSA) was purchased from Alpine Biologics (Blauvelt, NY), and fresh human serum was obtained from healthy volunteers.

Radiolabeling

For in vitro stability studies, portions of ^{90}Y-chloride (34.1–35.3 MBq), buffered with 0.25 mol/L ammonium acetate, pH 5.4, were mixed with 300 μg of each of the conjugates—hLL2-DOTA, hLL2-Mx-DTPA, and hLL2-Bz-DTPA. The DOTA conjugate was labeled at 45°C for 0.5 h and quenched with 10 mmol/L DTPA (final concentration) for 10 min at the same temperature. The labeling and the quenching durations were the same for the DTPA conjugates, but the labeling was performed at room temperature and the quenching was with 10 mmol/L ethylenediaminetetraacetic acid (EDTA). The incorporation of ^{90}Y into conjugates was measured by size-exclusion high-performance liquid chromatography (SE-HPLC) (SEC-250 column [Bio-Rad Laboratories Inc., Hercules, CA] run at 1 mL/min in 0.2 mol/L phosphate buffer, pH 6.9). The incorporation was 77.7% for DOTA-hLL2 conjugate, 88.8% for Bz-DTPA-hLL2, and 79.4% for Mx-DTPA-hLL2. Each of these materials was purified 2 successive times on 3-mL spin columns of Sephadex G50/80 (Sigma Chemical) equilibrated in 0.1 mol/L sodium acetate, pH 6.5. Analyses of the purified preparations by SE-HPLC did not show the presence of low-molecular-weight moieties; the aggregate contents were 3.4% for DOTA-hLL2, 8.7% for Bz-DTPA-hLL2, and negligible for Mx-DTPA-hLL2.

^{88}Y labeling for animal studies was performed as follows. ^{88}Y-chloride was buffered with 6 times its volume of 0.5 mol/L ammonium acetate, pH 5.4. DOTA-hLL2 (0.93 mg) was mixed with 2.89 MBq of ^{88}Y acetate, heated at 45°C for 1 h, and quenched with 10 mmol/L DTPA (final concentration) for 0.25 h at the same temperature. Instant thin-layer chromatography analysis showed an incorporation of 89%. The labeling mixture was made 1% in HSA and was purified 2 successive times on 1-mL spin columns of Sephadex G50/80 equilibrated in 0.1 mol/L sodium acetate, pH 6.5. The procedure for Mx-DTPA-hLL2 was essentially the same, except that the radiolabeling was performed at room temperature, and the quenching was done using a final concentration of 10 mmol/L EDTA. The incorporation of ^{88}Y into Mx-DTPA-hLL2 was 96%. Purified materials contained 5% aggregate (DOTA-hLL2) or 1% aggregate (Mx-DTPA-hLL2) and no low-molecular-weight entities by SE-HPLC analysis. Instant thin-layer chromatography showed that 100% of the ^{88}Y radioactivity was associated with the conjugate in each case. Also, in each case, mixing with a 40-fold molar excess of anti-idiotypic MAb, WN, resulted in a practically complete shift (>98%) of the SE-HPLC peak position to that near the void volume, corresponding to the higher molecular weight of ^{88}Y-hLL2-WN complex. A diluted solution for in vivo work was prepared in 50 mmol/L sodium acetate and 150 mmol/L sodium chloride, pH 6.5, containing 1% HSA and 1 mmol/L EDTA. In the case of ^{88}Y-DOTA-hLL2, the injection volume of 0.1 mL contained ~0.037 MBq of activity, at a specific activity of 2.79 MBq/mg, and an antibody dose of 13.2 μg. The injection volume of 0.1 mL of ^{88}Y-Mx-DTPA-hLL2 contained ~0.037 MBq of activity, at a specific activity of 3.00 MBq/mg, and an antibody dose of 12.3 μg.

In Vitro Stability

The specific activities of purified radiolabeled preparations used for in vitro stability assays were as follows: 78.07 MBq/mg for ^{90}Y-DOTA-hLL2, 76.22 MBq/mg for ^{90}Y-Mx-DTPA-hLL2, and 88.06 MBq/mg for ^{90}Y-Bz-DTPA-hLL2 conjugates. Purified radiolabeled samples were added to fresh human serum (with sodium azide added to 0.02%) at a final concentration of the radiolabeled conjugate in the range of 110–116 nmol/L. For the DTPA/HSA challenge experiment, samples were added to a solution of 1% HSA in saline, containing 0.02% azide and DTPA such that the molar excess of DTPA to radiolabeled conjugate was maintained at 9,434 to 1 (DOTA-hLL2), 8,857 to 1 (Bz-DTPA-hLL2), or 7,168 to 1 (Mx-DTPA-hLL2). The samples were then incubated at 37°C and were analyzed periodically over 10 d (single measurements). Radioanalyses consisted of incubating an aliquot of serum sample with EDTA (10 mmol/L final concentration) to chelate any loosely bound ^{90}Y and then mixing with an 80-fold molar excess of anti-idiotypic mAb to hLL2, WN. This secondary complex formation of the radiolabeled hLL2 species resulted in SE-HPLC elution profiles with normal serum protein regions (HSA, transferrin, etc.; appearing later than 10 min after injection under the SE-HPLC operating conditions) separated completely from any peak composed of an immunoreactive hLL2 component. The total percentage of ^{90}Y not associated with hLL2 was thus computed readily from ^{90}Y peak areas at retention times greater than 10 min (composed of ^{90}Y associated with serum proteins, or present as

free ^{90}Y and analyzed as ^{90}Y-EDTA), in relation to ^{90}Y eluting at less than 10 min as the ^{90}Y-hLL2-WN immune complex.

In Vivo Experiments

Animal studies described in this article were performed under an approved Institutional Animal Care and Use Committee protocol using procedures already described in detail (*18*,*21*). Briefly, female NCr athymic nude mice and normal BALB/c mice at 3–4 wk of age were obtained from Taconic (Germantown, NY) and were quarantined for at least 1 wk before use. For the tumor xenograft model, 5×10^6 cells of the human B-cell lymphoma cell line, Ramos, were injected into athymic mice, with xenografts being present about 2 wk later. hLL2 mAb labeled with 37–74 kBq of the ^{88}Y-hLL2 conjugates was injected intravenously into both athymic and normal animals, with necropsies performed up to 10 d after injection of the reagents. Blood, liver, spleen, kidney, lungs, stomach, small and large intestines, and bone (femurs), along with xenografts, if present, were excised, weighed, and counted for ^{88}Y activity by γ-scintillation. In addition, 1 femur from each animal was washed clean of marrow with 0.9% sodium chloride solution to obtain a sample of cortical bone for counting. All tissues were counted in a well-type scintillation counter against appropriate standards using energy windows set for ^{88}Y. Dosimetry calculations were performed using previously described methods (*26*,*27*), and total radiation absorbed doses were calculated with corrections for organ mass and for self-organ and cross-organ beta doses.

RESULTS

Figure 2 shows the results of incubating ^{90}Y-Bz-DTPA-hLL2, ^{90}Y-Mx-DTPA-hLL2, and ^{90}Y-DOTA-hLL2 in 1% HSA against a challenge of a 10,000-fold molar excess of free DTPA. The ^{90}Y-DOTA-hLL2 chelate withstands the challenge of the massive excess of the free DTPA, essentially unchanged over the 9-d course of the experiment, whereas the ^{90}Y-Bz-DTPA-hLL2 and ^{90}Y-Mx-DTPA-hLL2 chelates lose around 4% of initially bound ^{90}Y over the first 40 h of the incubation and between 7% and 14% of initially bound ^{90}Y at later times, between 5 and 9 d, respectively, after the initial challenge. Interestingly, for these hLL2 conjugates, the ^{90}Y-Bz-DTPA-hLL2 conjugate looked as stable as, if not a little more stable than, the later-generation ^{90}Y-Mx-DTPA-hLL2 conjugate.

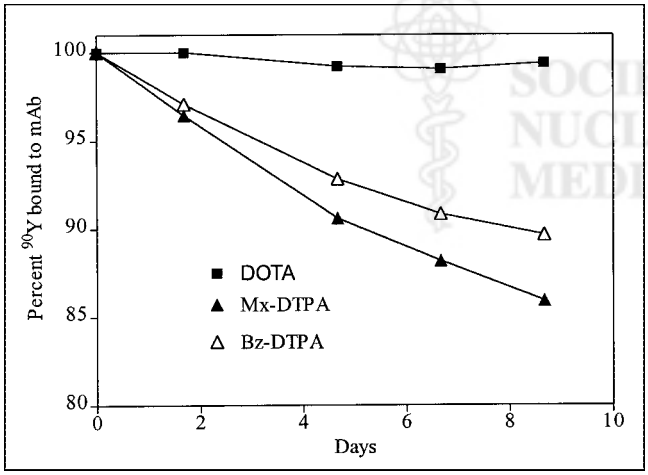

FIGURE 2. Comparative in vitro stabilities of ^{90}Y-DOTA-, ^{90}Y-Mx-DTPA-, and ^{90}Y-Bz-DTPA-hLL2 conjugates in presence of excess of free DTPA chelate in 1% HSA over nearly 9 d at 37°C. Compressed *y*-axis covers range only from 80% to 100% of initially bound ^{90}Y remaining bound to hLL2 mAb.

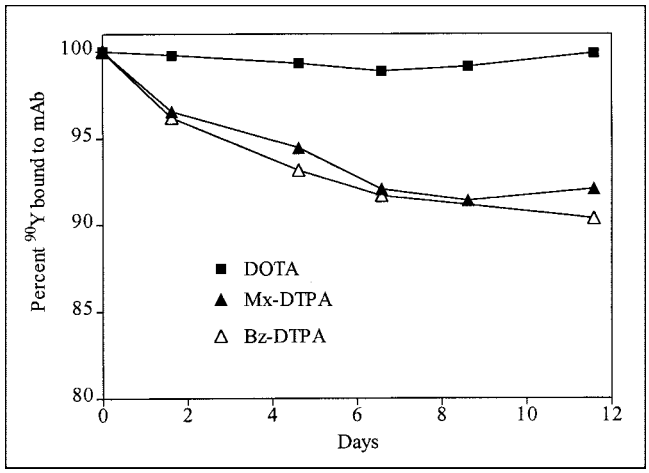

FIGURE 3. Comparative in vitro stabilities of 100 nmol/L amounts of ^{90}Y-DOTA-, ^{90}Y-Mx-DTPA-, and ^{90}Y-Bz-DTPA-hLL2 conjugates in normal human serum over nearly 12 d at 37°C. Compressed *y*-axis covers range only from 80% to 100% of initially bound ^{90}Y remaining bound to hLL2 mAb.

The in vitro stability data for the ^{90}Y-DOTA-hLL2, ^{90}Y-Bz-DTPA-hLL2, and ^{90}Y-Mx-DTPA-hLL2 conjugates, when tested in normal human serum, are shown in Figure 3. A similar pattern emerges, with the ^{90}Y-DOTA-hLL2 essentially inert to the serum challenge whereas the ^{90}Y-Bz-DTPA-hLL2 and ^{90}Y-MxDTPA-hLL2 conjugates progressively shed ^{90}Y over time. Again, for each DTPA chelate about 4% of the initially bound activity is lost over the first 40 h, and between 6% and 9% of initially bound radioactivity is lost between 5 and 10 d after the initial incubation. Similarly to the DTPA challenge, the data showed little difference in ^{90}Y stability between either variation of the 2 DTPA conjugates of the hLL2 mAb.

Figure 4 shows the comparative data for ^{88}Y-DOTA-hLL2 and ^{88}Y-Bz-DTPA-hLL2 in nude mice bearing Ramos lymphoma. Although both agents perform well in this animal xenograft model, with tumor xenograft uptakes and blood clearance rates being similar, it is evident that uptake in bone for ^{88}Y-DOTA-hLL2 is lower than that for ^{88}Y-Bz-DTPA-hLL2. The bone uptake difference is highlighted in the bottom trace, with bone and washed bone uptakes initially being similar at 24 h but uptake increasing for ^{88}Y-Bz-DTPA-hLL2 and decreasing for ^{88}Y-DOTA-hLL2 over the 10 d of the experiment. In a separate in vivo experiment, ^{88}Y-DOTA-hLL2 was compared with ^{88}Y-Mx-DTPA-hLL2, a conjugate prepared using a later-generation DTPA-type chelate (Fig. 1, R = methyl). This experiment was performed on normal mice to remove the possibility that variable tumor uptake might confound the primary data of interest related to bone uptake. Use of ^{88}Y-Mx-DTPA-

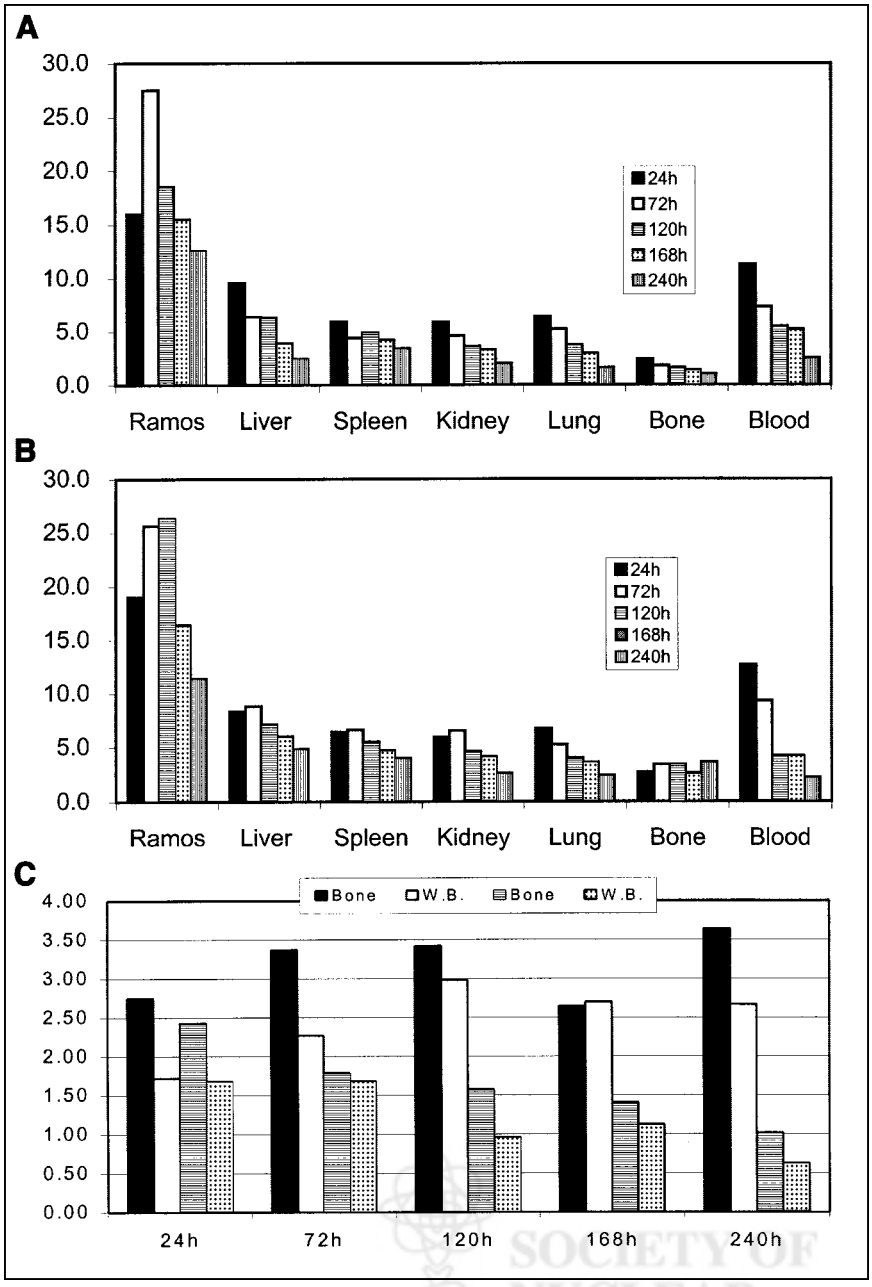

FIGURE 4. Biodistribution of ^{88}Y-DOTA-hLL2 (A) and ^{88}Y-Bz-DTPA-hLL2 (B), in nude mice bearing Ramos tumors, until 240 h after injection. (C) Uptake of ^{88}Y-Bz-DTPA-hLL2 in bone (first column) and washed bone (second column) versus uptake of ^{88}Y-DOTA-hLL2 over same period in bone (third column) and washed bone (fourth column). Data are in terms of %ID/g of tissue. W.B. = washed bone.

hLL2, compared with use of ^{88}Y-DOTA-hLL2 (Table 1), resulted in much higher bone and washed-bone yttrium uptake in the mice from 72 h after injection of the reagents, with 2- to 4-fold less radiolabeled yttrium in these tissues when the macrocyclic chelate was used.

Statistically (2-tailed test), bone uptake ($P = 0.0218$), but not washed-bone uptake ($P = 0.3867$), differed significantly between the DOTA and Mx-DTPA groups of animals as soon as 24 h after injection. Yttrium uptake differences for both bone ($P = 0.0002$) and washed bone ($P = 0.0005$) became highly significant by 72 h after injection, and these differences remained throughout the 240 h of the experiment for both tissues ($P \leq 0.001$).

Dosimetric calculations were performed using the dataset in Table 1, and these calculations are reported in Table 2. Radiation absorbed doses (cGy/37 MBq administered) were broadly similar in most tissues for either chelate conjugate, although both liver and lungs received higher doses, 8,098 versus 6,988 cGy and 8,454 versus 7,175 cGy, respectively, from the ^{90}Y-Mx-DTPA-hLL2 conjugate than from the ^{90}Y-DOTA-hLL2 conjugate. Of most significance were the cGy/37 MBq absorbed doses for bone, rising from 3,555 for ^{90}Y-DOTA-hLL2 to 5,405 for ^{90}Y-Mx-DTPA-hLL2, and for washed bone, rising from 2,664 for ^{90}Y-DOTA-hLL2 to 4,524 for ^{90}Y-Mx-DTPA-hLL2. These findings indicate 52.0% and 69.8% higher absorbed doses to bone and

TABLE 1
Comparative Biodistributions of ^{88}Y-DOTA-hLL2 and ^{88}Y-Mx-DTPA-hLL2 for All Major Organs in Normal Female BALB/c Mice Until 10 Days After Injection of Conjugates

Tissue	Label	%ID/g of tissue ± SD (n = 5)				
		24 h	72 h	120 h	168 h	240 h
Liver	^{88}Y-DOTA	7.25 ± 0.53	6.32 ± 0.31	5.62 ± 0.19	4.93 ± 0.34	4.27 ± 0.43
	^{88}Y-Mx-DTPA	7.92 ± 0.99	7.71 ± 0.90	6.40 ± 0.45	6.44 ± 0.36	4.82 ± 0.30
Spleen	^{88}Y-DOTA	6.30 ± 0.31	7.56 ± 1.86	7.15 ± 0.57	8.68 ± 0.49	6.15 ± 2.33
	^{88}Y-Mx-DTPA	7.69 ± 1.33	6.80 ± 1.02	6.56 ± 1.06	7.64 ± 1.21	7.62 ± 0.65
Kidney	^{88}Y-DOTA	5.55 ± 1.06	4.18 ± 0.40	4.46 ± 1.02	4.76 ± 0.08	3.48 ± 0.17
	^{88}Y-Mx-DTPA	5.38 ± 1.28	5.93 ± 0.70	5.67 ± 1.36	5.16 ± 0.30	4.53 ± 0.31
Lung	^{88}Y-DOTA	5.98 ± 1.16	5.10 ± 0.71	5.27 ± 0.66	5.02 ± 0.68	4.27 ± 1.09
	^{88}Y-Mx-DTPA	7.25 ± 1.96	6.47 ± 0.61	5.54 ± 0.84	6.15 ± 0.65	5.74 ± 0.48
Blood	^{88}Y-DOTA	24.41 ± 1.7	20.86 ± 2.1	17.26 ± 0.5	14.30 ± 0.5	13.28 ± 2.1
	^{88}Y-Mx-DTPA	24.33 ± 1.6	20.72 ± 1.5	15.90 ± 0.7	14.12 ± 0.7	10.56 ± 0.6
Stomach	^{88}Y-DOTA	1.26 ± 0.51	1.04 ± 0.29	1.51 ± 0.11	1.31 ± 0.18	1.16 ± 0.37
	^{88}Y-Mx-DTPA	1.78 ± 0.26	1.36 ± 0.40	1.59 ± 0.11	0.90 ± 0.23	1.01 ± 0.08
Small intestine	^{88}Y-DOTA	2.30 ± 0.24	2.08 ± 0.28	2.32 ± 0.18	1.97 ± 0.14	1.66 ± 0.17
	^{88}Y-Mx-DTPA	2.82 ± 0.35	2.55 ± 0.09	2.53 ± 0.17	1.88 ± 0.09	1.65 ± 0.11
Large intestine	^{88}Y-DOTA	1.59 ± 0.19	1.67 ± 0.17	1.69 ± 0.32	1.22 ± 0.11	0.89 ± 0.24
	^{88}Y-Mx-DTPA	1.78 ± 0.18	2.05 ± 0.51	1.56 ± 0.07	1.32 ± 0.15	1.04 ± 0.11
Bone	^{88}Y-DOTA	3.23 ± 0.46	3.12 ± 0.63	3.08 ± 0.46	2.95 ± 0.40	2.63 ± 0.38
	^{88}Y-Mx-DTPA	4.41 ± 0.81	5.68 ± 0.60	6.12 ± 0.78	8.43 ± 1.94	6.82 ± 0.57
Washed bone	^{88}Y-DOTA	2.22 ± 0.90	1.64 ± 0.52	1.58 ± 0.25	1.72 ± 0.22	1.36 ± 0.33
	^{88}Y-Mx-DTPA	2.64 ± 0.50	4.69 ± 1.10	5.26 ± 0.60	7.75 ± 1.85	5.30 ± 0.84

washed bone, respectively, when the yttrium chelating agent was changed from macrocyclic to open chain.

DISCUSSION

Several groups have examined the relative stabilities of cyclic and acyclic chelating agents used for yttrium, including various DOTA and DTPA analogs, respectively. Harrison et al. (*20*) described the dramatic superiority of ^{90}Y-mAb conjugates made using backbone-substituted DOTAs and DTPAs over the earlier-generation mAb conjugates made with cyclic anhydride DTPA. They also noted the smaller apparent superiority (~0%–30% lower bone uptake over the first 120 h after injection, with an increasing difference in favor of DOTA conjugates over the next 80 h) when using the same ^{90}Y-mAb prepared with backbone-substituted DOTA versus backbone-substituted DTPA. In their work, the backbone substituents used as the mAb conjugation site were maleimidoalkyl groups alpha to a DOTA nitrogen or alpha to an *N*-terminal iminodiacetate subunit of the DTPA. More intensively investigated series of backbone-substituted derivatives have used *para*-benzyl derivatives attached in the same relative positions as the maleimidoalkyl groups reported by Harrison et al. Thus, 2-benzyl-DTPA derivatives (e.g., Bz-DTPA, Mx-DTPA, and cyclohexyl [CHX]-DTPA) and 2-acetamidobenzyl-DOTA derivatives (e.g., 2-IT-BAD, where IT is iminothiolane and BAD is 2-[*p*-(bromoacetamido)-benzyl]-DOTA) have been independently prepared and more lately compared for yttrium-binding properties. Both series require complex multistep organic syntheses to generate bifunctional chelates for mAb conjugation. The availability of appropriate starting materials together with the practical ability to follow reaction intermediates during synthetic processing probably account for much of the popularity of benzyl-substituted derivatives.

TABLE 2
Dosimetry Calculations for Dataset in Table 1

Tissue	^{90}Y-DOTA-hLL2	^{90}Y-Mx-DTPA-hLL2
Liver	6,988	8,089
Spleen	5,283	5,567
Kidney	7,494	8,498
Lung	7,175	8,454
Stomach	3,272	3,651
Small intestine	3,837	4,392
Large intestine	1,851	2,069
Bone	3,555	5,405
Washed bone	2,664	4,524

Data are doses, in cGy/37 MBq of ^{99}Y-hLL2.

Camera et al. (*28*) compared biodistribution data for ^{88}Y-labeled B3 mAb when yttrium was conjugated through an Mx-DTPA or a 2-Bz-DOTA chelate. They found larger differences in bone uptake of radiolabeled yttrium than those reported previously by Harrison et al. (*20*), with

approximately 0.5- to 5-fold more yttrium in the bones of animals receiving the Mx-DTPA-mAb conjugates, and with these differences increasing consistently between 24 and 168 h after injection of the agents. From these data, an area-under-the-curve calculation for the femurs of the animals indicated values (percentage injected dose [%ID] × h/g) of 391 and 289 for the Mx-DTPA and the 2-Bz-DOTA conjugates, respectively, or a 35% increase in area under the curve (and, by implication, radiation dose) if one were substituting a 2-Bz-DOTA chelate for an Mx-DTPA chelate. DeNardo et al. (*29*) compared ^{90}Y-Mx-DTPA- and ^{90}Y-BAD-DOTA-BrE-3 mAb, but did so in terms of comparative toxicity, giving groups of mice increasing doses of mAb labeled with ^{90}Y and using each chelate to determine maximum tolerated dose differences. For the ^{90}Y-Mx-DTPA-mAb conjugate they found a median lethal dose of 8.2 MBq, whereas for the ^{90}Y-BAD-DOTA-mAb a median lethal dose of 11.4 MBq was determined, meaning that 39% more ^{90}Y could be tolerated when the BAD-DOTA chelate was used in lieu of an Mx-DTPA chelate.

Our data suggest an even larger difference between the 2 chelation methods, from 52% to almost 70% higher bone absorbed doses resulting from bone-deposited ^{90}Y with the Mx-DTPA conjugate instead of the corresponding DOTA conjugate. These dose estimates were derived using appropriate correction factors for tissue self-absorbed doses and tissue crossover doses. The nearly 70% increase in ^{90}Y uptake with regard to cortical bone might be of particular importance because, as DeNardo et al. (*29*) cogently point out from their own work, differences in toxicity found in mice are likely to be magnified in humans because of the size difference and the negligible amount of marrow dose to be derived from whole-body ^{90}Y in humans compared with mice, in which most of the bone dose actually comes from whole-body ^{90}Y decay.

An additional important issue is that since ^{90}Y is a pure β-emitter, regulatory authorities are likely to insist that it be traced with an alternate nuclide to ensure that normal tissues receive acceptable doses. The 2 nuclides most suitable are ^{86}Y and ^{111}In, the former being a positron emitter and the latter a γ-emitting nuclide. ^{86}Y produces high-energy emissions, is short lived at 14.7 h, and is difficult to produce and obtain on a widespread basis, whereas ^{111}In is readily available. ^{86}Y would be expected to predict ^{90}Y-mAb biodistribution accurately when either a DOTA- or an Mx-DTPA-type chelate is used. Further, the easily obtainable ^{111}In nuclide is not fully accurate in predicting ^{90}Y-mAb conjugate distribution when open-chain DTPA-type chelates are used for preparing conjugates, as has been shown in several studies using different DTPA-type chelates and different mAbs (*30*,*31*). Of particular note are the patient studies by Pai-Scherf et al. (*32*), using an Mx-DTPA chelate-mAb conjugate. They took bone marrow biopsies for quantitation of both ^{111}In and ^{90}Y uptake and reported 0.0038 %ID/g and 0.0046 %ID/g, respectively, for each nuclide. However, the site of highest excess uptake of ^{90}Y over ^{111}In, as shown by the preclinical data presented in the current article, is in cortical bone rather than in bone marrow, and the differences reported by Pai-Scherf might not be fully descriptive of the absolute dose underestimation to be expected from using ^{111}In as a ^{90}Y-surrogate with Mx-DTPA chelate-mAb conjugates.

For Mx-DTPA-mAbs, these considerations present one with a dilemma as to whether to rely on ^{86}Y for true accuracy in predicting ^{90}Y-mAb biodistribution or to use ^{111}In knowing that inaccurate estimations could result. It is clear from this work, and from previous work (*21*), that the carboxyl-linked form of DOTA-hLL2 can bind ^{111}In and ^{90}Y (or ^{88}Y) with equal strength and a maximum resistance to in vivo challenges, as demonstrated by equivalent and low bone uptake of indium/yttrium nuclides. Such findings should allow future use of either ^{86}Y or ^{111}In, through PET or SPECT, respectively, for dose estimations with an expectation of equivalent accuracy, in a similar manner to backbone-substituted DOTA analogs (*33*). The in vivo performance results of our study with DOTA-hLL2 are in every sense equivalent to the best data seen with 2-IT-BAD-mAb conjugates, with the added practical advantage that the carboxyl-linked DOTA-hLL2 used here is produced without the need for a complicated synthesis of a backbone-substituted DOTA.

It would be interesting to directly compare backbone-substituted and carboxyl-substituted DOTA-mAb conjugates on the same antibody, since these 2 groups of DOTA-mAb conjugates have both been proven to be completely stable to ^{90}Y dissociation in vivo. Backbone-substituted DOTA-mAbs that have been tested in patients have recently been reported to display rather high liver uptake (*34*), and it remains to be seen whether carboxyl-substituted DOTA-mAbs will show that same property and to the same extent. One might then speculate on how additional improvements might be made to ^{90}Y radioimmunotherapy. Future attempts to further mitigate the absolute uptake of ^{90}Y in bone after administration of ^{90}Y-Bz- (or Mx- or CHX-) mAb conjugates are likely to involve concomitant or early administration of an excess of free chelate, such as EDTA or DTPA (*35*), but such an approach would likely not be useful given the high-stability profiles of the ^{90}Y-DOTA conjugates. One approach for DOTA-mAb conjugates is to vary the chemical linkage between chelate and mAb, and studies on backbone-substituted DOTA-mAb conjugates prepared with cleavable linkers spaced between the 2-IT-BAD and the mAb to which it is attached have been reported (*36*,*37*). However, a more substantive improvement in achievable efficacy will probably require the adoption of some sort of pretargeting protocol (*38*–*40*).

CONCLUSION

The DOTA-hLL2 conjugate that we have described is easy to prepare and to radiolabel with ^{90}Y at high stability. When the DOTA chelate is used to attach ^{90}Y to hLL2, the

radioimmunoconjugate shows a significantly improved stability profile compared with that of ^{90}Y-hLL2 conjugates prepared with bifunctional DTPA-type chelates. This improvement not only will ensure optimal ^{90}Y-conjugate tissue localization and clearance properties but also should minimize toxicity while providing confidence about the accurate prediction of ^{90}Y doses from imaging studies using ^{111}In- or ^{86}Y-radiolabeled hLL2.

REFERENCES

1. Goldenberg DM. The role of radiolabeled antibodies in the treatment of non-Hodgkin's lymphoma: the coming of age of radioimmunotherapy. *Crit Rev Oncol Hematol.* 2001;39:195–201.
2. Wiseman GA, White CA, Sparks RB, et al. Biodistribution and dosimetry results from a phase III prospectively randomized controlled trial of Zevalin radioimmunotherapy for low-grade, follicular, or transformed B-cell non-Hodgkin's lymphoma. *Crit Rev Oncol Hematol.* 2001;39:181–194.
3. Kaminski MS, Estes J, Zasadny KR, et al. Radioimmunotherapy with iodine ^{131}I tositumomab for relapsed or refractory B-cell non-Hodgkin's lymphoma: updated results and long-term follow-up of the University of Michigan experience. *Blood.* 2000;96:1259–1266.
4. Stein R, Belisle E, Hansen HJ, Goldenberg DM. Epitope specificity of the anti-B cell lymphoma monoclonal antibody, LL2. *Cancer Immunol Immunother.* 1993; 37:293–298.
5. Leung SO, Goldenberg DM, Dion AS, et al. Construction and characterization of a humanized, internalizing, B-cell (CD22)-specific, leukemia/lymphoma antibody, LL2. *Mol Immunol.* 1995;32:1413–1427.
6. Juweid M, Sharkey RM, Markowitz A, et al. Treatment of non-Hodgkin's lymphoma with radiolabeled murine, chimeric, or humanized LL2, an anti-CD22 monoclonal antibody. *Cancer Res.* 1995;55(suppl):5899s–5907s.
7. Juweid ME, Stadtmauer E, Hajjar G, et al. Pharmacokinetics, dosimetry, and initial therapeutic results with ^{131}I- and ^{111}In-/^{90}Y-labeled humanized LL2 anti-CD22 monoclonal antibody in patients with relapsed, refractory non-Hodgkin's lymphoma. *Cancer Res.* 1999;5(suppl):3292s–3303s.
8. Leonard JP, Coleman M, Chadburn A, et al. Epratuzumab (hLL2, anti-CD22 humanized monoclonal antibody) is an active and well-tolerated therapy for refractory/relapsed diffuse large B-cell non-Hodgkin's lymphoma (NHL) [abstract]. *Blood.* 2000;96(suppl 1):578a.
9. Leonard JP, Coleman M, Matthews JC, et al. Combination monoclonal antibody therapy for lymphoma: treatment with epratuzumab (anti-CD22) and rituximab (anti-CD20) is well tolerated [abstract]. *Blood.* 2001;98:844a.
10. Murthy S, Sharkey RM, Goldenberg DM, et al. Lymphoma imaging with a new technetium-99m labelled antibody, LL2. *Eur J Nucl Med.* 1992;19:394–401.
11. Mattes MJ, Shih LB, Govindan SV, et al. The advantage of residualizing radiolabels for targeting B-cell lymphomas with a radiolabeled anti-CD22 monoclonal antibody. *Int J Cancer.* 1997;71:429–435.
12. Stein R, Govindan SV, Chen S, Reed L, Spiegelman H, Griffiths GL. Successful therapy of a human lung cancer xenograft using MAb RS7 labeled with residualizing radioiodine. *Crit Rev Oncol Hematol.* 2001;39:173–180.
13. Stein R, Govindan SV, Chen S, et al. Radioimmunotherapy of a human lung cancer xenograft with monoclonal antibody RS7: evaluation of ^{177}Lu and comparison of its efficacy with that of ^{90}Y and residualizing ^{131}I. *J Nucl Med.* 2001;42:967–974.
14. Wessels BW, Rogus RD. Radionuclide selection and model absorbed dose calculations for radiolabeled tumor associated antibodies. *Med Phys.* 1984;11: 638–645.
15. Watanabe N, Oriuchi N, Tanada S, et al. Effect of edetate calcium disodium on yttrium-90 activity in bone of mice. *Ann Nucl Med.* 1999;13:397–400.
16. Hnatowich DJ, Childs RL, Lanteigne D, Najafi A. The preparation of DTPA-coupled antibodies radiolabeled with metallic radionuclides: an improved method. *J Immunol Methods.* 1983;65:147–157.
17. Kozak RW, Raubitschek A, Mirzadeh S, et al. Nature of the bifunctional chelating agent used for radioimmunotherapy with yttrium-90 monoclonal antibodies: critical factors in determining in vivo survival and organ toxicity. *Cancer Res.* 1989;49:2639–2644.
18. Sharkey RM, Motta-Hennessy C, Gansow OA, et al. Selection of a DTPA chelate conjugate for monoclonal antibody targeting to a human colonic tumor in nude mice. *Int J Cancer.* 1990;46:79–85.
19. Li M, Meares CF, Zhong GR, Miers L, Xiong CY, DeNardo SJ. Labeling monoclonal antibodies with ^{90}yttrium- and ^{111}indium-DOTA chelates: a simple and efficient method. *Bioconjug Chem.* 1994;5:101–104.
20. Harrison A, Walker CA, Parker D, et al. The in vivo release of ^{90}Y from cyclic and acyclic ligand-antibody complexes. *Int J Rad Appl Instrum B.* 1991;5:469–476.
21. Govindan SV, Shih LB, Goldenberg DM, et al. ^{90}Yttrium-labeled complementary-determining-region-grafted monoclonal antibodies for radioimmunotherapy: radiolabeling and animal biodistribution studies. *Bioconjug Chem.* 1998;9:773–782.
22. Losman MJ, Hansen HJ, Dworak H, et al. Generation of a high-producing clone of a humanized anti-B-cell lymphoma monoclonal antibody (hLL2). *Cancer.* 1997;80(suppl):2660–2666.
23. Losman MJ, Leung SO, Shih LB, et al. Development and evaluation of the specificity of a rat monoclonal anti-idiotype antibody, WN, to an anti-B-cell lymphoma monoclonal antibody, LL2. *Cancer Res.* 1995;55:5987s–5982s.
24. Cummins CH, Rutter EW, Fordyce WA. A convenient synthesis of bifunctional chelating agents based on diethylenetriaminepentaacetic acid and their coordination chemistry. *Bioconjug Chem.* 1991;2:180–186.
25. Meares CF, McCall MJ, Reardan DT, Goodwin DA, Diamanti CI, McTigue M. Conjugation of antibodies with bifunctional chelating agents: isothiocyanate and bromoacetamido reagents, methods of analysis, and subsequent addition of metal ions. *Anal Biochem.* 1984;142:68–78.
26. Hui TE, Fisher DR, Kuhn JA, et al. A mouse model for calculating cross-organ beta doses from ^{90}Y-labeled immunoconjugates. *Cancer.* 1994;73(suppl 3):951–957.
27. Beatty BB, Kuhn JA, Hui TE, Fisher DR, Williams LE, Beatty JD. Application of the cross-organ beta dose method for tissue dosimetry in tumor-bearing mice treated with a ^{90}Y-labeled immunoconjugate. *Cancer.* 1994;73(suppl 3):958–965.
28. Camera L, Kinuya S, Garmestani K, et al. Comparative biodistribution of indium- and yttrium-labeled B3 monoclonal antibody conjugated to either 2(p-SCN-Bz)-6-methyl-DTPA (1B4M-DTPA) or 2-(p-SCN-Bz)-1, 4, 7, 10-tetraazacyclododecane tetraacetic acid (2B-DOTA). *Eur J Nucl Med.* 1994;21:640–646.
29. 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.
30. Lövquist A, Humm JL, Sheikh A, et al. PET imaging of ^{86}Y-labeled anti-Lewis Y monoclonal antibodies in nude mouse model: comparison between ^{86}Y and ^{111}In radiolabels. *J Nucl Med.* 2001;42:1281–1287.
31. Carrasquillo JA, White JD, Paik CH, et al. Similarities and differences in ^{111}In- and ^{90}Y-labeled 1B4M-DTPA antiTac monoclonal antibody distribution. *J Nucl Med.* 1999;40:268–276.
32. Pai-Scherf LH, Carrasquillo JA, Paik CH, et al. Imaging and phase I study of ^{111}In- and ^{90}Y-labeled anti-Lewis^{Y} monoclonal antibody B3. *Clin Cancer Res.* 2000;6:1720–1730.
33. DeNardo GL, O'Donnell RT, Shen S, et al. Radiation dosimetry for ^{90}Y-2IT-BAD-Lym-1 extrapolated from pharmacokinetics using ^{111}In-2IT-BAD-Lym-1 in patients with non-Hodgkin's lymphoma. *J Nucl Med.* 2000;41:952–958.
34. Wong JYC, Chu DZ, Yamauchi DM, et al. A phase I radioimmunotherapy trial evaluating ^{90}yttrium-labeled anti-carcinoembryonic antigen (CEA) chimeric T84.66 in patients with metastatic CEA-producing malignancies. *Clin Cancer Res.* 2000;6:3855–3863.
35. Watanabe N, Oriuchi N, Endo K, et al. CaNa2EDTA for improvement of radioimmunodetection and radioimmunotherapy with ^{111}In and ^{90}Y-DTPA-anti-CEA MAbs in nude mice bearing human colorectal cancer. *J Nucl Med.* 2000; 41:337–344.
36. DeNardo GL, Kroger LA, Meares CF, et al. Comparison of DOTA-peptide-ChL6, a novel immunoconjugate with catabolizable linker, to 2IT-BAD-ChL6 in breast cancer xenografts. *Clin Cancer Res.* 1998;4:2483–2490.
37. Kukis DL, Novak-Hofer I, DeNardo SJ. Cleavable linkers to enhance selectivity of antibody-targeted therapy of cancer. *Cancer Biother Radiopharm.* 2001;16: 457–467.
38. Press OW, Corcoran M, Subbiah K, et al. A comparative evaluation of conventional and pretargeted radioimmunotherapy of CD20-expressing lymphoma xenografts. *Blood.* 2001;98:2535–2543.
39. DeNardo DG, Xiong CY, Shi XB, DeNardo GL, DeNardo SJ. Anti-HLA-DR/anti-DOTA diabody construction in a modular gene design platform: bispecific antibodies for pretargeted radioimmunotherapy. *Cancer Biother Radiopharm.* 2001;16:525–535.
40. Karacay H, McBride WJ, Griffiths GL, et al. Experimental pretargeting studies of cancer with a humanized anti-CEA x murine anti-[In-DTPA] bispecific antibody construct and a (99m)Tc-/(188)Re-labeled peptide. *Bioconjug Chem.* 2000;11: 842–854.