ABSTRACT

A major disadvantage of 131I-iodinated monoclonal antibodies (MAbs) for radioimmunotherapy has been the rapid diffusion of iodotyrosine from target cells after internalization and catabolism of the radioiodinated MAbs. We recently reported that a radioiodinated, diethylenetriaminepentaacetic acid-appended peptide, designated immunomedics’ residualizing peptide 1 (IMP-R1), was a residualizing iodine label that overcame many of the limitations that had impeded the development of residualizing iodine for clinical use. To determine the factors governing the therapeutic index of the labeled MAb, as well as the factors required for production of radioiodinated MAb in high yield and with high specific activity, variations in the peptide structure of IMP-R1 were evaluated. A series of radioiodinated, diethylenetriaminepentaacetic acid-appended peptide moieties (IMP-R1 through IMP-R8) that differed in overall hydrophilicity and charge were compared. Radioiodinations of the peptides followed by conjugations to disulfide-reduced RS7 (an anti-epithelial glycoprotein-1 MAb) furnished radioimmunonoconjugates in good overall incorporations, with immunoreactivities comparable to that of directly radioiodinated RS7. Specific activities of up to 8 mCi/mg and yields >80% have been achieved. In vitro processing experiments showed marked increases in radioiodination with all of the adducts; radioiodine retention at 45 h was up to 86% greater in cells than with directly iodinated RS7. Each of the 125I-peptide-RS7 conjugates was compared with 131I-RS7 (labeled by the chloramine-T method) in paired-label biodistribution studies in nude mice bearing human lung tumor xenografts. All of the residualizing substrates exhibited significantly enhanced retention in tumor in comparison to directly radioiodinated RS7, but the non-target uptakes differed significantly among the residualizing labels. The best labels were IMP-R4 and IMP-R8, showing superior tumor-to-nontumor ratios by virtue of high tumor uptake and retention and low normal organ uptake, as well as superior radiochemical properties. The therapeutic efficacy of 131I-IMP-R4-RS7 was compared with that of conventionally 131I-labeled RS7 and 90yttrium-RS7 in the nude mice lung cancer model. The therapeutic efficacy of 131I-IMP-R4-RS7 and 90yttrium-RS7 were equivalent, and both agents yielded significantly improved control of tumor growth compared with conventional 131I-labeled RS7.

INTRODUCTION

In the optimization of methods to deliver therapeutic doses of radiation to tumor cells via MAbs conjugates, the fate of the MAb after binding to the tumor cell is a factor that must be considered. If the MAb is internalized and catabolized rapidly, then the fate of the catabolic products is important. For MAbs labeled with radioiodine by conventional methods, the catabolic product is iodotyrosine (1, 2), which rapidly diffuses from the cell. In contrast, most radioiodinated conjugates have catabolic products that are trapped within lysosomes and therefore are better retained at the tumor site. Such intracellularly retained labels are referred to as residualizing radiolabels. Although it is often considered that only a minority of MAbs is rapidly catabolized, considerable data suggest that this may not be the case and that this issue may be relevant to a large fraction of MAbs binding to the cell surface. First, in vitro experiments demonstrate that all MAbs binding to the cell surface are gradually internalized, probably because of bulk turnover of cell surface constituents (1–3). Although the rate of turnover varies depending on the particular cell line, the typical half-life is ~2 days. Catabolism at this rate will have a major impact on radionuclide accumulation in tumors. Second, we compared different radiolabels for their localization to human tumor xenografts, using a rapidly internalized MAb, RS7, in comparison to a slowly internalized MAb, RS11, with the stated internalization rate being based on in vitro studies (4). Unexpectedly, the use of a residualizing label proved greatly advantageous for both MAbs, with no significant difference between them in this respect. Thus, the catabolic rate of RS11 was fast enough such that use of a residualizing label provided a large advantage. Third, we recently investigated MAb accumulation in B-cell lymphoma xenografts in SCID mice, and in this model, detection of significant MAb accumulation was entirely dependent on the use of residualizing radiolabels (5). This occurred with all MAbs tested, which included MAbs that are known to be slowly internalized. With a conventional iodine label, the radioactivity appeared to leave the tumor as rapidly as it bound so that no specific uptake was detected. Similar results were obtained in two B-lymphoma model systems, and it is likely that similar results would occur with the other B-lymphoma xenograft models that have been described. Thus, it appears that the use of residualizing radiolabels may generally provide an advantage.

Residualizing labels are trapped not only in the lysosomes of tumor cells but also in the lysosomes of any other cells that catabolize the MAbs. This includes the cells normally responsible for MAb catabolism, which includes primarily the liver, spleen, and possibly the kidney. In fact, residualizing labels do accumulate in these tissues more than a conventional iodine label (4). However, in most of the cases investigated, this uptake is at a relatively low level and does not outweigh the large increase in tumor uptake. The extent of this problem also depends on the rate of blood clearance of the particular MAb.

We recently reported the development of two radiiodinated, DTPA-appended peptides, designed to circumvent the short retention time of conventional iodine labels after tumor targeting. These peptides, IMP-R1 and IMP-R2, were conjugated to rapidly internalizing antibodies for evaluation of radioiodinated MAb processing, tumor targeting, and therapeutic efficacy (6–8). Briefly, this new method resulted in residualizing labels, which were produced in higher overall radioiodination yields and specific activities, with preservation of IMR
and with negligible protein aggregation, and the method itself was operationally simple. This method thus overcame many of the practical limitations associated with early approaches to the production of residualizing labels that were based on carbohydrate adducts and other technologies. These previous approaches have been addressed elsewhere (6) and include dilactitolylamine (4, 9), tyraminecillobiose (10), and N-succinimidyl 3-iodo-3-pyridinecarboxylate (11).

Using IMP-R1, residualizing 131I-labeled antibodies were prepared, which were demonstrated to deliver a greatly elevated radiation dose-to-tumors. IMP-R1-based label was used in preclinical therapy studies in the Calu-3 human lung adenocarcinoma model in nude mice. 131I-IMP-R1-labeled MAb therapy mediated by the internalizing anti-EGP-1 MAb RS7, led to a marked therapeutic advantage in comparison to conventional 131I-labeled RS7 (8).

IMP-R2 is a variation of IMP-R1 designed to increase the MAb radioiodination yields by increasing the tyrosine and maleimide contents of the peptide (see Fig. 1 for structures). In in vitro analyses, IMP-R2-based label was superior to IMP-R1-based label, attributable possibly to the large number of D-amino acids in the structure. The intracellular retention of the 131I-label derived from IMP-R2 was similar to that of 111indium. The presence of two tetrapeptides and the conjugation of IMP-R2 via NH$_{2}$-terminal d-alanine to a cross-linker possibly contributed to the formation of a relatively large, lysosomally trapped, 131I-labeled moiety. Radiolabeling using IMP-R2 gave ~90% overall yield and specific activity up to 12 mCi/mg, compared with ~40% overall yield and specific activity up to 2 mCi/mg using IMP-R1. These excellent characteristics associated with IMP-R2 were exploited in single-cell kill experiments using Auger electron emitters (12). However, IMP-R2-based label resulted in high nontarget uptake of the label in animal in vivo biodistribution studies (7). Thus, whereas IMP-R2 is superior in terms of achievable radiochemical yield and the extent of cellular retention in vitro, it is unusable for in vivo applications.

In this study, we focused on developing a method to improve the yield and specific activity of the IMP-R1-based procedure while maintaining or surpassing the positive attributes of IMP-R1. The data generated using IMP-R2 provided a basis for improving radiochemical yield and revealed the necessity for modifying structural elements that caused the high nontarget uptake of the IMP-R2 label. The improved yields were apparently because of two protein-binding groups and four tyrosines. A report by Roger et al. (13) on the biodistributions of radiocopper-labeled antibodies concluded that non-target uptake increased with increasing hydrophobicity of the chelate. IMP-R2 can be empirically seen to have more hydrophobic groupings relative to hydrophilic carboxylic acids, and this could result in an overall greater hydrophobicity of IMP-R2 compared with IMP-R1, where there are six carboxylic acids (including COOH terminus) relative to just a tripeptide. With this working hypothesis, we set out to design an improved bifunctional peptide, keeping the elements of IMP-R2 needed for better overall radiochemical yields while increasing the overall hydrophobicity to improve the in vivo biodistribution of the label. A series of radioiodinated, DTPA-appended peptide moieties (IMP-R1 through IMP-R8) that differed in overall hydrophilicity and charge were compared. These moieties are comprised of D-amino acids to render the peptide bonds relatively resistant to the action of proteases in lysosomes; DTPA, for inclusion of negative charges and because the bond between DTPA and the e-amino group of lysine is stable within lysosomes; and maleimide as a cross-linking agent for attachment to the MAb.

Variations in the peptide structure of the residualizing adduct that were studied included (a) increasing the number of maleimide cross-linking groups of IMP-R1 from one to two to obtain better conjugation yield and (b) manipulation of hydrophilicity by including additional DTPA groups, use of a less hydrophobic cross-linker, and amino acid substitutions. Because four tyrosine residues should not be necessary for obtaining adequate radioiodination yield, we also lowered the number of tyrosine groups that were present in IMP-R2, thereby lowering overall hydrophilicity. We report here the identification of IMP-R4 as an adduct meeting the requirements for clinical development. When conjugated to MAb RS7, this adduct yields superior radiochemical properties, as well as excellent tumor targeting and therapeutic efficacy in an animal model of human lung cancer.

### MATERIALS AND METHODS

#### General Procedures

MAbs were obtained from Immunomedics, Inc., and the Garden State Cancer Center, 2-Chlorotrityl chloride resin and protected D-amino acids were obtained from Advanced ChemTech (Louisville, KY) and used as received. Sulfo-SMCC and succinimidyl 2-maleimidocysteate were purchased from Molecular Biosciences, Inc. (Boulder, CO). Aminobenzyl-DTPA penta Bu ester and isothiocyanatobenzyl-DTPA were purchased from Macroyclics, Inc. (Richardson, TX). All other chemicals and solvents, of high-purity grade, were obtained from Advanced ChemTech, Aldrich Chemical Company (Milwaukee, WI), Fisher Scientific (Pittsburgh, PA), and JT Baker (Phillipsburg, NJ), Na$^{125}$I, Na$^{131}$I, and $^{90}$YCl$_3$ were purchased from NEN Life Science Products (Boston, MA). All non-aqueous reactions were carried out under argon or nitrogen. Electrospray mass spectral data were obtained from HT Laboratories (San Diego, CA) and were recorded using either a Hewlett-Packard 1100 MSD or an API 1 Perkin-Elmer SCIEX electrospray mass spectrometer. Analytical HPLC analyses of peptides were carried out using a Waters PrepLC 4000 using a Nova Pak C-18 (4 μm, 8 × 100 mm) Radiol Pak cartridge, equipped with a UV detector (220 nm), and using a gradient elution consisting of buffers A (0.1% aqueous TFA) and B (9:1 acetonitrile/water, 0.1% TFA). [The gradient was: 100% A changing to 100% B in 10 min (linear gradient) at 3 ml/min flow, and changing to 5 ml/min flow at 10.1 min and remaining isocratic at 100% B at this flow-rate for the next 5 min.] Preparative HPLC was performed on a Waters PrepLC 4000 using a Delta Pak C18 (15 μm, 100 A, 4 × 31 cm) or Nova Pak C-18 (6 μm, 60 A, 2.5 × 20 cm) preparative HPLC columns. HPLC analyses of radiolabeled MAbs were carried out on an analytical Bio-Sil 250 size-exclusion column in series with a guard column (both from Bio-Rad Laboratories, Hercules, CA), using 0.2 mM sodium phosphate buffer (pH 6.8) as the mobile phase at a flow-rate of 1 ml/min, with in-line UV (280 nm) and radioactivity detection.

#### Chemical Syntheses

The substrates IMP-R3 through IMP-R8, shown in Fig. 1, were synthesized by the solid-phase peptide synthesis methodology, additional derivatizations, and preparative HPLC purifications, along the lines used in the preparations of IMP-R1 and IMP-R2 (6).

#### Common Step for all Peptides (Step-1). Fmoc-D-Lysine(Aloc), 0.488 g (1.08 mmol), was dissolved in anhydrous dichloromethane (7.5 ml) and mixed with DIEA (0.83 ml). The solution was stirred with 0.75 g of 2-chlorotrityl chloride resin at room temperature for 18 h. This initial attachment to resin was done in duplicate. The combined resin was filtered, washed with solvents, and dried in a stream of nitrogen. Fmoc-deprotection was done using 40 ml of 20% piperidine in DMP for 15 min, followed by a resin-wash sequence. The peptide was elaborated using Fmoc-D-lysine(tBu) (2.48 g; 5.4 mmol), which was preactivated with 1-hydroxybenzotriazole (0.96 g; 6.2 mmol) and 0.93 ml of diisopropycarbodiimide (5.9 mmol) in 12 ml of NMP for 20 min, and the coupling reaction was carried out overnight, followed by the addition of 1.86 ml of DIEA and the continuation of coupling for 20 min. This resin (0.665 mmol peptide/g of resin), in addition to additional lots of the resin generated

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1 The evaluation of IMP-R7 is incomplete. It is expected to be similar or slightly better than IMP-R5; however, because the radiochemical yields and specific activities obtained with IMP-R4 and IMP-R8, both containing two protein cross-linkers, were superior to that with IMP-R5 and the in vivo characteristics obtained were comparable or superior to that with IMP-R5, the choice was between IMP-R4 and IMP-R8. Therefore, there was no immediate urgency to evaluate IMP-R7.
during the course of the work, was used in the preparation of IMP-R3 through IMP-R8 as described below. Resin-wash protocol was used after every step of the peptide synthesis.

Synthesis of IMP-R3. Using the resin from the common step above, Fmoc-deprotection was first carried out as before. This was followed by a sequence involving a second coupling with activated Fmoc-lysine(Al(o)c) and Fmoc-deprotection, and finally coupling with Boc-lysine(Boc)-Osu. The Aloc protecting group was removed by adding a solution of 0.1547 g of tetraakis(triphenylphosphine) palladium (0) in a mixture consisting of dichloromethane (40 ml), acetic acid (2 ml), and DIEA (5 ml), followed by the addition of tributyltinhydride (5 ml). After 1 h, the filtered resin was washed with solvents, dried, and treated with 10 ml of 1:1:8 acetic acid-trifluoroacetic acid-dichloromethane, containing 3% TFA, for 1 h. The peptide, cleaved from the resin, was collected by filtration of the reaction mixture. Solvent removal furnished the protected peptide, which was purified by preparative HPLC. Retention time (HPLC): 9.04 min. Mass spectrum: m/e 1042 (M + H), 1042 (M – H). This product (60 mg: 0.06 mmol) was derivatized with excess N-(3-isothiocyanatobenzyl)DTPA in water (pH 8.5) at 37°C for 4.5 h. HPLC analysis indicated ~65% conversion. Preparative purification on reverse-phase HPLC followed by lyophilization furnished 33 mg of DTPA-derivatized product. Retention time (HPLC): 9.28 min. Mass spectrum showed the M – H peak at m/e 2121 (M – H + C4H11, negative ion mode). This material was then treated with 0.8 ml of a mixture consisting of TFA (2 ml), dichloromethane (0.5 ml), 0.12 ml of 1:3 v/v ethanol/dimethylformamide, and 0.06 ml of water, stirred for 1 h at the room temperature, and the product was precipitated with diethyl ether. Retention time (HPLC): 6.58 min. Mass spectrum: M + H + C13H11O11N3 1811. This intermediate (13.3 mg) was reacted with a 12-fold molar excess of sulfo-SMCC in 0.1 M sodium phosphate, pH periodically adjusted to be in the 7.0–7.2 range, for 1 h. Preparative reverse-phase HPLC yielded IMP-R3. Mass spectrum (m/e): 2248 (M + H), 2247 (M + H + C4H11, negative ion mode).

Synthesis of IMP-R4. A total of 1.35 g of resin from step-1 (0.90 mmol of peptide) was used. Fmoc-deprotection was followed by reaction with an excess of activated Fmoc-lysine (Aloc) in NMP. Fmoc-deprotection, and finally coupling with an excess of BOC-lysine(BOC)-Osu in NMP. The Aloc protecting group was removed, and the peptide was cleaved from the resin as described above for IMP-R3. Mass spectrum: m/e 823 (M + H) and 821 (M – H). This peptide (0.14 g; 0.17 mmol) was reacted with 2.2 equivalent (0.374 mmol) of isothiocyanatobenzyl-DTPA penta Bu ester [generated from commercially available aminobenzyl-DTPA using a procedure adapted from Szalecki (14)] in DMF (1.3 ml) and triethylamine (0.2 ml) for 3 h. The crude product, after solvent removal, was subjected to TFA-mediated cleavage of BOC and tBu groups, as for IMP-R3. Preparative HPLC purification and lyophilization furnished 58.4 mg of Lys-Lys(Bn-DTPA)-n-Tyr-Lys(Bn-DTPA)-OH, HPLC retention time: 6.06 min. Mass spectrum: m/e 1645 (M + H). Additional derivatization of the latter (0.025 g; 0.015 mmol) with sulfo-SMCC (0.24 mmol) in 0.1 M sodium phosphate, pH adjusted to be in 7.1–7.2 range for ~1 h, followed by preparative HPLC yielded the title product (0.011 g), retention time: 7.41 min. Electrospray mass spectrum (m/e): 2108 (M + Na), 2084 (M – H).

Synthesis of IMP-R5. A total of 0.23 g of resin from step-1 (0.15 mmol of peptide) was used. Fmoc-deprotection was followed by reaction with Boc-Asp(O-Bu)-ONP (0.92 mmol) and subsequent Aloc-deprotection. The retention time of the peptide, determined after cleaving the peptide from an analytical sample of the resin, was 8.11 min. Derivatization of the N-lysine side chain of the peptide was carried out on the resin using excess of isothiocyanatobenzyl-DTPA penta Bu ester, and the peptide was cleaved off the resin. The material was subjected to TFA-mediated cleavage of BOC and tBu groups, and the product was purified by preparative HPLC using 0.1 M sodium phosphate, pH 7.2 range, for 1 h. The filtered resin was washed with solvents, dried, and treated with 10 ml of 1:1:8 acetic acid-trifluoroacetic acid-dichloromethane, containing 3% TFA, for 1 h. The peptide, cleaved from the resin, was collected by filtration of the reaction mixture. Solvent removal furnished the protected peptide, which was purified by preparative HPLC. Retention time (HPLC): 6.58 min. Mass spectrum: M + H + C13H11O11N3 1811. This intermediate (13.3 mg) was reacted with a 12-fold molar excess of sulfo-SMCC in 0.1 M sodium phosphate, pH periodically adjusted to be in the 7.0–7.2 range, for 1 h. Preparative reverse-phase HPLC yielded IMP-R5. Mass spectrum (m/e): 2248 (M + H), 2247 (M + H + C4H11, negative ion mode).

Synthesis of IMP-R7. The intermediate peptide Asp-n-Tyr-o-Lys(Bn-DTPA)-OH (0.031 g: 0.032 mmol), used for IMP-R5 preparation, was derivatized with 0.016 g (0.063 mmol) of succinimidyl 2-maleimidoacetate in DMF (0.15 ml) and DIEA (0.016 ml) for 1.3 h. The product was precipitated from ether and purified by preparative HPLC to obtain 0.003 g of lyophilized IMP-R8, retention time: 6.34 min. Mass spectrum (m/e): 1367 (M + H) and 1389 (M + Na), 1365 (M – H).

Radiiodination and Conjugation

These were carried out as detailed earlier (6). Disulfide reduction of RS7 MAb, using DTT, resulted in 8–9 thios/igG molecule as determined by Ellman’s assay (15). The peptide was first radiiodinated, any unused reactive iodoine was quenched by adding excess 4-hydroxyphenylacetic acid followed by potassium iodide, and then conjugated to disulfide-reduced MAb. The overall incorporations were described as described previously (6).

MAbs and Cell Lines

The production and characterization of MAbs have been described previously. MAB RS7, an IgG1 murine MAbs, reacts with the integral membrane glycoprotein EGP1 (16). Antibodies were purified from ascites fluid by passage through a protein A-immunoadsorbent column. ME180, a human cervical carcinoma cell line, and Calu-3, a human adenocarcinoma of the lung cell line, were purchased from the American Type Culture Collection (Manassas, VA). Cell lines were cultured in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and l-glutamine (2 mm) as described previously (7, 8) and were routinely tested for Mycoplasma by the Mycetox assay (Life Technologies, Inc.).

IMR

Assessment of IMR after radiolabeling was performed using a direct cell binding assay (17). The percentage of IMR, calculated according to Lindmo et al. (18), of the radiolabeled RS7 preparations for each study reported here was ≥67% for all preparations.

In Vitro MAb Retention Studies

These studies were carried out as previously described (4, 19) using ME180 cells. All incubations were carried out in tissue culture medium at 37°C. Confluent cells in 96-well plates were incubated with 5 x 10^5 cpm of labeled antibody for 2 h and then washed four times with media by centrifugation. Tissue culture medium (0.2 ml) was added, and incubation continued for 3 days. At the indicated times, 0.1 ml of the medium was removed, and after washing via centrifugation, the cells were solubilized with 2.0 N NaOH to determine the amount of cell bound radioactivity. After the total counts/min were determined in the culture medium, iodinated MAbs were precipitated with 5 ml of cold 10% trichloroacetic acid. The amount of precipitable radioactivity and soluble radioactivity in the supernatant were thus determined. Each MAb processing experiment was carried out in triplicate.

In Vivo Studies

Tumors were propagated in female nu/nu mice (Taconic Farms, German- town, NY) at 6–8 weeks of age by s.c. injection of 2 x 10^5 Calu-3 cells, which had been propagated in tissue culture. The mice were used for in vivo biodistribution studies ~3 weeks after the injection of cells when tumors...
reached a weight of ~0.2 g (generally in the range of 0.1–0.5 g). Radiiodinated antibodies were injected i.v., via the lateral tail vein, into the tumor-bearing animals. Details on the quantities of radiodinated injected are indicated in the “Results” section for each study. For biodistribution studies, the animals were sacrificed at the times indicated, and the radioactivity in the tumor, liver, spleen, kidneys, lungs, stomach, small and large intestines, muscle, bone (whole femur), and blood was determined after correction for physical decay in a γ-scintillation counter. Results are given as the mean ± SD of 4–5 animals/time point on days 1, 3, and 7, and 3–5 animals/time point for day 14.

For MTD and RAIT experiments, tumor size was monitored by weekly measurements of the length, width, and depth of the tumor using a caliper. Weekly follow-up continued for 15–25 weeks. Tumor volume was calculated as the product of the three measurements. In all studies, there was only one tumor/animal. The MTD was defined as the highest dose that will allow 100% of the animals to survive with no >20% loss in body weight. Reversible myelotoxicity was acceptable. Studies were performed using 9–10 animals/group. Toxicity was monitored principally by loss of body weight and WBC counts.

**Dosimetry**

Radiation dose estimates delivered to the tumor and normal organs were calculated from the biodistribution data as described previously (4). Briefly, radiation dose estimates were determined by first integrating the trapezoidal regions defined by the time activity data (corrected for physical decay). A zero-time value of zero is assumed for the trapezoidal fit. The resulting integral for each organ is converted to cGy/MBq using S values calculated for each isotope by assuming uniformly distributed activity in small unit-density spheres, which do not assume 100% absorption of β-particles (20). For the blood, the absorbed dose was calculated for a sphere the size of the total blood volume of the mouse (assumed to be 1.5 ml). This model of the blood is useful in that the blood dose calculated has been found to correlate well with experimentally determined bone marrow toxicity, which is the dose-limiting toxicity (21).

**Statistical Analyses**

For the in vitro and biodistribution experiments, statistical significance was ascertained by Student’s t test. For the therapy studies, statistical analyses were performed to compare different treatment groups using the Student’s t test on the area under the growth curves. Two-sided tests were used throughout.

### Table 1

<table>
<thead>
<tr>
<th>Run no.</th>
<th>Peptide</th>
<th>mCi of 125I</th>
<th>Recovery*</th>
<th>Specific activity (mCi/mg)</th>
<th>Aggregation (by HPLC)</th>
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<tr>
<td>1</td>
<td>IMP-R1</td>
<td>1.78</td>
<td>24.1%</td>
<td>0.85</td>
<td>0%</td>
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<td>20.4%</td>
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<td>0%</td>
</tr>
<tr>
<td>3</td>
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<td>0%</td>
</tr>
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<td>4</td>
<td>IMP-R4</td>
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<td>0%</td>
</tr>
<tr>
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<td>0%</td>
</tr>
<tr>
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<td>7</td>
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<tr>
<td>8</td>
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<td>0%</td>
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<td>9</td>
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<td>0%</td>
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<tr>
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<td>0%</td>
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<td>0.94</td>
<td>0%</td>
</tr>
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<td>12</td>
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<td>19.0%</td>
<td>0.94</td>
<td>0%</td>
</tr>
<tr>
<td>13</td>
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<td>19.0%</td>
<td>0.94</td>
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<tr>
<td>14</td>
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<td>19.0%</td>
<td>0.94</td>
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<tr>
<td>15</td>
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<tr>
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<tr>
<td>18</td>
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<td>1.12</td>
<td>19.0%</td>
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*Overall isolated yield.

**RESULTS**

**Chemistry.** To optimize both yield and in vivo pharmacokinetics of residualizing radioiodinated MAbs, a series of DTPA-appended peptide substrates was generated. This was done by modifying the general IMP-R1 structure. Modifications of the structure were generated by changing the number of tyrosine, maleimide, and DTPA residues. A limited number of substitutions and additions of other amino acid residues were also evaluated. The structures of IMP-R1 through IMP-R8 are shown in Fig. 1. Whereas IMP-R1 contains one D-tyrosine, one maleimide, and one benzyl-DTPA group, IMP-R2 contains four D-tyrosines and two maleimide groups and one DTPA group. IMP-R3 contains two D-tyrosines, two maleimides, and two benzyl-DTPAs. The extra benzyl-DTPA moiety in IMP-R3 was used to offset the hydrophobicity because of two hydrophobic cross-linkers (two MCC residues) and one extra D-tyrosine. IMP-R4 contains one less D-tyrosine than IMP-R3, and thus its hydrophilicity profile is enhanced over that of IMP-R3. The structure of IMP-R5 is similar to IMP-R1 with aspartic acid in place of glycine to modify the charge as well as the hydrophilicity. IMP-R6 is a modification of IMP-R5 with an added lysine at the NH2 terminus that, in turn, carries two maleimide-containing cross-linkers. IMP-R7 was synthesized but was not included in the evaluations. IMP-R8 is a modification of IMP-R6 in which the cross-linker used in IMP-R1 through IMP-R6 has been substituted by a less hydrophobic maleimide-containing cross-linker (MA rather than MCC). The structures of IMP-R4 and IMP-R8 represent different approaches to modulating the hydrophilicity profiles. In the former, one extra benzyl-DTPA was added to increase the polar groups; in the latter, the hydrophilicity of the protein cross-linker is reduced by resorting to an ultra short linker.

Table 1 details overall incorporation, specific activity, and aggregate content of the purified 125I-peptide-RS7 conjugates. Incorporation of 125I using the adducts containing one maleimide group ranged from 24 to 31% with IMP-R1 and up to 45% with IMP-R5. Improved incorporations were achieved using IMP-R2, IMP-R3, IMP-R4, and IMP-R8, where recoveries of 125I ranged from 65 to 80%. All four of these moieties contain two maleimides, indicating that the presence of two maleimides is important for improved labeling yield. Synthesis of 125I-IMP-R6 resulted in low yield because of low solubility of the product. The solubility of IMP-R6 in aqueous medium pH ~5–6 was less than that of other adducts, probably attributable to its hydrophobic nature (only one benzyl-DTPA but two hydrophobic cross-linkers). The increased incorporation using IMP-R2, IMP-R3, IMP-R4,
IMP-R8 led to increases in specific activities. These substrates resulted in <2% aggregation in antibody labeling.

In Vitro Antibody Retention Experiments. Cellular retention of radioisotope, after labeled MAbS are bound to the cell surface, was studied using an in vitro assay. Retention of MAb RS7 labeled with ¹²⁵I via the various residualizing adducts was evaluated by measuring the percentage of originally bound isotope still bound by ME180 cells at indicated times after removal of excess-labeled MAb. Comparisons were made between the retention of ¹²⁵I delivered using RS7 labeled with the residualizing adducts IMP-R1 to IMP-R8 and RS7 labeled by the conventional CT method and ¹¹¹In-benzyl-DTPA. The amount of cell-bound radioactivity remaining associated with the cells over a 69-h time course is shown in Fig. 2. A close similarity was observed among all of the residualizing substrates, except ¹²⁵I-IMP-R2-RS7. At 45 h, the amount of cell-bound radioactivity for ¹²⁵I-IMP-R1, ¹²⁵I-IMP-R3, ¹²⁵I-IMP-R4, ¹²⁵I-IMP-R5, ¹²⁵I-IMP-R6, and ¹²⁵I-IMP-R8 ranged from 57.5 to 60.4% of the initially bound value compared with 39.2% with directly iodinated RS7, an increase of 47–54%. With ¹²⁵I-IMP-R2-RS7, the tumor cell retention was higher and comparable with that observed with ¹¹¹In-labeled RS7, 72.8 and 73.9%, respectively. At 69 h, retention with ¹²⁵I-IMP-R1, ¹²⁵I-IMP-R3, ¹²⁵I-IMP-R4, ¹²⁵I-IMP-R5, ¹²⁵I-IMP-R6, and ¹²⁵I-IMP-R8 was increased by 64–91% compared with directly iodinated RS7, whereas IMP-R2 and ¹¹¹In yielded increases of 129 and 139%, respectively.

In Vivo Animal Biodistributions. Targeting of MAb RS7 labeled with residualizing adducts IMP-R1 through IMP-R8 was studied in a series of paired labeled studies in which RS7 iodinated by each residualizing ¹²⁵I-labeled adduct was directly compared with conventionally iodinated ¹³¹I-RS7 in nude mice bearing Calu-3 tumor xenografts. The percentage of ID/g resulting from these experiments are shown in Fig. 3. All of the residualizing substrates exhibited significantly enhanced retention in tumor in comparison to directly radioiodinated RS7, but the nontarget uptakes differed significantly among the residualizing labels. The IMP-R2 label showed higher uptake in spleen and liver and an increasing kidney level over the 14-day period of the experiment. Although not as severe as with IMP-R2, the IMP-R3-based label showed increased uptakes in liver and kidney. Radioidinated RS7 prepared with IMP-R4, IMP-R5, and IMP-R8 showed normal tissue clearance similar to that of conventionally iodinated RS7. Fig. 4 shows representative data of percentage of ID/g with the conventionally iodinated RS7; paired graphs of the conventionally iodinated RS7 are not shown for each study because of space considerations.

Tumor:nontumor ratios on day 7 after injection of RS7 are shown in Table 2. Using IMP-R2, IMP-R3, IMP-R4, IMP-R5, and IMP-R8, tumor:blood ratios increased 10–12-fold compared with the conventionally iodinated RS7. Increased uptake in liver, spleen, and kidney using IMP-R2 and IMP-R3 led to relatively low tumor:nontumor...
MAbs were calculated using the 125I-labeled MAb biodistribution data. Cumulative absorbed doses for the 131I-labeled IMP adducts lead to increases in tumor:nontumor ratios for liver, spleen, and kidney ranging from 1.3- to 3.4-fold. The highest increases in the tumor: nontumor ratios were observed using IMP-R4, which yielded 8.9- and 10.5-fold increases, respectively, for the muscle and lungs.

Cumulative absorbed radiation doses were calculated from the biodistribution data. Cumulative absorbed doses for the 131I-labeled MAb comparisons (22). For therapy, it is the relative dosimetry, not percent-values are estimated as the administered dose that would yield an equal tumor dose.

As shown in Fig. 5, it is estimated that the residualizing iodine labels lead to approximately a 4-fold increase in dose-to-tumor compared with conventional iodine-labeled 131I-RS7 (at the estimated MTD). Except for IMP-R2-RS7, these doses of residualizing 131I-labeled RS7 are estimated to deliver doses to all normal tissues that are below toxic levels. Toxic levels for these organs are taken to be:

<table>
<thead>
<tr>
<th>IMP-Rx</th>
<th>Liver</th>
<th>Spleen</th>
<th>Kidney</th>
<th>Lungs</th>
<th>Blood</th>
<th>Muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMP-R1</td>
<td>10.5 ± 4.1</td>
<td>7.5 ± 1.5</td>
<td>3.9 ± 1.3</td>
<td>7.7 ± 2.7</td>
<td>4.1 ± 1.6</td>
<td>45.2 ± 17</td>
</tr>
<tr>
<td>IMP-R2</td>
<td>2.0 ± 0.5</td>
<td>2.5 ± 0.4</td>
<td>0.7 ± 0.3</td>
<td>10.0 ± 1.0</td>
<td>7.5 ± 1.1</td>
<td>36.0 ± 6.4</td>
</tr>
<tr>
<td>IMP-R3</td>
<td>2.3 ± 0.8</td>
<td>3.5 ± 1.1</td>
<td>3.5 ± 1.4</td>
<td>13.7 ± 5.1</td>
<td>9.1 ± 4.3</td>
<td>84.3 ± 31</td>
</tr>
<tr>
<td>IMP-R4</td>
<td>5.8 ± 2.1</td>
<td>7.0 ± 2.7</td>
<td>6.3 ± 1.1</td>
<td>24.1 ± 17</td>
<td>9.7 ± 4.9</td>
<td>105.7 ± 61</td>
</tr>
<tr>
<td>IMP-R5</td>
<td>11.5 ± 2.3</td>
<td>9.2 ± 2.0</td>
<td>7.0 ± 1.7</td>
<td>16.0 ± 6.4</td>
<td>7.9 ± 3.2</td>
<td>101.4 ± 33</td>
</tr>
<tr>
<td>IMP-R6</td>
<td>5.4 ± 2.4</td>
<td>3.9 ± 1.4</td>
<td>3.7 ± 1.4</td>
<td>3.6 ± 1.5</td>
<td>1.6 ± 0.7</td>
<td>25.5 ± 17</td>
</tr>
<tr>
<td>IMP-R8</td>
<td>4.4 ± 1.4</td>
<td>10.6 ± 2.5</td>
<td>7.3 ± 2.1</td>
<td>19.0 ± 7.9</td>
<td>7.9 ± 3.2</td>
<td>74.3 ± 7.4</td>
</tr>
<tr>
<td>131I-RS7 (CT)</td>
<td>3.4 ± 1.1</td>
<td>3.8 ± 1.0</td>
<td>3.0 ± 0.8</td>
<td>2.3 ± 0.9</td>
<td>0.8 ± 0.2</td>
<td>11.9 ± 3.9</td>
</tr>
</tbody>
</table>

* Calculated from data in Fig. 2.

Fig. 5. Mean cumulative absorbed dose in tissues after injection of radiolabeled RS7 in nude mice bearing Calu-3 tumors. Results are calculated from data described in Fig. 3 and Fig. 4 and are presented as cGy to tissue normalized to 1500 cGy cumulative absorbed dose-to-blood.

Therapy with 131I-IMP-R4 RS7. On the basis of the superior radiophysical properties (>85% yield) and superior tumor:nontumor ratios achieved in the animal model (tumor:blood ratio of 9.7 day-7 after MAb injection), IMP-R4 was the adduct selected for use in a RAIT study. The therapeutic efficacy of 131I-IMP-R4-RS7 was compared with that of conventionally 131I-labeled RS7 and 90Y-RS7 in nude mice bearing Calu-3 lung cancer xenografts at equitoxic doses. Growth of tumors in an untreated group was compared with tumor growth compared with conventionally 131I-labeled RS7. Results of this study are summarized in the tumor growth curves shown in Fig. 6. Eleven weeks posttreatment mean tumor volumes were 2000 cGy to lung and kidney and 3000 cGy to liver (23). 131I-IMP-R2-RS7 would not yield as great an improvement on the therapeutic efficacy of 131I-RS7(CT) because of the large doses delivered to kidney, liver, and spleen by 131I-IMP-R2-RS7. These data indicate that in a dose escalation study of 131I-IMP-R2-RS7, the kidneys would receive a dose-limiting toxicity before bone marrow, thus the dose that delivers 2000 cGy to the kidney would be the estimated MTD. The dose-to-tumor at the MTD is estimated to be 1536 cGy, which although still higher than the tumor dose obtained at the MTD of 131I-RS7(CT), is only 30-40% of the dose-to-tumor obtained at the MTD of the other 131I-labeled IMP adducts, IMP-R1, IMP-R3, IMP-R4, IMP-R5, and IMP-R8.
Radioimmunotherapy with Residualizing Iodine

90Y-RS7, respectively. Tumors in untreated animals reached ~10 times the starting volume (~3.0 cm³) at 6 weeks. Toxicity of the treatments was comparable, as measured by WBC counts and body weight loss.

DISCUSSION

To develop a method for linking radioiodine to MAbs in such a fashion as to cause the radioiodine to remain trapped inside tumor target cells after uptake and catabolism, a series of DTPA-appended peptide moieties was synthesized. These moieties, referred to as IMP-R1 through IMP-R8, differ in overall hydrophilicity and charge. IMP-R1 through IMP-R8 were then used to link radioiodine to MAb. The aminopolycarboxylate DTPA was selected as a building block for these adducts because it is known that radiometal chelates of DTPA are retained in cells in the form of their lysine adduct (4). We hypothesized that if DTPA was attached to the ε-amino of d-lysine and the peptide was additionally elaborated by sequential coupling to one or more d-amino acids, including d-tyrosine, the result would be a DTPA-attached peptide wherein the peptide bonds would be relatively resistant to the action of proteases in lysosomes. The NH₂ terminus of the peptide could be coupled to a maleimide-containing amine-reactive, cross-linker such as sulfo-SMCC. Radiodiation of such an entity, followed by conjugation to thiol-containing antibody would be expected to produce a residualizing iodine label. The use of maleimide as MAb-reactive group is advantageous because the maleimide-to-thiol conjugation yields can be rendered near quantitative in aqueous solutions.

We evaluated this radiodination methodology using MAB RS7 in a human lung cancer xenograft model in nude mice. MAB RS7 is an IgG1 murine MAB that reacts with the integral membrane glycoprotein EGP-1 (16, 24). A high frequency of EGP-1 expression has been observed in a variety of tumor types, including tumors of the lung, stomach, bladder, breast, ovary, uterus, and prostate, with limited observed expression on normal human tissue. Radiolabeled RS7 has been used extensively for localization and therapy studies in animal models and has demonstrated tumor targeting and significant antitumor efficacy (4, 7, 8, 25–29).

RS7 has been demonstrated to rapidly internalize after binding to target cells. The rapid rate of internalization was initially shown by in vitro studies on cultured human lung cancer cells using unconjugated MAB followed by fluorescence labeling and by binding of 125I-RS7 followed by acid removal of surface bound MAB (24). Antibody internalization was noted at 30 min, and by 2 h, virtually all of the MABs were internal. Subsequent studies demonstrated internalization of radiolabeled RS7 in human breast and cervical carcinoma cell lines (26). Because RS7 is rapidly internalized by the target cells, it has been used as a model MAB to demonstrate that the use of radiouclides that are retained inside the cell after antibody catabolism maximizes the effectiveness of RAIT. 90Y-, 177Lu-, and various residualizing forms of 131I-labeled RS7 have all yielded therapeutic advantages over 131I-labeled RS7 labeled by conventional methodology (8, 27–29). RS7 was also used in a study that compared the targeting advantage gained from using residualizing radioiodine on a rapidly internalizing MAB to the effects of using residualizing radioiodine on a slowly internalized MAB (4). Although in vitro experiments demonstrated a substantial increase in retention of the residualizing iodine only for the rapidly internalizing MAB, the use of a residualizing label provided a great advantage in the tumor accretion of the radiolabel for both MABs in vivo. Thus, the catabolic rate of a MAB that binds to the cell surface and is internalized at the relatively slow rate of normal cell membrane turnover is fast enough to enable a residualizing label to provide a large advantage.

Although our previous studies demonstrated the advantage of residualizing iodine for RAIT, improvements in radioiodine incorporation were necessary to make the technology feasible for clinical use. Radioiodinations of the IMP-R1 through IMP-R8 peptides followed by conjugations to disulfide-reduced RS7 yielded radioimmunoconjugates that varied in overall incorporation of radioiodine. The presence of two maleimides was found to be important for improved labeling results. Whereas the first generation DTPA-appended peptide, IMP-R1, led to radioiodine recoveries of ~30%, yields >80% have been achieved with the adducts synthesized with two maleimide residues.

In addition to high incorporation of radioiodine, it is also important to provide a conjugate that does not cause elevated accretion in nontarget tissues. In vivo biodistribution studies in the Calu-3 lung tumor xenograft model demonstrated that all of the residualizing substrates exhibited significantly enhanced retention in tumor in comparison to directly radioiodinated RS7. However, accretion of radioiodine in nontarget organs differed significantly among the residualizing labels. The less hydrophobic labels, IMP-R1, IMP-R4, IMP-R5, and IMP-R8, yielded superior tumor-to-nontumor ratios by virtue of high tumor uptake and retention and low normal organ uptake. Structures that maintained low hydrophobicity while incorporating two maleimide groups for increased radioiodination yield led to the desired combination of high tumor-to-nontumor ratios and high radioiodination yield. Low hydrophobicity was achieved in combination with inclusion of two maleimide groups by the use of two benzyl-DTPAs in IMP-R4. Alternatively, one benzyl-DTPA could be used if the cross-linking group was modified with less hydrophobic moieties such as MA rather than MCC, as in the structure of IMP-R8. In the clinical setting, improved intracellular retention of the radiodine in antigen-expressing cells may also lead to increased retention in normal tissues that express low concentrations of the target antigen. This is an issue that cannot be evaluated in the nude mouse model and will have to be assessed in clinical trials. The use of MAbs recognizing antigens with low normal tissue expression or which are located in sites inaccessible to MAbs such as the luminal edge of glands and ducts and the upper layers of squamous epithelia will minimize this concern.

In light of the radiolabeling results and results of the biodistribution/dosimetry analyses, IMP-R4 appeared to be suitable for developing as a clinical agent and was evaluated for therapeutic efficacy in nude mice bearing lung tumor xenografts. The results of this study demonstrate the superiority of 131I-IMP-R4-RS7 over 131I-RS7 labeled by the conventional CT method. Although both forms of 131I-labeled RS7 yielded a therapeutic effect, 11 weeks posttreatment mean tumor volumes were less than half the pretreatment volume in the group treated with 131I-IMP-R4-RS7 compared with an increase of >3-fold the pretreatment volume in the mice treated with conventionally 131I-labeled RS7. A mean tumor volume nadir of 29% of the pretreatment volume was reached 9 weeks posttreatment in the group treated with 131I-IMP-R4-RS7 compared with a mean tumor volume nadir of 68% 4 weeks posttreatment in the mice treated with conventionally 131I-labeled RS7. These results confirm the predicted advantage of 131I-IMP-R4-RS7 made by dosimetry calculations based on the biodistribution studies.

The radioisotopes from radiometal-labeled MAbs are also residualizing in the lysosomes in the form of the lysine adducts of the respective metal chelates (30). 90Y-labeled MAbs were previously shown to be superior to conventionally 131I-labeled MAbs for RAIT in this model, due at least, in part, to the longer retention of 90Y in the target cells (8, 28). In comparing residualizing 131I and 90Y, each has potential advantages and disadvantages. Dosimetric considerations favor 131I over 90Y because of the longer physical half-life of 131I.
which more closely matches the biological half-life of the MAb in circulation. The longer half-life of $^{131}$I enables more of the dose to be delivered after the antibody has localized in the tumor. The shorter path length of $^{131}$I relative to $^{90}$Y may also be an advantage for treatment of minimal residual disease and disseminated micrometastatic disease because more of the dose is delivered to the targeted tumor cells and less to surrounding normal tissue. This is especially important for bone marrow, considering that myelotoxicity has been the major noted toxicity with nonmyeloablative doses. A major advantage of $^{90}$Y is that it is a pure $\beta$-emitter. The lack of $\gamma$-emissions allows less stringent isolation and hospitalization requirements for patients treated with this isotope. In the comparative study reported here, the therapeutic efficacy of $^{131}$I-IMP-R4-RS7 and $^{90}$Y-RS7 were equivalent. It is likely that the murine model used in these studies is unable to expose therapeutic differences that could become apparent in the clinical setting.

Although IMP-R4 appears to be suitable for developing as a clinical agent, having the properties of high labeling yield, high tumor accretion, and low normal organ uptake, there may be room for improvement to further increase labeling yield and target cell accretion. It is interesting to note that the in vitro processing studies indicate that cellular retention of radioisotope after labeled MAbs were bound to the cell surface was higher with $^{131}$I-IMP-R2-RS7 and $^{111}$In-RS7 than with the other residualizing adducts. These in vitro processing studies are not necessarily predictive of suitability for in vivo use as evidenced by the unforeseen high level of kidney accretion with IMP-R2. In addition, the requirement for low overall hydrophobicity and/or an increase in charge was not predictable from in vitro data.

In conclusion, we have succeeded in making a practical residualizing iodine agent, having the properties of high labeling yield, high tumor accretion, and low normal organ uptake, that may be readily used for clinical level radiolabelings and therapeutic applications.

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Improved Iodine Radiolabels for Monoclonal Antibody Therapy

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