Advantage of a Residualizing Iodine Radiolabel for Radioimmunotherapy of Xenografts of Human Non-Small-Cell Carcinoma of the Lung

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Attachment of ^{131}I to MAbs through adducts such as dilactitol-tyramine (DLT), which remain lysosomally trapped after catabolism of the labeled MAb, can greatly increase the residence time of the radiolabel at the tumor site. Our previous studies demonstrated a marked increase in accretion of ^{131}I in a human lung-cancer xenograft model, using ^{131}I-DLT in comparison to ^{131}I linked to MAb by the conventional chloramine-T methodology. **Methods:** In this study, biodistribution experiments were performed to evaluate the effect of protein dose on the accretion of ^{131}I-DLT-labeled MAb RS11 in tumor and nontumor tissues, and in vivo radioimmunotherapy experiments compared the effect of single injections of ^{131}I-DLT-labeled MAb RS11 to conventional ^{131}I-labeled RS11 and an untreated control group. **Results:** Dosimetry calculations based on the biodistribution data indicate only small changes in absorbed dose-to-tumor and nontumor tissues with increasing protein dose up to 100 μg, with a predicted absorbed dose to tumor of from 21,000 to 25,000 cGy/mCi. A single dose of 100 μCi of ^{131}I-DLT-RS11 was found to cause tumor regression. At 7 wk postinjection of the radiolabeled MAbs, tumor volume in 73% of the animals administered ^{131}I-DLT-labeled RS11 remained smaller than at the time of MAb injection. This is compared to 14% of the tumors in the conventionally labeled ^{131}I-RS11 group and none in the untreated group. **Conclusion:** The use of the residualizing radiolabel DLT provides a therapeutic advantage in comparison to conventional ^{131}I-labeled RS11.

Key Words: residualizing radiolabels; monoclonal antibodies; dilactitol-tyramine; radioiodine; lung cancer xenograft

J Nucl Med 1997; 38:391–395

Iodine-131 has been the primary radionuclide for radioimmunotherapy, in part because labeling methods are simple and efficient, it is inexpensive and abundant, and its gamma emissions permit tracing of in vivo distribution by external camera imaging (*1*). However, it is acknowledged that there are disadvantages associated with conventional iodine labels. A major drawback is the short retention time of the isotope within target cells that is observed with many MAbs (*2,3*). After cellular uptake and catabolism of the radioiodinated monoclonal antibody (MAb), the radioisotope diffuses freely out of the cell as monoiodotyrosine (*4–6*). This behavior has been observed with a broad range of MAbs. In our studies with 12 adherent tumor cell lines, including carcinomas of diverse histological origins and melanomas, we found that all MAbs tested were internalized and catabolized with a half-life of no more than 2–3 days (*2,3,7*). We suggested that this catabolism is a result of normal turnover of cell surface constituents. Other types of tumors, specifically B-cell lymphomas, appear to have a lower rate of catabolism of many cell surface-bound MAbs (*8,9*).

To circumvent this effect, novel methodologies for the incorporation of iodine to protein have been developed in which the iodine residue is conjugated to MAb via adducts that become lysosomally trapped (*10*). When the MAb labeled by this type of technique is taken into the cell and catabolized, the iodine remains trapped inside the cell. These radioiodine constructs have been called "residualizing" labels. Radiometal chelates also behave as residualizing labels (*3,11,12*). Other novel methods of indirect protein iodination have also been developed to improve retention of the isotope at the tumor site, using derivatives of iodobenzene (*13,14*). However, these particular methods were designed to prevent deiodination, which now appears to be a minor factor (*12*); the labeled moieties would not be expected to be lysosomally trapped, and the biodistribution data strongly suggest that these labels are not residualizing (*13,14*).

Residualizing radiolabels have been shown to accumulate within tumor cells to a greater extent than conventional iodine labels in several in vitro studies (*4,12,15–17*). In a recent report we demonstrated a marked increase in accretion of ^{131}I in Calu-3, a human non-small-cell carcinoma of the lung xenograft model, using the residualizing label, dilactitol-iodo-tyramine (DLT), in comparison to ^{131}I linked to MAb by the conventional chloramine-T methodology (*16*). These results were demonstrated with two MAbs, RS7 and RS11, which react with the integral membrane glycoproteins EGP1 and EGP2, respectively (*18,19*). RS7 has been shown to be relatively rapidly catabolized (*18*), at a rate compatible with either a coated or noncoated vesicle pathway (*20*), whereas RS11 is catabolized more slowly in the same cells at a rate similar to that for most MAbs binding to the cell surface (*2,3*). Results of biodistribution studies in this model system predicted that the prolonged retention of ^{131}I-DLT-labeled MAbs in tumor cells, the 8-day half-life of the ^{131}I, and the relatively low levels of accretion in normal tissues would combine to make radioiodinated DLT an adduct of great potential for radioimmunotherapy.

In this study we show that ^{131}I-DLT-labeled MAb RS11 is therapeutically more effective in the Calu-3 xenograft model than conventionally radioiodinated antibody.

MATERIALS AND METHODS

Monoclonal Antibodies, Cell Lines and Radiolabeling

The production and initial characterization of RS11 (also referred to as RS11-51), growth of Calu-3, human adenocarcinoma of the lung cells and radioiodination by the chloramine-T method or via dilactitol-tyramine have been previously described (*16*). To equalize the specific activity of the labeled MAbs, unlabeled MAb was added to the chloramine-T label. This method is appropriate because the chloramine-T label, with a specific activity of 10–15

Received Feb. 12, 1996; revision accepted Jun. 15, 1996.
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mCi/mg, has a molar conjugation ratio of 1–2; and the DLT label, with a specific activity of approximately 1 mCi/mg, has a molar conjugation ratio of approximately 0.14. Thus, the DLT label consists of a mixture of labeled and unlabeled molecules. Assessment of immunoreactivity after radiolabeling was performed using a direct cell binding assay (*21*).

In Vivo Studies

Tumors were propagated in female nu/nu mice at 6–8 wk of age by s.c. injection of 2×10^7 washed Calu-3 cells, which had been propagated in tissue culture. The mice were used for in vivo biodistribution studies approximately 3 wk after the injection of cells (tumors generally in the range of 0.1–0.5 g). Radioiodinated antibodies were injected intravenously via the lateral tail vein into the tumor-bearing animals. For biodistribution studies, the animals were killed at the times indicated and the radioactivity in the tumor, nontumor organs and blood were determined after correction for physical decay in a gamma scintillation counter. Results are given as the mean ± s.d. of 5 to 13 animals per time point, representing combined data from two or three independent studies. For radioimmunotherapy experiments, tumor size was monitored by weekly measurements of the length, width and depth of the tumor using a caliper. Tumor size was calculated as the product of the three measurements. Toxicity was monitored principally by loss in body weight. Results are the mean ± s.d. of 9–14 animals per group, representing combined data from two independent studies. Statistical differences were calculated using the Student's t-test. Radiation dose estimates were determined as previously described (*16,22*).

RESULTS

Biodistribution Studies: Effect of Protein Dose

Since antibody protein dose is a parameter that affects tumor targeting (i.e., percent injected dose/g (%ID/g), microdistribution of antibody in tumors and tumor-to-nontumor ratios) we determined the effect of increasing antibody protein dose in this model system (*23–26*). In addition to using the results of these studies for optimization of the model, the studies were necessary prerequisites to therapy with ^{131}I-DLT-labeled RS11 due to the practical limitation imposed by the specific activity of the ^{131}I-DLT-labeled MAb. The DLT labeling method described in the Materials and Methods section yielded an iodinated DLT product with a specific activity in the range of 0.5–1.0 mCi/mg, which is approximately 15-fold lower than the specific activity obtained by conventional iodination. At this specific activity, 100–200 μg of MAb must be injected per mouse in order to administer 100 μCi of ^{131}I. Previous biodistribution studies of RS11 in nude mice bearing Calu-3 tumor xenografts had been performed at a protein dose of 10 μg/animal.

In preliminary experiments, biodistribution studies were performed with a protein dose of 500 μg RS11. As expected, this high protein dose resulted in a decrease in the %ID/g accreted in tumor, and in tumor-to-nontumor ratios, especially at time points of 1–7 days. For example, at day 7 postinjection, the %ID/g in tumor was 35.31% ± 12.54% with 10 μg and 21.61% ± 5.39% with 500 μg. The tumor-to-liver ratios were 9.96 ± 3.68 and 5.41 ± 1.26, and the tumor-to-blood ratios were 9.42 ± 5.07 and 2.64 ± 0.48 for the 10 μg and 500 μg doses, respectively, on day 7. Moreover, dosimetry calculations indicated that, when normalized to a 1500-cGy dose to blood, the tumor radiation dose was approximately 2.5 times lower with 500 μg than with 10 μg. We concluded that 500 μg was an excessive protein dose for radioimmunotherapy in this model system.

Subsequent experiments were designed to determine the highest possible protein dose at which biodistribution results were still favorable, in terms of radiation dosimetry. Protein doses of 50 and 100 μg RS11 were tested together with the 10 μg dose. Figure 1 summarizes the effect of protein dose on the biodistribution of this conjugate. At days 3 and 7 postinjection, the mean %ID/g of RS11 accreting in tumor at the 10 μg dose was somewhat higher than that observed with 50 μg ($p < 0.01$, day 3) or 100 μg ($p < 0.05$, day 3; $p < 0.01$, day 7) (Fig. 1A). These differences, however, did not translate into marked differences in tumor-to-blood or tumor-to-nontumor organ ratios, as shown in Figures 1C and 1D. Results with the liver are only shown in Figure 1, but generally similar results were obtained with the other tissues examined (kidney, spleen, lungs, muscle, small intestine, large intestine and bone). Thus, increasing the protein dose from 10 to 100 μg had little effect. The amount of antibody that accumulated in the tumor (μg/g tumor) is shown in Figure 1B. Antibody uptake per gram of tumor increased with increasing injected dose, suggesting that saturation of binding sites did not occur, and supporting the other evidence that increasing the protein dose to 100 μg does not impair targeting.

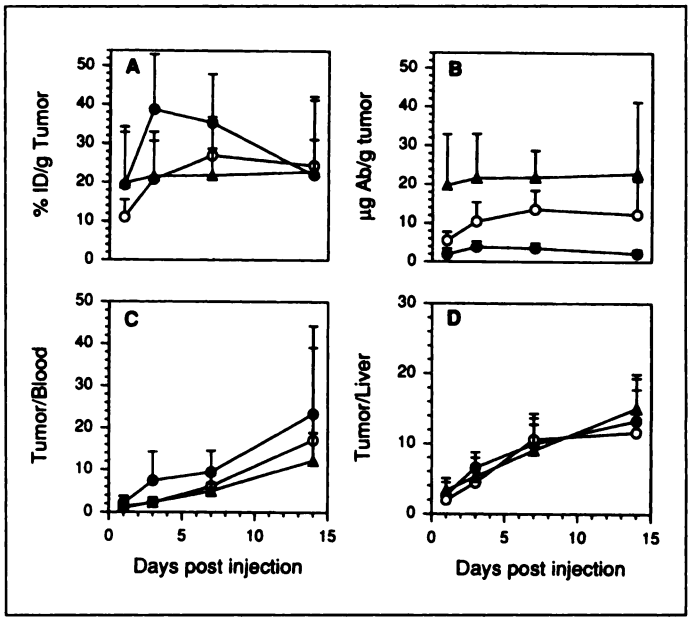

FIGURE 1. Comparative biodistribution of ^{131}I-labeled DLT-RS11 (10 μCi) at three protein doses. (A) The percentage of the injected dose per gram of tumor. (B) Micrograms MAb/g tumor, calculated as μg MAb injected × % ID/g tumor × 100. (C) Tumor-to-blood ratios. (D) Tumor-to-liver ratios. The error bars represent s.d. and are shown only above the symbol for clarity. ● = 10 μg; ○ = 50 μg; ▲ = 100 μg.

To aid in the selection of the optimal therapeutic dose, we calculated the absorbed doses to tumor and blood as a function of injected dose, from 10 μCi to 250 μCi, at protein doses of 10 μg, 50 μg, 100 μg and 500 μg (Fig. 2). Previous studies using this model (*27,28*) have shown that doses in the range of 1500 cGy to tumor have a therapeutic effect. Similarly, an absorbed dose of 1500 cGy to blood approximates the maximally tolerated dose (MTD) for radioiodinated MAbs. (The blood dose is a reliable estimate of the dose to bone marrow, the dose limiting organ for ^{131}I-labeled MAbs). A dashed line at the 1500 cGy level is therefore included in the figure for reference. Effective therapy is predicted for conditions in which the tumor dose is >1500 cGy, and the blood dose is <1500 CGy.

Figure 2 displays clear trends in dosimetry results: as protein dose increases there is a gradual decrease in the dose delivered to the tumors and a gradual increase in the dose delivered to the blood. It appears, therefore, that higher specific activities are

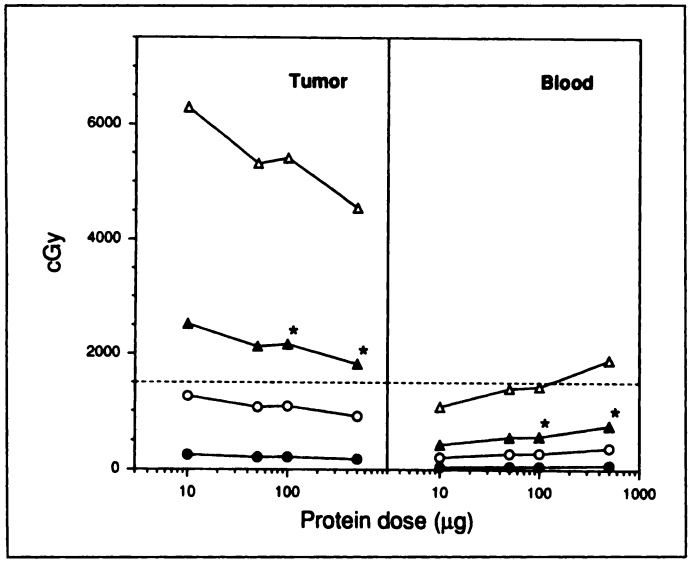

FIGURE 2. Mean cumulative absorbed dose as a function of protein dose. Dosimetry calculations were performed on the biodistribution data. A dashed line at the 1500 cGy level is included for reference. ● = 10 μCi; ○ = 50 μCi; ▲ = 100 μCi; △ = 250 μCi. Points indicated by asterisk are conditions in which therapeutic efficacy is predicted and in which the specific activity is attainable.

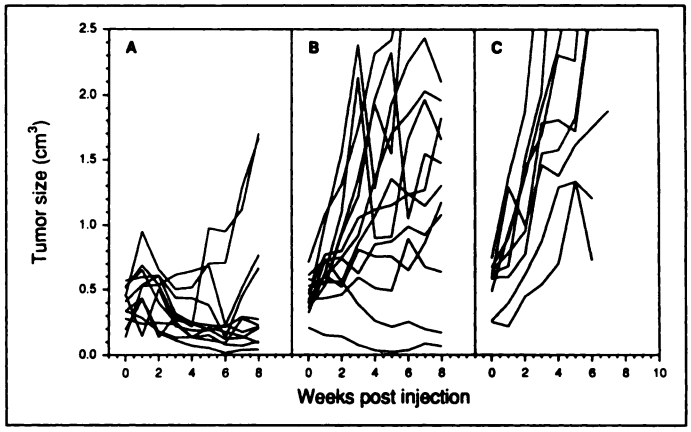

FIGURE 3. Treatment of mice bearing Calu-3 adenocarcinoma of the lung xenografts with ^{131}I-labeled RS11. Animals were given intravenous injections of 100 μCi ^{131}I-labeled DLT-RS11 (A), 100 μCi conventional ^{131}I-labeled RS11 (B), or were left untreated (C). Radiolabeled MAbs were administered 3 wk after injection of tumor cells. Each group consisted of the following number of animals: (A) ^{131}I-labeled DLT-RS11, 11; (B) conventional ^{131}I-labeled RS11, 14; and (C) untreated, 9. Each line represents the tumor growth in an individual mouse; measurements were taken weekly.

preferable, up to the point at which immunoreactivity is impaired. From Figure 2, the highest therapeutic index would be predicted at the highest specific activity shown, 25 mCi/mg, which is far above the specific activity that can be achieved with DLT labeling at the current time. Of the conditions plotted, only a dose of 250 μCi on 500 μg of MAb would be predicted to cause toxicity, because the dose to blood is over 1500 cGy. A 500 μCi injected dose was calculated to be above the MTD at all protein doses tested (not shown). The doses received by the normal organs using ^{131}I-DLT-labeled RS11, under all conditions shown, are below the levels expected to cause toxicity.

Although the therapeutic index should increase markedly if more efficient labeling can be achieved, it seemed worthwhile to perform some therapy experiments, in order to further support the potential value of this approach. Figure 2 predicts that a 100 μCi dose should be therapeutically effective using MAbs labeled to a specific activity of ≤1 mCi/mg (points marked by asterisks), which is attainable, and these are the conditions we evaluated. We note that use of higher doses of radioactivity also would be preferable, but this is limited by the low conjugation efficiency. Therefore, we tested 100 μCi doses, using 20–25 mCi ^{131}I for conjugation.

Radioimmunotherapy with Iodine-131-Labeled DLT-MAb

To compare the therapeutic efficacy of ^{131}I-labeled DLT-RS11 to that of conventionally chloramine-T ^{131}I-labeled RS11, and to confirm the dosimetry calculations described above, in vivo radioimmunotherapy experiments studying single injections of the labeled IgG molecules were performed in nude mice bearing Calu-3 xenografts. The immunoreactivities of the MAbs labeled by either method were indistinguishable, and ranged from 62%–69% for the chloramine-T label, and 58%–65% for the DLT label. Mice were used for the radioimmunotherapy studies approximately 3 wk after the injection of tumor cells, when the tumors had reached a size of approximately 0.3–0.75 cm^3. Mean tumor volumes at the time of treatment were 0.38, 0.45 and 0.53 cm^3 for the ^{131}I-DLT-RS11, ^{131}I-CT-RS11 and untreated groups, respectively. The administered dose of ^{131}I-DLT-RS11 (100 μCi) was selected based on the specific activity of labeling which was achieved. For comparison, 100 μCi of conventional chloramine-T labeled ^{131}I-RS11 was administered to a control group and the protein dose was adjusted to 100 μg by addition of unlabeled MAb RS11. The biodistribution study described above predicted that an absorbed dose of approximately 2200 cGy would be delivered to tumor in the DLT-labeled group. This dose is greater than the absorbed dose-to-tumor generated in our previous radioimmunotherapy studies (*28*) with intact ^{131}I-RS7-IgG labeled with the conventional chloramine-T methodology (1661 cGy/250 μCi ^{131}I-RS7-IgG), and the dose-to-blood is calculated to be well below the MTD. We note that the MTD could not be approached due to the relatively low specific activity of the labeled MAb produced. The therapeutic effect of ^{131}I-DLT-RS11 on individual animals is shown in Figure 3. A single dose of 100 μCi of ^{131}I-DLT-RS11 was found to cause tumor regression. Between weeks 3 to 7 postinjection of the radiolabeled MAbs, the tumor size in 8 of 11 animals (73%) administered ^{131}I-DLT-labeled RS11 remained smaller than at the time of MAb injection. This is compared to 2 of 14 (14%) of the tumors in the conventionally labeled ^{131}I-RS11 group and 0 of 9 in the untreated group. Figure 4 shows the mean tumor volumes of the three treatment groups. The mean tumor volumes at 7 wk postinjection were 107% and 358% of the volume at the time of injection, for the ^{131}I-DLT- and conventionally iodinated-RS11 groups, respectively. The doubling time of the tumors in the untreated animals was 1.9 wk. The untreated animals were killed at the 6-wk time point since tumors had increased to a mean volume of greater than 3 cm^3. These results demonstrate that the therapeutic efficacy observed after treatment with 100 μCi ^{131}I-DLT-labeled RS11 is superior to the 4 to 5 wk of growth suppression observed previously using 250 μCi of ^{131}I-conventionally labeled RS7, and to the 100 μCi ^{131}I-conventionally labeled intact RS11 IgG examined in this study.

Toxicity was monitored by weekly measurement of body weight. The animals did not experience any detectable weight loss from the radioantibody doses administered. Blood counts were monitored in a subset of the animals without evidence of toxicity in any of the groups.

DISCUSSION

This study demonstrates that the residualizing radioiodine label yields improved radioimmunotherapy in comparison to

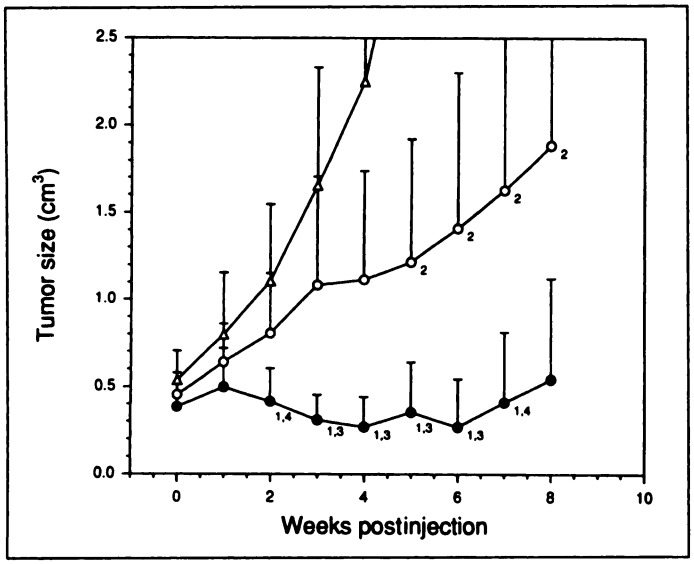

FIGURE 4. Mean tumor growth. Points represent the mean tumor size of the three treatment groups presented in Figure 3. Error bars represent s.d. and are shown only above the symbol for clarity. (●) = ^{131}I-labeled DLT-RS11; (○) = conventional ^{131}I-labeled RS11; (△) = untreated. $^{1}p < 0.001$ between treated and untreated groups; $^{2}p < 0.01$ between treated and untreated groups; $^{3}p < 0.001$ between the two treatment groups; $^{4}p < 0.01$ between the two treatment groups.

the MAb labeled by the conventional chloramine-T procedure. A single injection of 100 μCi ^{131}I-DLT-labeled RS11 caused tumor regression in vivo. Seven weeks after the administration of ^{131}I-DLT-labeled RS11 to animals with established tumors, 73% of the tumors were smaller than at the time of MAb injection. The findings in the present study extend our previous observations, in which marked improvements in tumor accretion of the radiolabel and in tumor-to-nontumor localization ratios were seen with ^{131}I-labeled DLT-MAbs (*16*), and support the validity of the dosimetry calculations made previously.

The activity level injected in our therapy experiments is predicted to be approximately 3–4-fold below the MTD. Our current focus is to improve the conjugation efficiency with DLT, or a similar residualizing label, to allow full evaluation of the therapeutic potential which is predicted by the dosimetry analysis. The low conjugation efficiency with DLT makes it difficult to determine the MTD directly, since the protein dose required would be excessive in the mouse model. In preliminary experiments, we have demonstrated an MTD for ^{131}I-DLT-RS11 of approximately 350 μCi in nude mice bearing Calu-3 xenografts, but these experiments required a protein dose of approximately 1.0 mg/mouse (unpublished data). This is consistent with our dosimetry calculations, which predict an MTD for both ^{131}I-DLT and conventional ^{131}I of approximately 300–400 μCi. These values are expected to be similar because this calculation depends on the blood clearance rate, which is similar for both labels.

Chelated radiometals are also trapped within cells, and thus represent an alternate source of residualizing labels available for radioimmunotherapy (*12*). Our previous studies predict an increase of approximately 2-fold in absorbed dose to tumor using the ^{131}I-DLT-labeled MAbs in comparison to ^{90}Y, due to the longer physical half life of ^{131}I (*16*). This advantage would also be predicted using radiometals of longer half-life, such as ^{177}Lu [which has a 6.7 day half-life (*29*)], assuming that they are trapped within cells as well as DLT or indium- or yttrium-DTPA.

Although promising results have now been reported with two different residualizing iodine labels, differences exist between the products. The tyramine-cellobiose labeling method is somewhat simpler and more efficient, but it has a consistent tendency to generate a significant level of antibody aggregates, because of the cyanuric chloride used to conjugate tyramine-cellobiose to protein (*15,17*). Although we have not tested tyramine-cellobiose conjugation, we have used cyanuric chloride in a similar conjugation, with 5-([4,6-dichlorotriazin-2-yl]amino) fluorescein (DTAF), and also observed a similar problem with aggregation (*7*). In contrast, DLT labeling has never resulted in detectable levels of aggregates, which would have been seen in the gel filtration HPLC that is routinely performed (unpublished data). We note that DLT has two potentially reactive sites, which might yield protein cross-linking. The fact that this does not occur cannot currently be explained, but constitutes an advantage of this method over the use of tyramine-cellobiose. The only direct comparison between DLT and tyramine-cellobiose was performed by Strobel et al. (*30*). They did not detect cross-linking induced by tyramine-cellobiose, but they were labeling proteins other than antibodies, and the extent of cross-linking is likely to be protein-dependent. Another difference between these two labels may also be significant, namely the extent of biliary secretion, which is higher with tyramine-cellobiose labeled proteins (*30*). It is likely that neither of these labeling methods is optimal. The advantage of residualizing radiolabels, demonstrated here, should stimulate the development of improved labeling methodology having greater efficiency and simplicity.

CONCLUSION

By increasing the residence time of radioiodine at the tumor site, residualizing labels such as DLT allow an increased radiation dose to tumor, while maintaining a lower dose to the dose-limiting organs and thus promise to improve the therapeutic index of radioimmunotherapy. We showed that ^{131}I-DLT-labeled RS11 provides a therapeutic advantage in comparison to conventionally labeled ^{131}I-labeled RS11 for tumor therapy in vivo. Since dosimetry calculations predict that longer half-life isotopes will be advantageous if biodistribution is unchanged, we predict that residualizing iodine should be better than ^{90}Y, but direct therapeutic comparisons must be made.

ACKNOWLEDGMENTS

We thank the excellent technical assistance of Susan Chen, Rosarito Aninipot, Philip Andrews and Rincel Jusino. This work was supported in part by USPHS grants CA60039, CA63624 and CA39841 from the NIH.

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Pharmacokinetics and Experimental PET Imaging of a Bromine-76-Labeled Monoclonal Anti-CEA Antibody

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Bromine-76 is potentially useful as a radiolabel for monoclonal antibodies (MAbs) in PET imaging. The purpose of the present study was to evaluate the ^{76}Br-labeled anticarcinoembryonic antigen (-CEA) MAb 38S1 as a tumor imaging agent in an experimental tumor model and to study the pharmacokinetics of ^{76}Br-38S1 in comparison with ^{125}I-38S1. **Methods:** Nude rats carrying human colon carcinoma xenografts were co-injected with directly labeled ^{76}Br-38S1 and ^{125}I-38S1. Biodistribution of labeled 38S1 was monitored for 4 days after administration, in the case of ^{76}Br activity, including PET imaging. In addition, catabolism of radiolabeled MAbs was analyzed by gel filtration chromatography of blood plasma and homogenized tissues. **Results:** Tumor sites could be readily identified by PET imaging from 46 hr after administration of ^{76}Br-38S1 and onwards. The concentration of ^{76}Br activity in tumors, blood and most normal tissues was higher than the corresponding ^{125}I concentration at all time points. This was mainly due to catabolism of radiolabeled MAb, resulting in free radiohalides, of which $^{76}Br^-$ was retained in contrast to the rapidly excreted $^{125}I^-$ ion. **Conclusion:** Bromine-76-labeled anti-CEA MAbs may be applied for experimental tumor imaging with PET.

Key Words: bromine-76; MAbs; PET; radioimmuno PET

J Nucl Med 1997; 38:395–401

For the imaging of monoclonal antibodies (MAbs) using PET, the choice of radiolabel is of major importance. The PET technique offers a higher resolution than SPECT and also allows radionuclide quantification with high accuracy. However, the production and use of suitable positron-emitting radionuclides for this purpose are less well-established than those of the standard SPECT radionuclides.

The physical half-life of a positron emitter in relation to the pharmacokinetics of the selected antibody and the labeling methods available for each nuclide are examples of factors determining the choice of radionuclide. Short-lived positron-emitters such as the routinely available PET nuclide ^{18}F ($T_{1/2}$ 110 min) have been used as radiolabels for rapidly clearing MAb fragments (*1–4*), whereas intact MAbs require radionuclides with longer half-lives, such as ^{66}Ga ($T_{1/2}$ 9.4 hr) (*5*), ^{64}Cu ($T_{1/2}$ 13 hr) (*6*), ^{76}Br ($T_{1/2}$ 16 hr) (*7*), ^{55}Co ($T_{1/2}$ 18 hr) (*8*) and ^{124}I ($T_{1/2}$ 4 days) (*9–15*).

To date, ^{124}I has been the nuclide most extensively utilized for clinical and experimental radioimmuno PET. However, the decay properties of ^{124}I are not ideal for PET imaging purposes since the positron abundance is only 23%. Combined with the long physical half-life, this may result in unnecessarily high patient radiation doses. In the halogen group, the only other positron emitter potentially useful for labeling of intact MAbs is ^{76}Br (Table 1), which was previously used mainly for labeling of various receptor ligands (*16*). The majority of the positrons emitted in the ^{76}Br decay have a high maximum energy (3.4 MeV) which affects both image resolution and radiation dose, but 55% of the decays do result in positron emission. As a radiolabel for radioimmuno PET, ^{76}Br is therefore an interesting alternative to ^{124}I.

Similar principles may be applied for direct bromination as for iodination of proteins, i.e., oxidation of the halide, although a more powerful oxidant may be required for sufficient yield in

Received Feb. 26, 1996; revision accepted Jun. 13, 1996.
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