Development of a Stable Radioiodinating Reagent to Label Monoclonal Antibodies for Radiotherapy of Cancer

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A method of radioiodinating monoclonal antibodies such that the labeled antibodies do not undergo in vivo deiodination has been studied. The method utilizes conjugation of succinimidyl *para*-iodobenzoate to the antibody. The iodobenzoate was radiolabeled by using an organometallic intermediate to facilitate the reaction. Thus, succinimidyl *para*-tri-*n*-butylstannylbenzoate was radiolabeled in 60–90% radiochemical yield and subsequently conjugated to the antibody in 80–90% yield. Animal biodistribution studies were carried out with two separate anti-melanoma antibodies (9.2.27 and NR-M1-05) labeled by this method, and examined in nude mice bearing human melanoma tumor xenografts. Very large differences in the localization of radioactivity were observed in the thyroids and stomachs of mice when the iodobenzoyl-labeled antibodies were compared with the same antibodies labeled using the chloramine-T method of radioiodination. Few other significant differences in the tissue distribution of the radioiodinated antibodies were seen.

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Radioiodinated monoclonal antibodies are of interest for diagnosis and radiotherapy of cancer (*1,2,3*). The specific localization and retention of radioactivity at tumors by using radioiodinated antibodies developed against antigenic sites on cancer cells holds great promise (*4,5*). Indeed, this promise prompted studies using radioiodinated polyclonal antibodies some three decades ago (*6*). Since that time many investigations into the use of radiolabeled antibodies for diagnosis and therapy have been carried out. In the majority of the previous studies the radionuclide used has been iodine-131 (^{131}I). While ^{131}I may not be the ideal radionuclide for radiotherapy (*7*), its medium-energy beta emissions can kill cells and it has been proven to be useful in the therapy of thyroid cancer (*8,9*). Its radiotherapeutic potential and its very good commercial availability make ^{131}I an attractive radionuclide for radiotherapy.

The chemistry associated with radioiodination of monoclonal antibodies and other proteins has been studied extensively (*10–14*). For example, many scientific investigations have been carried out to determine which oxidant might be used with radioiodide to obtain efficient labeling without damaging the biologic properties of proteins. These studies have pointed out that the first radioiodinating reagents, iodine and iodine monochloride (*15*), could be replaced by iodide and oxidizing agents like chloramine-T (*16*) and tetrachlorodiphenylglycouril (IODO-GEN) which presumably generate iodine monochloride in situ (*17*). However, it has been noted that even under very mild oxidizing conditions some proteins lose a portion of their biologic activity, most likely from oxidation of thiols or from iodination of a tyrosine that is involved in a specific interaction such as binding. This fact prompted Bolton and Hunter to develop a reagent (*18*), N-succinimidyl *para*-hydroxyphenylpropionate **1** which could be reacted with (or conjugated to) a protein under milder conditions than those found in direct radioiodinations. Other reagents such as *para*-hydroxybenzimidate **2** (Wood's reagent) (*19*), *para*-hydroxybenzaldehyde **3** (*20*), and *para*-hydroxybenzacetaldehyde **4** (*21*) have also been studied as radioiodinating reagents (Fig. 1).

Although these radioiodinating reagents, **1–4**, do not appear to alter the biologic activity of the labeled proteins, the in vivo use of the reagents has been found to be limited in some cases as a result of in vivo deiodi-

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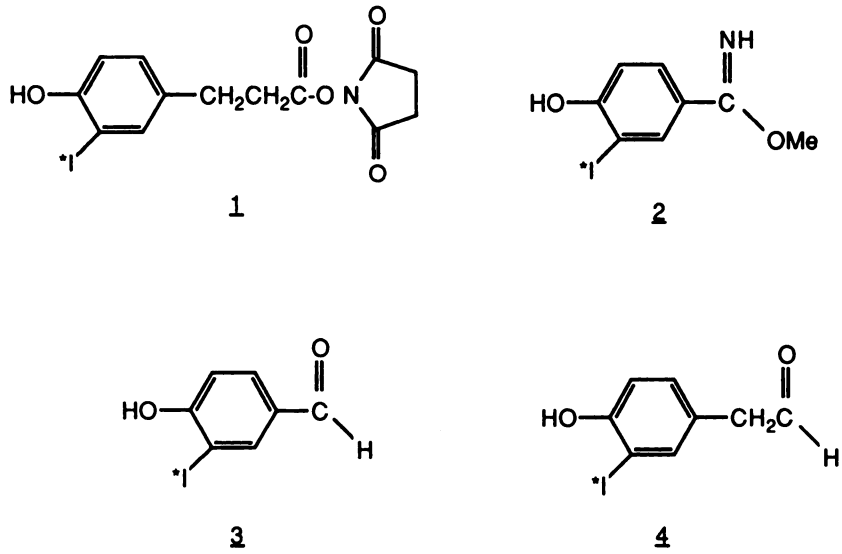


FIGURE 1
Reagents used for protein radioiodinations.

nation (*22*). Although an antibody may be efficiently radioiodinated and retains its immunoreactivity toward the antigen containing tumor cells, enzymatic deiodination (*23–25*) of the labeled antibody or antibody catabolites may decrease the concentration of radioiodinated antibody available for tumor binding.

Reasoning that a protein labeled with radioiodine attached to a nonactivated (nonphenolic) aromatic ring might not be susceptible to in vivo dehalogenase activity, the radioiodination of small molecules that could be conjugated to the protein via a covalent bond analogous to the Bolton-Hunter reagent was investigated. Further, the studies were limited to the evaluation of the *para*-substituted aromatic ring containing radioiodinating reagents because of a concern about potential enzymatic hydroxylation which occurs on phenylalanines to produce tyrosine (*26*). Such *para*-hydroxylation might render the radioiodine susceptible to dehalogenase activity. A generalized scheme for these reagents is depicted in Figure 2.

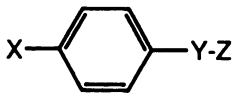
Reported herein are the results of studies employing a radioiodinated conjugating reagent, succinimidyl *para*-iodobenzoate **9**, which is a simple representative of compounds depicted in Figure 2. A number of other reagents were studied (*27*), but the final choice of **9** was determined by its stability, radioiodination yields, protein conjugation yields and the knowledge that the benzoate should be rapidly cleared from all tissues (*28*).

MATERIALS AND METHODS

Method of Labeling

The initial task in the development of **9** was to identify which method of radioiodination could be used. Iodide exchange reactions using copper with iodo- and bromobenzoic acids have been described (*29*), but isotopic exchange with iodine-127 (^{127}I) would not give high enough specific activity for protein labeling. Similarly, nonisotopic exchange with bromobenzoic acid would be of low specific activity unless difficult high performance liquid chromatography (HPLC) separations were made. Furthermore, the reaction conditions of the exchange, would not be compatible with our targeted "active ester", compound **9**. Likewise the use of diazonium salts (*30*) or modifications thereof (*31*) did not appear to be compatible with the desired compound. An alternate approach to these standard labeling methods was sought.

In recent years, many studies of incorporating radioiodine and other radiohalogens (*32*) into aromatic compounds have been carried out using organometallic intermediates. These studies have shown that organothallium (*33*), organomercury (*34*), organoboranes (*35*), organosilanes (*36*), organosilicates (*37*), organogermanes (*38*), and organostannanes (*39,40*) can be used to rapidly and efficiently incorporate radioiodine into



Where:

X = radioiodine or group exchangeable for radioiodine (precursor)

Y = linking functionality

Z = functional group capable of reacting with protein

FIGURE 2
General scheme for protein conjugates.

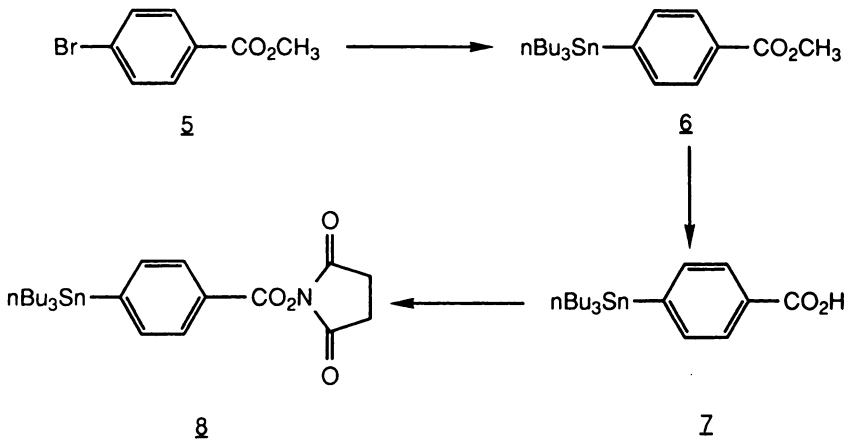


FIGURE 3
Syntheses of the organometallic intermediate, **8**.

many different aromatic compounds. Of these organometallic intermediates, the organostannanes have been particularly attractive due to their ease of preparation and handling, relative stability, and broad reactivity with radiohalogens. Based on these considerations, succinimidyl *para*-tri-*n*-butylstannylbenzoate, **8**, was targeted for synthesis and radioiodination studies. The syntheses leading to preparation of **8** are shown in Figure 3, and the subsequent steps to obtain radioiodinated proteins by employing **9** are depicted in Figure 4.

General

IR spectra were obtained on a Perkin-Elmer 1310 Infrared Spectrophotometer. Nuclear magnetic resonance (NMR) spectra were obtained on a Varian EM360 60 MHz instrument or a Varian Gemini-200 200 MHz instrument. Mass spectral data were obtained on a VG 7070H mass spectrometer. Plasma emission spectral analyses were obtained from AMTEST in Redmond, WA. Radioiodine was obtained from Dupont/NEN (North Bellerica, MA). Iodine-125 (^{125}I) was obtained as a 0.1N NaOH solution, high concentration, at 17 Ci/mg in 2 and 5 mCi quantities. Iodine-131 was obtained as a high concentration 0.1N NaOH solution in 5 mCi quantities with specific activities of 7–12 Ci/mg.

All reagents used were reagent grade or better and were used as purchased. Methyl 4-bromobenzoate was obtained from Alfa Products, Danvers, MA. Anhydrous THF was prepared by distillation from Na/benzophenone and used immediately. HPLC solvents were obtained as HPLC grade and were filtered (0.2 μm) prior to use. For gradient mixtures, pure solvents were mixed with a minimum (2%) of the opposing solvent and degassed prior to use. The phosphate buffered saline was purchased from Gibco Labs as Dulbecco's phosphate buffered Saline-(#310-4190).

High performance liquid chromatography was conducted with Beckman Model 110B pumps, Beckman Model 153 uv detector, Rheodyne Model 7125 injector, and Beckman Model 170 radioisotope detector. Spectrophotometric analyses of the samples were performed by uv detection at 254 nm, and radiometric analyses were achieved by NaI scintillation optimized to the radionuclide used. Integration

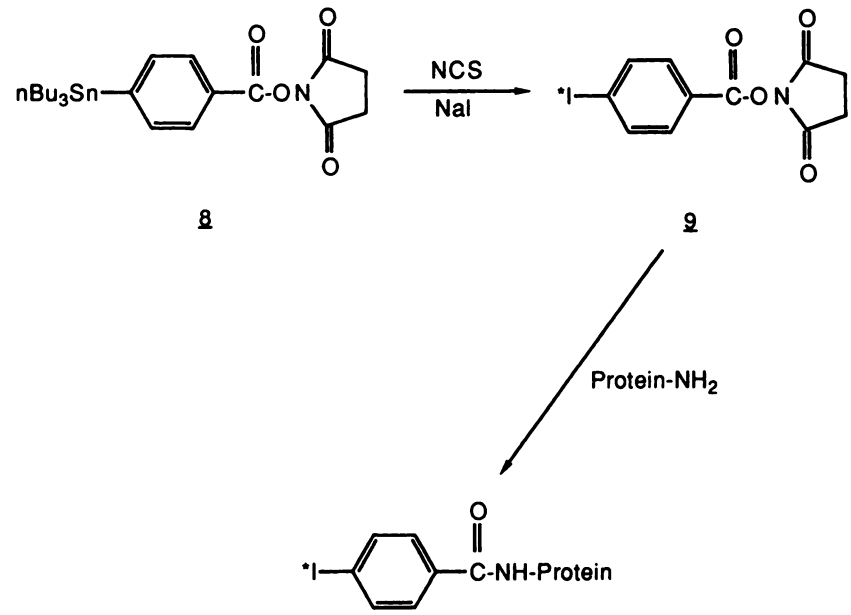


FIGURE 4
Scheme for radioiodinating proteins.

and plotting of the chromatograms was accomplished by either an integrator/plotter (Hewlett-Packard, Model 3390) or computer with a chromatographic software package (Dynamic Solutions, Maxima 820 workstation).

Size exclusion, chromatography was performed on a Zorbax Bio Series GF-250 9.4 mm × 24 cm column (Dupont) with 0.2*M* phosphate, pH 6.8 as the solvent system, eluting at a flow rate of 1.0 ml/min. Using these conditions, the retention time for whole antibody was 8.5 min, $F(ab')_2$ was 9.0 min, Fab was 10.5 min, and the iodobenzoate or iodide was 13.0 min.

Reverse-phase chromatography was run on a Partisphere, 4.6 mm × 12.5 cm, C-18 column (Whatman) using a gradient solvent system. Solvent A in the gradient was 98% MeOH with 2% of a 1% HOAc/H_2O solution. Solvent B was 10% MeOH in a 90% solution of 1% HOAc/H_2O. The gradient was begun at a 50:50 mixture of solvents A and B. After 3 min the gradient was decreased in % B over the next 7 min to 2% B and was held there for 10 min. Using this gradient, retention time for iodide was 1.2 min (solvent front), *para*-iodo succinimidyl ester **9** was 6.0 min, *para*-iodobenzoic acid was 7.3 min, and *para*-stannyl succinimidyl ester **8** was 17.2 min.

Thin layer chromatography (TLC) was performed on silica gel impregnated glass fiber strips (ITLC-Gelman). The silica plates were activated by heating at 110°C for 30 min prior to use. After elution, the plates were cut into small strips (horizontal) and were counted to detect radioactive samples. Radioactive samples were counted in a dose calibrator (Capintec, CRC-7) for μCi (37 kBq) or larger quantities or in a gamma counter (Packard, Autogamma 5650) for smaller quantities. Iodine-125 (^{125}I) samples were counted in plastic tubes routinely. Iodine-125 was counted using a 15-80 keV window and ^{131}I was counted in a 260-470 keV window. In dual labeling experiments, the ^{131}I counts were measured by standards and the contribution to the ^{125}I window was subtracted from the ^{125}I counts.

Nude mice were obtained from Dr. Giovanella (Houston, TX) under a contract.

Preparation of methyl 4-tri-n-butylstannylbenzoate, 6

Methyl 4-bromobenzoate (5 g, 23 mmol), **5**, was dissolved in anhydrous toluene (100 ml). The resulting solution was degassed by bubbling nitrogen (N_2) through the solution. Hexabutylditin (27.3 ml, 54 mmol) was added via syringe, followed by tetrakis(triphenylphosphine)palladium(0) (0.269g, 0.23 mmol). The solution was refluxed under N_2 for 24 hr. After cooling to room temperature, the solvent was evaporated under reduced pressure to afford ~30 ml. This sample was applied to a silica gel column (45 × 5 cm pre-equilibrated with 100% hexanes) and eluted with 100% hexanes collecting 25 ml fractions. After eluting with 1200 ml, the column was eluted with 10% ethyl acetate/90% hexanes, collecting 25 ml fractions. Fractions which contained the product were combined, and the solvent was evaporated under reduced pressure to afford 8.6 g (88%) of **6**. NMR ($CDCl_3$,δ) 8.00 (d,2H,J=8Hz), 7.55 (d,2H,J=8Hz), 3.90 (s,3H), 0.50–2.20 (m,27H). IR (neat, cm^{-1}) 2960, 2920, 2870, 2850, 1725, 1585, 1445, 1420, 1270, 1110.

Preparation of 4-tri-n-butylstannylbenzoic acid, 7

To a solution of **6** (3.0 g, 7 mmol) in absolute ethanol (60 ml) was added 0.25 g KOH (9 mmol). The resulting solution was refluxed for 2 hr. The solution was cooled and poured into an ice-cold solution of acetic acid (0.64 g, 10.6 mmol) and water (100 ml). The solution was then extracted with diethyl ether (80 ml). The ether phase was washed with water (30 ml), dried over $MgSO_4$ (5 g), filtered and evaporated under reduced pressure to afford 2.6 g (88%) of **7**. This material was used without further purification. NMR ($CDCl_3$,δ) 8.00 (d, 2H, J=8 Hz), 7.50 (d, 2H, J=8Hz), 0.5–2.20 (m,27H).

Preparation of N-succinimidyl 4-tri-n-butylstannylbenzoate, 8

To a solution of **7** (2.0 g, 4.9 mmol) in anhydrous THF (50 ml) was added N,N′-dicyclohexylcarbodiimide (1.2 g, 5.8 mmol) followed by N-hydroxysuccinimide (0.67 g, 5.8 mmol). The resulting solution was kept at 5°C for 18 hr. The solution was then filtered and concentrated under reduced pressure. A solution of 10% ethyl acetate/90% hexanes (25 ml) was added and the resulting solution was again filtered. The solution was concentrated to ~5 ml under reduced pressure. This material was applied to a silica gel column (19 × 2.5 cm, pre-equilibrated with 25% ethyl acetate/75% hexanes) and eluted with 25% ethyl acetate/75% hexanes collecting 5 ml fractions. The fractions were analyzed by HPLC to assess the purity of the samples. Fractions which were greater than 99% pure were combined. The solvents were removed under reduced pressure to afford 0.9 g (36%) of **8**. ^1H NMR ($CDCl_3$,δ) 0.50–2.20 (m,27H), 2.90 (s,4H), 7.65 (d, 2H, J=8Hz) 8.06 (d, 2H, J=8Hz). IR (neat cm^{-1}) 1,780, 1,750, 1,190, 1,055, 980. ^{13}C NMR ($CDCl_3$,δ), 169.6, 162.6, 153.5, 137.0, 129.2, 124.5, 28.9, 27.2, 25.5, 13.5, 9.5. MS (CI, CH_4) 510 (1.67%, M + 1), 508 (1.34%, M + 1), 452 (57.4%), 450 (43.5%), 395 (100%), 393 (75.3%), 291 (82.4%), 289 (61.7%).

Radioiodination of 8 and antibody labeling

Into a reaction vial fitted with a septum was placed 50 μl of a 0.25 mg/ml solution of **8** in 1% HOAc/MeOH (0.025 μmol), 10 μl of a 1.0 mg/ml solution of N-chlorosuccinimide in MeOH (0.075 μmol), and 10 μl of phosphate buffered saline. The desired quantity of $Na^{125}I$ or $Na^{131}I$ was added (up to 5 mCi, volume not to exceed 10 μl if diluted with PBS). After 5 min at room temperature the reaction was quenched by the addition of 10 μl of a 0.72 mg/ml aqueous solution of $NaHSO_3$ (10 μl, 0.075 μmol). An aliquot was removed for HPLC analysis. The MeOH was evaporated by passing a stream of N_2 gas through the reaction vial by means of a needle inlet and a needle outlet which was connected to a drying tube packed with granulated charcoal to trap any volatile iodine. After the MeOH has been evaporated (~8–10 min) a solution of the protein in 0.5*M* sodium borate buffer, pH 9.3, was added. The protein solution was prepared by mixing the protein (minimum of 500 μg protein at 5 mg/ml) with 100 μl borate buffer. The conjugation reaction was allowed to proceed for 5 min at room temperature. The crude labeled protein was purified by either gel filtration (Sephadex G-25, PD-10) or by ultrafiltration (Centricon, 10,000 molecular weight cutoff). The radiochemical purity of the labeled protein product was assessed by ITLC.

Immunoreactivity Assessment

The immunoreactivities of the radioiodinated antibodies were determined from a cell-binding assay using a fixed antibody concentration and a varied number of cells (*41*). Dupli-

cate 110-μl aliquots of 25, 20, 15, and 10 × 10^6 cells/ml were added to eight 0.5 ml tubes containing 55 μl of the assay buffer (PBS 11% BSA/0.1% sodium azide). To a ninth tube containing 55 μl assay buffer was added 110 μl of the 25 × 10^6 cells/ml solution, followed by 55 μl of a 200-μg/ml solution of unlabeled antibody. The tubes were mixed and incubated at 4°C for 1 hr. Then 55 μl of a 160-ng/ml solution of the radioiodinated antibody was added to each of the nine tubes. The tubes were incubated (with mixing) for 2 hr at 4°C. Aliquots of 200 μl of the labeled antibody/cell reaction mixtures were withdrawn from the tubes and overlayed onto an oil mixture (*42*). The overlayed oil tubes were cooled to 4°C, centrifuged for 30 min, and cut through the oil layer to separate the cell pellet from the aqueous layer. The cut tubes were placed into 12 × 75 mm glass tubes and counted in a radioisotope counter. The percent bound was calculated from the counts in the cell pellet divided by total counts (×100). The duplicates were averaged and the nonspecific bound obtained from tube nine was subtracted to give the percent of specific binding. Adjustments to the specific binding were made by the ITLC purities.

Values obtained for immunoreactivities of radiolabeled whole NR-ML-05 have been 87 ± 8% (n = 14), for 9.2.27 $F(ab')_2$ values of 89 ± 10% (n = 10) have been obtained, and for the monovalent NR-ML-05 Fab values of 72 ± 11% (n = 50) have been obtained. The measured immunoreactivities of the radioiodinated antibodies used in animal biodistributions described herein were within these bounds.

Animal Studies

The biodistribution studies employed whole or fragments of one of two different anti-melanoma antibodies, 9.2.27 (*43*) or NR-ML-05, labeled with **9**. The purified conjugates were injected into nude mice bearing Belovsky (*44*) melanoma xenografts.

In all experiments, nude mice were injected intravenously via the lateral tail vein and sacrificed by cervical dislocation at preselected times postinjection. Blood samples were obtained by retro-orbital bleeding. Syringes were weighed before and after injection to determine the volume delivered. The activity per unit volume was obtained from standards. The animals were weighed (typically 25 g) and marked for identification in all the experiments. A total of 13 different tissues were excised, weighed, and counted. These tissues were: blood; tail (injection site); skin; bone (femur); lung; liver; spleen; neck including thyroid (the major portion of the muscle from the front of the neck); kidneys; intestine; muscle (thighs); stomach; and the tumor. The excised tissues were blotted, weighed, and counted in a scintillation well counter. The raw counts were collected and decay corrected. Summarized data included mean ± s.d. of the percent injected dose; the percent injected dose per gram; the tissue-to-blood ratios; and the tumor-to-tissue ratios.

Challenge Experiments

Procedure. To test tubes containing 1.0 ml in each of 12% TCA, 6*M* urea, 10 m*M* dithiothreitol (DTT), 1.0*M* pH 9.5 carbonate buffer, 95% ethanol, cysteine, or PBS (as a control), and 1*M* lysine in pH 9.5 carbonate buffer was added 25 μg of the antibody conjugate **10**. The samples were lightly vortexed, an aliquot was removed and analyzed by ITLC (eluting with 80% MeOH) as the t = 0 time point. The tubes were then incubated at 37°C for 24 hr. Aliquots were periodically removed and analyzed by ITLC.

Serum Stability

Procedure. Samples of freshly isolated human serum were filtered twice through sterile 0.2-μ filters and placed in sterile, capped culture tubes. Twenty-five micrograms of antibody conjugate **10** were added to each 1.0 ml serum sample. Triplicate samples were incubated at 37°C (after vortexing and removal of zero time point for ITLC analysis, as above) for a total of 7 days (168 hr). Samples were analyzed by ITLC (in 80% MeOH) and HPLC. Time points taken for analysis were 1, 4, 24, 48, 72, 96, 120, 144, and 168 hr.

RESULTS

Chemistry

The synthetic steps to prepare the organotin intermediate **8** are shown in Figure 3. The synthetic pathway to obtain **7** involved the use of the methyl *para*-bromobenzoate **5** in a metal-halogen exchange reaction using hexabutylditin (*45*), followed by base hydrolysis of the intermediate methyl ester **6**. The conversion of the benzoate **7** to the desired succinimide ester **8** was accomplished by reaction with dicyclohexylcarbodiimide (DCC) and N-hydroxysuccinimide (NHS) in anhydrous tetrahydrofuran at 7–12°C overnight.

Radioiodination of **8** was accomplished in methanol or ethanol using N-chlorosuccinimide (NCS) and sodium iodide to give 60–95% radiochemical yields. Studies of the stability of the NHS ester in alcohol solvent indicated that 1–5% acetic acid added to the reaction medium stabilized the ester toward alcoholysis for long periods of time, with no alcoholysis of the ester detectable during the radioiodination reaction. The purity of the NHS ester **8** was found to be very important in the radioiodination reactions as the benzoate **7** reacted much faster than the ester **8**. Purification of the ester **8** to >99% (by HPLC analysis) was required to minimize the amount of iodobenzoic acid formed during the radioiodination reaction. Radioiodination of purified **8** with no-carrier-added radioiodine was found to be very facile and efficient resulting in 85–95% radiochemical yields in <5 min at room temperature with optimized reaction conditions. Under the optimized conditions, addition of phosphate buffered saline (PBS) to the radioiodine solution prior to the radioiodination reaction gave consistently higher yields. It is not clear whether this finding is due to the abundance of chloride ions in the PBS or whether traces of nonradioactive iodide (^{127}I) which are introduced from the PBS facilitated the reaction. However, the former is suspected as slightly better yields were obtained when PBS was added even at carrier-added levels of radioiodine.

The final reaction step in the preparation of **9** was the addition of sodium metabisulfite to reduce any unreacted electrophilic iodine species to iodide. The

reduction to iodide assures that there will be no reaction of radioiodine with protein tyrosines upon mixing. The quenching of excess NCS with metabisulfite is also beneficial as no oxidizing agent is presented when the radioiodination reaction mixture is combined with the protein to be labeled. The quantity of metabisulfite is kept to a minimum as higher quantities have been observed to be deleterious to some antibody preparations.

Conjugation of the radioiodinated **9** with antibodies afforded yields varying from 40% to 90% of the adduct **10**. The conjugation efficiency of **9** appears to be very dependent on the concentration of antibody. For example, a 1-mg protein sample in 0.9 ml of reaction solution (1.1 mg/ml) of borate buffer at pH 9.0 gave a conjugation yield of 54%, whereas under the identical reaction conditions, 5 mg of protein gave a conjugation yield of 92%.

A study was carried out to determine if purification of **9** was necessary prior to its reaction with an antibody. In the study, identical labeling yields were obtained when the reaction mixture was purified by HPLC and when it was mixed directly with the buffered protein. Additionally, insignificant differences were observed when purified and nonpurified preparations were examined in animal distributions. Since these observations offered a simplification of the labeling procedure, most subsequent labelings have been carried out without purification of **9** from the other radioiodination reaction components prior to conjugation. However, it is anticipated that when small quantities of protein (<100 μg) are utilized, a purification step may be needed for improved yields.

The initial investigations employed a 0.5*M* borate buffer pH 9.3, giving a final protein conjugation mixture pH of 8.5 to 9.0. Later experiments were conducted with 1.0*M* sodium carbonate/bicarbonate buffer to alleviate in vivo toxicity concerns. The high concentrations of the buffers were necessary to keep the protein solution pH ~9, as the radioiodinated reagent **9** was in an acetic acid solution during the radioiodination step. The effect of the pH on the conjugation reaction yields was studied. While a linear relationship was found with increasing pH, a conjugation pH of 9 was routinely used because very good conjugation yields could be obtained at that pH when other reaction parameters such as protein concentration, were optimized.

In Vitro Analyses

After the conjugation reaction had proceeded for 10 min, the reaction product was purified by gel filtration (Sephadex G-25, PD-10). Radioiodinated antibody conjugate purities of 95% to 99% were normally obtained.

The labeled antibodies were routinely analyzed by HPLC using size exclusion columns, isoelectric focusing (IEF), and radiolabeled cell binding assay. In virtually all of the examples, the HPLC retention times and peak shapes were identical to those obtained prior to the radiolabeling reactions. They were also identical to those for the direct labeling with chloramine-T. In a few experiments, it was noted that when the concentration of methanol or ethanol was >20% in the buffered protein solution, there was an HPLC peak with a shorter retention time, indicating that protein aggregates were being formed. The immunoreactivities of the antibodies labeled with **9** equivalent to those obtained for the same antibody labeled directly via chloramine-T.

Concern for toxicity that might be imparted by having the aryltin **8** remaining in the labeled protein preparation, either attached to or associated with the protein, led to an evaluation of how much aryltin was present in the final preparation. Surprisingly, analysis by plasma emission spectroscopy of the labeled protein after digestion has shown that there was no detectable tin associated with the protein even when ten equivalents of aryltin **8** was reacted with the protein under the usual conjugation reaction conditions. The detectability of organotin was estimated to be 3 ng or ~100 times less than the amount which would have been present from the aryltin added in the reaction.

In vitro assessment of whether any of the radioiodinated active ester **9** was associated with the protein without being bonded to it, or bonded to a group other than an amine, was carried out by challenge experiments. In the experiments radioiodinated antibodies were subjected to denaturation with 12% TCA, 6*M* urea, or 95% ethanol. Other reagents which might free associated **9** and/or denature the protein were also used in the challenge experiments. These included dithiothreitol, cysteine, lysine, and 1.0*M* pH 9.5 carbonate buffer. In the challenge experiments the postchallenge purity was essentially the same as the prechallenge purity. These results confirmed that a very stable attachment of the radioactivity was being obtained.

Incubation of antibodies labeled with **9** in freshly isolated human serum was also carried out. Monitoring the purity of the radioiodinated antibodies by ITLC demonstrated that it was very stable in human serum, with essentially no change in purity over a seven day period.

Storage of ^{125}I-labeled antibodies would permit radioiodinated preparations to be used as standards over extended periods of time. Thus, in vitro stability of refrigerated (4°C) antibodies labeled with **9** was compared to that of the in vitro stability of the same antibody labeled with chloramine-T, using the immunoreactivity as the indicator of relative stability with time. The results indicated that exceptional stabilities are obtained for the storage of up to a 1-mo period of an anti-melanoma antibody (9.2.27) labeled with either method.

In Vivo Evaluations

Evaluation of radioiodinated conjugates which would resist in vivo deiodination was most readily accomplished by studying the distribution of the labeled antibody in nude mice. The in vivo stabilities toward deiodination of a whole antibody, a $F(ab')_2$ fragment, and a Fab fragment were of interest as the differences in metabolism are not well understood and each may have different rates of deiodination. The antibody chosen for the in vivo evaluations was NR-ML-05 an antimelanoma antibody which reacts with the 250 kD proteoglycan antigen on melanoma cells. While it would have been desirable to study the in vivo stability of NR-ML-05 and its fragments alone, NR-ML-05 which is an IgG_{2b} subclass antibody does not yield a $F(ab')_2$ fragment. This is typical for the IgG_{2b} subclass. Thus, the $F(ab')_2$ fragment of another anti-melanoma antibody 9.2.27, (*43*), an IgG_{2a} subclass antibody which reacts with the same antigen was employed.

Evaluation of the biodistributions were carried out in nude mice bearing human (Belovsky) tumor xenografts. Deiodination was evaluated by the accumulation of the radioactivity in the thyroid (neck) and stomach. As a control, a biodistribution of hydrolyzed radioiodinated benzoate, **9**, obtained from the normal labeling procedure without added protein, was carried out (Table 1). As expected all tissues decreased in radioactivity from 2 hr to 72 hr except for the neck, and the highest concentration of radioactivity at 2 hr was the stomach (presumably from free contaminating iodide).

An evaluation of the in vivo deiodination of whole NR-ML-05 labeled by using **9** or by direct labeling with chloramine-T was carried out. In the experiment 50 μg of labeled protein was injected into each mouse. Four nude mice were killed per timepoint for each of the two radioiodinated antibody preparations. The antibody labeled with **9** had a purity of 98.3%, a specific activity of 0.34 μCi/μg, and an immunoreactivity of 89%. The antibody labeled with chloramine-T had a purity of 99.6%, a specific activity of 0.48 μCi/μg, and an immunoreactivity of 85%. The data obtained from the biodistributions are presented in Table 2. It is important to note that while the thyroid activity decreased over the eight day study when labeled with **9**, it increased significantly over the same period in the animals injected with antibody labeled with chloramine-T.

In another experiment, 9.2.27 $F(ab')_2$ was labeled with **9** or via chloramine-T. A 5-μg quantity of the labeled antibody fragment was injected into each of 24 nude mice for each labeling method. The $F(ab')_2$ labeled with **9** had a purity of 99% and a specific activity of 0.10 μCi/μg. The $F(ab')_2$ labeled by using chloramine-T also had a purity of 99% and a specific activity of 0.10 μCi/μg. The biodistribution data for the two groups of animals are given in Table 3.

A dramatic difference was observed in the accumulation of radioactivity in the stomach and neck of the two sets of animals, but the distribution of activity in the other tissues was comparable. The large difference in the observed thyroid (neck) activity is more readily

TABLE 1
Biodistribution of Hydrolyzed **9** (^{125}I) in Nude Mice*

Tissue	2 hr	72 hr
Blood	0.33 ± 0.02	0.03 ± 0.00
Tail	0.68 ± 0.33	0.17 ± 0.20
Skin	0.25 ± 0.04	0.06 ± 0.01
Muscle	0.11 ± 0.01	0.02 ± 0.00
Bone	0.19 ± 0.02	0.04 ± 0.01
Lung	0.28 ± 0.03	0.05 ± 0.01
Liver	0.89 ± 0.14	0.26 ± 0.03
Spleen	0.57 ± 0.10	0.28 ± 0.04
Stomach	4.52 ± 1.43	0.07 ± 0.02
Neck	2.37 ± 1.02	2.99 ± 0.98
Kidney	0.38 ± 0.03	0.07 ± 0.00
Intestine	0.67 ± 0.11	0.05 ± 0.02

* Results were obtained from N = four mice per time point and are tabulated as mean ± s.d. % injected dose/g.

TABLE 2
Distribution of NR-ML-95 Labeled with Chloramine-T or **9** in Nude Mice Bearing Melanoma Xenografts*

Tissue	2 days	8 days
	Labeling with **9**	
Blood	15.10 ± 1.6	3.53 ± 0.95
Tail	1.91 ± 0.89	0.43 ± 0.09
Tumor	12.21 ± 1.05	5.07 ± 1.26
Skin	2.39 ± 0.50	0.77 ± 0.26
Muscle	2.56 ± 0.49	0.74 ± 0.24
Bone	1.23 ± 0.08	0.36 ± 0.07
Lung	3.70 ± 1.06	1.03 ± 0.18
Liver	2.34 ± 0.51	0.56 ± 0.12
Spleen	2.02 ± 0.29	0.60 ± 0.16
Stomach	0.55 ± 0.13	0.14 ± 0.05
Neck	3.10 ± 0.83	1.12 ± 0.29
Kidney	2.42 ± 0.45	0.65 ± 0.16
Intestine	0.61 ± 0.11	0.14 ± 0.02
	Labeling with chloramine-T	
Blood	17.43 ± 1.68	5.29 ± 0.96
Tail	1.96 ± 0.21	0.59 ± 0.21
Tumor	15.46 ± 4.22	7.19 ± 2.14
Skin	3.79 ± 0.14	1.03 ± 0.12
Muscle	2.89 ± 0.14	1.02 ± 0.45
Bone	1.39 ± 0.10	0.44 ± 0.17
Lung	5.62 ± 0.50	1.91 ± 1.03
Liver	3.28 ± 0.35	1.85 ± 2.09
Spleen	3.44 ± 0.38	0.93 ± 0.57
Stomach	1.96 ± 0.27	0.47 ± 0.12
Neck	5.79 ± 1.57	8.38 ± 4.26
Kidney	2.42 ± 0.49	0.95 ± 0.74
Intestine	0.75 ± 0.09	0.18 ± 0.07

* Results were obtained from N = four mice per time point and are tabulated as mean ± s.d. of % injected dose/g.

TABLE 3
Biodistribution of 9.2.27 F(ab')$_2$ Labeled with Chloromine-T or **9** in Nude Mice Bearing Melanoma Xenografts*

Tissue	4 hr	8 hr	12 hr	24 hr	48 hr	72 hr
			Labeling with **9**			
Blood	27.91 ± 2.48	11.48 ± 1.63	9.35 ± 0.85	2.44 ± 0.39	0.08 ± 0.02	0.05 ± 0.01
Tail	5.42 ± 0.84	3.35 ± .82	3.67 ± 1.80	1.49 ± 0.29	0.22 ± 0.05	0.26 ± 0.13
Tumor	23.52 ± 3.21	19.37 ± 2.37	18.76 ± 2.41	14.91 ± 14.91	3.14 ± 3.96	4.08 ± 2.24
Skin	4.53 ± 0.85	3.61 ± 0.52	2.60 ± 0.35	0.95 ± 0.17	0.05 ± 0.01	0.04 ± 0.01
Muscle	2.15 ± 0.20	1.56 ± 0.25	1.10 ± 0.15	0.41 ± 0.04	0.05 ± 0.03	0.04 ± 0.03
Bone	3.08 ± 0.34	2.07 ± 0.41	1.76 ± 0.15	0.75 ± 0.11	0.17 ± 0.05	0.18 ± 0.04
Lung	14.27 ± 1.25	7.89 ± 0.87	5.52 ± 1.56	2.12 ± 0.28	0.39 ± 0.20	0.18 ± 0.09
Liver	10.87 ± 1.59	7.37 ± 0.70	6.94 ± 1.34	2.19 ± 0.43	0.08 ± 0.01	0.04 ± 0.02
Spleen	7.42 ± 1.17	4.50 ± 0.51	4.55 ± 0.99	1.55 ± 0.19	0.14 ± 0.02	0.09 ± 0.04
Stomach	7.40 ± 1.76	4.90 ± 1.80	2.87 ± 0.75	1.12 ± 0.18	0.05 ± 0.00	0.05 ± 0.03
Neck	5.77 ± 0.88	4.66 ± 1.06	3.02 ± 0.51	1.86 ± 0.23	0.31 ± 0.16	0.75 ± 0.45
Kidney	42.82 ± 3.99	23.96 ± 3.60	16.53 ± 2.01	4.06 ± 0.49	0.46 ± 0.12	0.16 ± 0.05
Intestine	8.33 ± 1.14	7.05 ± 0.75	5.74 ± 1.28	2.65 ± 0.33	0.08 ± 0.02	0.04 ± 0.03
			Labeling with chloramine-T			
Blood	32.79 ± 2.09	21.73 ± 1.80	11.80 ± 3.88	4.12 ± 1.48	0.14 ± 0.02	0.06 ± 0.02
Tail	11.16 ± 4.72	8.24 ± 1.99	5.44 ± 1.41	3.41 ± 0.92	0.38 ± 0.02	0.18 ± 0.03
Tumor	23.97 ± 5.64	26.06 ± 2.55	21.15 ± 1.68	19.21 ± 5.10	5.81 ± 1.22	2.83 ± 1.27
Skin	7.33 ± 1.28	7.75 ± 0.64	5.79 ± 1.72	2.93 ± 0.92	0.13 ± 0.02	0.06 ± 0.02
Muscle	3.10 ± 0.61	2.97 ± 0.53	1.95 ± 0.50	1.01 ± 0.30	0.05 ± 0.01	0.05 ± 0.03
Bone	4.64 ± 0.53	4.41 ± 0.53	2.76 ± 1.07	1.64 ± 0.52	0.24 ± 0.10	0.18 ± 0.03
Lung	16.97 ± 2.09	14.87 ± 2.04	9.08 ± 1.65	5.27 ± 1.28	0.85 ± 0.38	0.40 ± 0.36
Liver	10.70 ± 1.53	9.23 ± 2.27	4.86 ± 0.55	2.90 ± 0.94	0.13 ± 0.02	0.05 ± 0.01
Spleen	9.96 ± 1.43	9.54 ± 1.66	5.43 ± 1.13	2.84 ± 0.60	0.21 ± 0.04	0.09 ± 0.03
Stomach	31.00 ± 9.82	32.15 ± 13.39	41.69 ± 69.73	26.39 ± 10.40	0.53 ± 0.20	0.31 ± 0.28
Neck	25.53 ± 6.79	45.20 ± 14.95	65.80 ± 38.73	116.41 ± 34.81	37.92 ± 12.51	21.46 ± 8.99
Kidney	45.30 ± 4.95	28.52 ± 5.07	11.81 ± 4.77	5.47 ± 2.79	0.23 ± 0.03	0.12 ± 0.03
Intestine	5.58 ± 0.91	5.50 ± 1.04	4.71 ± 0.98	2.49 ± 1.23	0.08 ± 0.02	0.04 ± 0.02

* Results were obtained from N = four mice per time point and are tabulated as mean ± s.d. % injected dose/g.

apparent when the data is plotted (Fig. 5). This data suggests that virtually no dehalogenation was occurring in the animals administered the antibody fragment labeled with **9**, but appreciable dehalogenation had occurred in the animals administered the antibody fragment labeled with chloramine-T.

Similar results were obtained in an animal biodistribution experiment in which Fab fragment of NR-M1-05 was labeled either with **9** or chloramine-T. The Fab labeled with **9** had a purity of 99% and a specific activity of 0.22 μCi/μg. The Fab labeled by using chloramine-T had a purity of 99% and a specific activity of 0.50 μCi/μg. In the experiment, nude mice were injected with 5 μg of radioiodinated Fab and were killed at 4 hr and 20 hr postinjection. The tissue biodistribution data for these experiments is given in Table 4.

Another animal biodistribution experiment compared whole NR-ML-05 labeled with **9** with an irrelevant antibody, MOPC-195, labeled with **9**. The radiochemical purity of the NR-ML-05 preparation was 98.5% with a specific activity of 0.81 μCi/μg. The radioiodinated MOPC-195 preparation had a 99.2% radiochemical purity and a specific activity of 0.82 μCi/μg. At 8 days postinjection (four animals/point), the labeled NR-ML-05 had a blood concentration of 3.38 ± 1.46% dose/g and the labeled MOPC-195 had a blood level of 2.76 ± 0.89% dose/g. The specific localization of the NR-ML-05 was clearly observed as it had a 13.45 ± 2.95% dose/g in the tumor at 8 days, whereas the MOPC-195 had a 1.68 ± 0.59% dose/g in the tumor.

DISCUSSION

The impetus to develop a new radioiodine labeling method came from the fact that in vivo deiodination of radioiodinated antibodies was seen as a potential problem in the development of radioimmunotherapy agents. This study focused on the question of whether or not the hypothesis that deiodination could be blocked by using the *para*-iodophenyl (PIP) substituted conjugate was correct. The results of our studies support that conclusion in that evidence of free radioiodide thyroid and stomach localization in animals was not seen over time periods of up to several days post injection.

The only limitations observed for the use of **9** in radioiodinating proteins were that an organic solvent

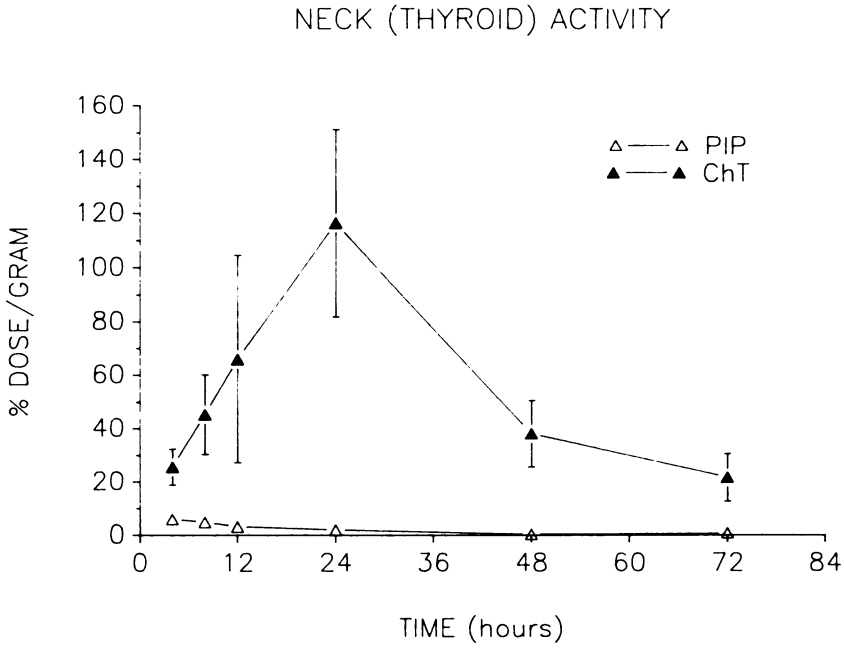


FIGURE 5
Comparison of radioactivity in the necks (thyroids) of mice injected with 9.2.27 F(ab')$_2$.

was needed in the radioiodination step and that concentrations of protein below 1 mg/ml did not give good conjugation yields. It was observed that no more than 25% (v/v) of water could be present in the radioiodination step to obtain high yields. Thus, high concentrations of radioactivity were necessary in the labeling procedure. Removal of all or part of the organic solvent (MeOH or EtOH) prior to conjugation was important, but it appeared as though up to 15% (v/v) could be maintained during the conjugation process with little or no detectable effect on the antibody. Our finding that low conjugation yields were obtained when dilute protein samples were reacted was not surprising for a biomolecular reaction where a competing hydrolysis reaction is rapid; however, it does exemplify a major limitation of using this method of radioiodination. In contrast, direct iodination of small quantities or dilute samples of protein can be accomplished in high yields. However, protein concentration is generally not a limitation as long as adequate quantities are available since methods of concentrating proteins are easily carried out. Using protein concentrations over 5 mg/ml, conjugation yields of 70–90% have been obtained consistently.

The application of an organometallic intermediate, **8**, in the radioiodination step was needed for efficient labeling at a high specific activity. Fortunately, the aryltin intermediate **8** was readily synthesized and was found to be quite stable to a variety of storage and reaction conditions. While radioiodine labeling of other organometallic derivatives of benzoic acid was also facile (*46*), the final choice of the aryltin was advantageous as the high lipophilicity of the tri-*n*-butyltin functionality caused a rapid partitioning in aqueous base, such that within detectable limits none of tin active ester **8** was found associated with the protein. This was true even though radiochemical yields up to 90% were obtained for the conjugations of the radioiodinated molecule, **9**. Further, labelings via the benzoate **9** gave conjugates which had very good retention of immunoreactivities.

The animal biodistribution studies clearly indicate that there is a difference in the distribution of the radioiodine label for whole antibody or antibody fragments when attached to protein tyrosines versus being attached to a *para*-iodobenzoyl moiety. However, the tumor uptake appears to be roughly equivalent for the antibodies labeled via either method. In a few of our animal studies selective clearance from the tumor has been noted, but it appears that this varies with the antibody and xenograft model studied. All of the results that we have obtained thus far using antibodies labeled with **9** indicate that the distribution of radioactivity directly reflects antibody distribution in animals. Due to this, the radioiodinated benzoate **9** is routinely used to label antibodies which are being evaluated.

Other investigators have described the use of halogenated benzoates for protein labeling (*47,48*). However, the method we have described here should be more adaptable to rapid and efficient labeling of antibodies. A similar investigation to our own has recently been reported by Zalutsky (*49*). Contrary to our results, he has observed an appreciably different tumor uptake at extended times when the *meta*-iodobenzoyl conjugate of an anti-ovarian antibody, OC125, was studied in nude mice bearing OVCAR-3 ovarian xenografts. We have not seen reproducible differences in the tumor uptake between chloramine-T labeled and iodobenzoyl

TABLE 4
Biodistribution of NR-M1-05 Fab Labeled with Chloramine-T or 9 (^{125}I) in Nude Mice*

Tissue	4 hr	20 hr
	Labeling with 9	
Blood	2.00 ± 0.73	0.07 ± 0.07
Tumor	5.20 ± 0.82	1.47 ± 0.37
Skin	0.89 ± 0.07	0.05 ± 0.01
Muscle	0.43 ± 0.02	0.10 ± 0.07
Bone	0.59 ± 0.03	0.19 ± 0.06
Lung	1.51 ± 0.09	0.31 ± 0.09
Liver	0.65 ± 0.06	0.09 ± 0.01
Spleen	0.46 ± 0.03	0.11 ± 0.03
Stomach	0.56 ± 0.33	0.09 ± 0.02
Neck	0.77 ± 0.08	0.10 ± 0.02
Kidney	7.99 ± 1.74	0.67 ± 0.23
Intestine	1.25 ± 0.19	0.14 ± 0.03
	Labeling with chloramine-T	
Blood	1.53 ± 0.33	0.09 ± 0.08
Tumor	3.22 ± 0.89	1.63 ± 0.38
Skin	0.92 ± 0.16	0.08 ± 0.02
Muscle	0.39 ± 0.06	0.11 ± 0.12
Bone	0.45 ± 0.08	0.05 ± 0.01
Lung	1.15 ± 0.14	0.21 ± 0.04
Liver	0.47 ± 0.05	0.09 ± 0.01
Spleen	0.57 ± 0.12	0.07 ± 0.01
Stomach	6.71 ± 1.82	0.69 ± 0.12
Neck	4.67 ± 0.45	10.81 ± 4.46
Kidney	3.40 ± 1.13	0.21 ± 0.03
Intestine	0.42 ± 0.04	0.07 ± 0.01

* Results were obtained from N = four mice per time pint and are tabulated as mean deviation % injected dose/g.

labeled antibodies in melanoma xenografts. The observed difference in the labeled OC125 is likely to be a factor of the antibody and animal model studied.

CONCLUSIONS

We have demonstrated that the *para*-iodobenzoyl conjugate does not deiodinate in vivo in an animal model. This fact makes the described radioiodine labeling approach a valuable alternative for in vivo assessment of labeled proteins in general. The relevance of the labeling method to clinical applications on radioiodinated antibodies for radioimmunotherapy is not known at this time. It is anticipated that there will be advantages in the treatment of some tumor types, but this must be tested in human clinical trials. In fact, we have begun clinical trials which should answer this. Of interest is whether there is selective stability at tumor sites; a significant difference in tissue localization/clearance; or difference in total body retention of activity.

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