Site-Specific Conjugation of a Radioiodinated Phenethylamine Derivative to a Monoclonal Antibody Results in Increased Radioactivity Localization in Tumor

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The preparation of a novel radioiodination reagent, the (aminooxy)acetyl derivative of (*p*-[^{125}]-iodophenyl)ethylamine, is described. Conventional radioiodination of proteins involves the formation of iodotyrosine residues, but for in vivo applications such as thyroid or stomach immunoscintigraphy, the susceptibility of these residues to tissue dehalogenases constitutes a serious disadvantage. Using our new compound, which has a particularly nonreactive aromatic ring, we confirm and extend studies published by other workers indicating the much greater in vivo stability of iodophenyl compounds compared to the more conventional iodophenolic ones. In addition, the aminooxy group of our reagent gives a stable and specific linkage to aldehyde groups formed by periodate oxidation on the sugar moiety of antibody molecules. In vitro, favorable binding activity and high stability was obtained with a ((([^{125}I]iodoaryl)amino)oxy labeled monoclonal antibody directed against carcinoembryonic antigen. In vivo, using paired labeling experiments in nude mice bearing colon carcinoma xenografts, the ((([^{125}I]iodoaryl)amino)oxy-MAb (MAb = monoclonal antibody) was compared with the same MAb ^{131}I-labeled by conventional chloramine-T method. Tumor ^{125}I concentration of (arylamino)oxy MAb (measured as percent injected dose per gram) was significantly higher as compared to values obtained with a conventionally labeled ^{131}I antibody. Additionally, thyroid uptake, an indicator of iodine release from the antibody, was up to 25 times lower after injection of ^{125}I-MAb obtained by the new method as compared to the conventionally iodinated ^{131}I-MAb.

Introduction

The use of monoclonal antibodies (MAbs) directed against tumor associated or differentiation antigens for immunoscintigraphy and radioimmunotherapy continues to be the subject of many experimental and clinical studies.[1-4] Of the different labeling possibilities, radioiodination enjoys a number of practical advantages, making it the standard against which new strategies are evaluated. However, while the tumor specificity of MAbs and MAb fragments amenable to radioiodination have improved, the problem of deiodination of the tyrosine-labeled antibody in vivo has remained.[5] This is presumably the consequence of enzymic attack by dehalogenases and of the intrinsic electronic instability of ortho-substituted iodine in the phenolic ring.[6]

Several workers have reported labeling studies that, in contrast to iodination on tyrosine residues, involve halogenated aromatic compounds that do not have a hydroxyl substituent on the aromatic ring.[7-11] Such compounds would be expected to be less susceptible to loss of halogen in vivo, and this has indeed proved to be the case. For example, Wilbur et al. described the use of *p*-iodobenzoate in this context,[10] while Zalutsky et al. chose *m*-iodobenzoate.[11] In both studies, which showed satisfactory increases in the in vivo stability of the labeled MAbs, the [^{125}I]iodobenzoic acid derivative was randomly coupled to primary amino groups of the proteins using a reagent made by forming the *N*-hydroxysuccinimide ester of the carboxylic acid group.

We wished to avoid the random nature of the substitution produced by such reagents, as well as the loss, for each amino group substituted, of a positive charge on the MAb. We therefore made the (aminooxy)acetyl derivative of *p*-[^{125}I]iodophenethylamine. The aminooxy group, as shown in a different context,[12] provides an excellent means for site-specific introduction of labeling reagents. It has long been recognized[13,14] that periodate oxidation of the sugar moiety of antibodies can produce a MAb with one or more aldehydic groups confined to the carbohydrate moiety. If care is taken, this oxidation does not impair the function of the antibody.

In addition, the aromatic ring of our reagent is electronically quite unactivated so that the chemical stability of the aryl iodide would be expected to be at a maximum,[15] even relative to that of the benzoic acid derived compounds cited above.[10,11] These latter compounds have an electron-deficient ring and so are expected to be more susceptible to loss of iodine by nucleophilic aromatic substitution.

Rea et al. have also used the oxidation of sugars to provide a specific site for radioiodination,[16] but the reagent they describe (2-hydroxy-5-iodo-3-methylbenzohydrazide) has an aromatic ring that is relatively reactive. Also, as a hydrazide, this reagent forms a hydrazone link to the protein's aldehyde group rather than the considerably more stable oxime link formed by our compound.

The in vitro and in vivo results described in the present paper demonstrate that we have produced a reagent which combines the favorable features of the approaches surveyed above.

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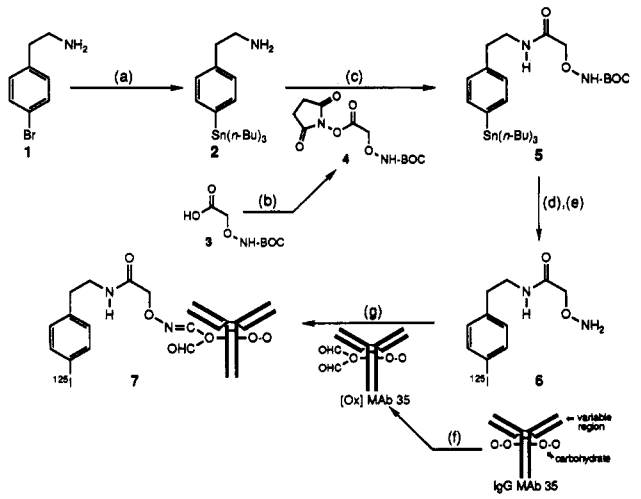

Figure 1. Phenethylamine iodination scheme. (a) $[(n\text{-}Bu)_3Sn]_2$, $Pd[(C_6H_5)_3P]_4$, toluene, 95 °C; (b) NHS, DCC, Et_3N, EtOAc; (c) 4, Et_3N, EtOAc; (d) (i) Na[^{125}I]I, MeOH–H_2O; (ii) chloramine-T; (iii) $Na_2S_2O_5$; (e) 90% aqueous TFA; (f) $NaIO_4$, PBS (pH 6); (g) [Ox]MAb 35, 0.15 M HOAc/NaOAc (pH 4).

Chemistry

The synthetic scheme for [^{125}I]iodophenethylamine labeling of the monoclonal antibody is shown in Figure 1. Starting material for our method is commercially available 4-bromophenethylamine (1) which, by palladium-catalyzed replacement of bromide with a tri-*n*-butyltin moiety at 95 °C in toluene, delivers aryltin **2** in 78% yield. Subsequent N-acylation of 4-(tri-*n*-butylstannyl)phenethylamine (2) with activated ester **4** gave compound **5** which is suitably disposed for sequential iodination via an aryltin to aryl iodide transformation and site-specific protein conjugation via oxime formation after removal of the BOC protecting group with aqueous trifluoroacetic acid. Using stable iodine (K[^{127}I]I) in the iodination of **5** gave [^{127}I]-**6** in 87% isolated yield, and subsequent removal of the BOC group produced the unlabeled analogue of ((iodoaryl)amino)oxy **6** in 91% yield. Using radioactive iodine (Na[^{125}I]I) instead of stable iodine produces [^{125}I]-**6** and subsequent deprotection/coupling to the carbohydrate moiety of our monoclonal antibody [MAb 35; directed against carcinoembryonic antigen (CEA)][17,18] yields **7**. This recently reported strategy[12] of using an aminooxy moiety to couple a radiolabeled substrate to the oxidized carbohydrate of MAbs is central to our approach. The requisite MAb oxidation proceeds by conversion of vicinal diols on MAb carbohydrate groups to form bis-aldehyde functionalities. Thus, the key synthetic intermediate, aryltributylstannyl derivative **5**, was synthesized in three steps from BOC-protected *O*-(carboxymethyl)hydroxylamine (3) and 4-bromophenethylamine (1). Subsequent radioiodination, removal of the BOC group, coupling with an oxidized MAb ([Ox]-MAb), and gel filtration on Sephadex G-25 delivers the [^{125}I]iodophenethylamine-labeled MAb (i.e., **7**) free from other reaction products (e.g., uncoupled **6**, free ^{125}I, chloramine-T, etc.).

Depending upon the quantity of [Ox]MAb 35 employed, the labeling efficiency at 6 h reproducibly ranged from 25–28% (three runs using 200 μg of [Ox]MAb 35) to 39–43% (three runs using 650 μg of [Ox]MAb 35). A 10-h time-course study of this step established that conjugation was essentially complete in 3 h at 4 °C as judged by bound versus free **6** (Table I). Extended incubation gave no meaningful increase in percent incorporation.

Table I. Aminooxy Reagent/Antibody Coupling: Time Dependence

time (h)	chemical incorporation (%)	immunological reactivity (%)
1	17[a]	76[b]
3	23	74
6	26	74
10	24	73
x	x	x

[a] [^{125}I]Iodophenethyl (aminooxy)acetamide was incubated for different times with [Ox]MAb 35 (200 μg) activated with 5 mM $NaIO_4$. Chemical Incorporation of ^{125}I on the antibody is given as percent overall yield based on the amount of starting Na[^{125}I]I. [b] [^{125}I]Iodophenethylamine-labeled MAb 35 (200 μg) binding capacity was measured by incubation with CEA-Sepharose in duplicates. Variance of double measurements was less than 5%. Nonspecific binding to control protein on Sepharose (below 1.5%) has been subtracted.

Table II. Periodate Activation of MAb 35 Carbohydrate Moieties: Labeling Efficiency and Immunological Reactivity

$NaIO_4$ (mM)[a]	labeling efficiency with iodogen (%)[b]	immunological reactivity (%)[c]
0	88	85
5	88	80
10	82	79
22	71	70

[a] Purified MAb 35 was incubated for 40 min at 4 °C with different concentrations of $NaIO_4$. [b] After dialysis of activated MAb, Iodogen (30 μg for 15 min at 4 °C) labeling was performed. Incorporation of ^{125}I on antibody is given in percent. [c] Binding capacity of ^{125}I-labeled MAb on CEA-Sepharose was measured in duplicates and is given in percent. Nonspecific binding to control protein on Sepharose (below 1.5%) has been subtracted.

Another important variable tested was the molarity of sodium periodate used in the oxidation of MAb 35. As an initial probe of this reaction parameter, MAb 35 was exposed to varying concentrations of oxidant and thereafter conventionally labeled with ^{125}I by the iodogen method. The percent CEA binding of the radiolabeled antibodies was then determined; these results are summarized in Table II. While up to 10 mM sodium periodate was tolerated with little loss of binding capacity, a 22 mM concentration began to affect not only the binding capacity, but also the Iodogen labeling efficiency.

Similarly, when MAb 35 was activated by 22 mM sodium periodate and then conjugated with reagent **6**, binding of [^{125}I]iodophenethylamine MAb 35 to CEA decreased to 45%. Reducing the concentration of oxidant to 5 mM provided repeatedly oxidized antibody with a high CEA binding capacity of 74–94% (mean 86.3% for 14 batches). Mean binding of [^{131}I]MAb 35 labeled by the chloramine-T method was 88.2% (range 76–94% for eight batches). For all labeled antibody preparations, nonspecific binding to irrelevant control protein-Sepharose was below 1.5%.

The phenethylamine-labeled MAb exhibited excellent in vitro stability as less than 8% of free iodine was found by chromatography on Sephadex G-200, and no detectable amounts of aggregates had been formed after storing a batch for 3 weeks at 4 °C. The 7S IgG peak of the batch refiltered on Sephadex G-200 gave again a binding capacity to CEA of 94%. Moreover, all labeled MAb solutions contained, after final chromatography and before injection to animals, less than 2% of aggregates as determined by molecular-size fractionation on Sephadex G-200.

Table III. Tissue Distribution of RadioIodine in Mice Bearing Large (Mean 1.0 g) Human Colon Carcinoma T380 Tumors following Injection of MAb 35 Labeled with ^{125}I Using the Phenethylamine Method and with ^{131}I Using the Chloramine-T Method

	1 day		3 days		5 days		7 days[a]
tissue	mouse 1	mouse 2	mouse 3	mouse 4	mouse 5	mouse 6	mouse 7
	% injected dose/gram tissue·[^{125}I]iodophenethylamine labeling method						
tumor	50.05	40.86	71.90	42.95	15.87	24.20	21.84
blood	4.77	9.29	0.34	1.29	0.25	0.31	0.32
liver	7.36	8.54	4.48	8.03	3.48	3.67	2.36
kidney	2.94	3.51	0.73	1.88	0.63	0.67	0.43
lung	2.42	5.20	0.31	0.89	0.24	0.33	0.25
spleen	3.66	3.97	2.14	2.62	2.24	2.54	1.15
heart	1.46	2.33	0.21	0.41	0.14	0.13	0.15
muscle	0.55	0.71	0.08	0.28	0.16	0.10	0.05
bone	0.59	1.27	0.19	0.43	0.14	0.19	0.19
carcass	2.17	1.83	0.22	0.50	0.24	0.22	0.15
thyroid	1.42	1.82	0.36	1.14	2.43	2.48	10.79
	% injected dose/gram tissue·[^{131}I]chloramine-T labeling method						
tumor	41.53	33.02	10.68	27.16	7.81	11.20	9.06
blood	6.87	10.70	1.24	3.03	0.97	1.21	1.16
liver	2.58	3.82	0.61	1.36	0.53	0.55	0.44
kidney	2.35	2.78	0.52	1.34	0.50	0.57	0.57
lung	2.92	5.36	0.48	1.36	0.47	0.69	0.47
spleen	1.56	1.98	0.35	0.66	0.39	0.44	0.29
heart	1.92	2.46	0.34	0.59	0.26	0.26	0.29
muscle	0.63	0.76	0.12	0.37	0.16	0.12	0.07
bone	0.66	1.16	0.14	0.40	0.15	0.15	0.17
carcass	2.03	1.81	0.30	0.64	0.29	0.31	0.21
thyroid	22.15	14.67	17.91	24.60	89.01	119.46	269.68

[a] A single animal at the 7-day time point.

Table IV. Tissue Distribution of Radioiodine in Mice Bearing Small (Mean 0.12 g) Human Colon Carcinoma T380 Tumors following Injection of MAb 35 Labeled with ^{125}I Using the Phenethylamine Method and with ^{131}I Using the Chloramine-T Method

	1 day		3 days		5 days		7 days	
tissue	mouse 8	mouse 9	mouse 10	mouse 11	mouse 12	mouse 13	mouse 14	mouse 15
tumor	69.91	71.19	66.98	85.57	62.47	71.25	77.88	75.63
blood	10.62	11.71	8.12	10.26	3.74	7.62	4.39	3.99
liver	5.05	5.37	4.07	5.72	2.29	3.69	2.53	2.19
kidney	4.17	4.28	3.2	3.92	1.48	2.57	1.54	1.32
lung	5.7	4.56	4.65	4.49	2.05	3.6	2.86	2.35
spleen	6.02	3.84	3.01	3.89	2.48	3.89	2.66	2.69
heart	2.75	3.95	2.4	2.61	0.97	1.7	1.16	1.09
muscle	1.12	0.93	0.99	1.04	0.28	0.65	0.35	0.30
bone	1.72	1.16	1.38	1.4	0.61	1.28	0.87	0.66
carcass	2.22	2.05	1.56	2.02	0.85	1.59	1.02	0.88
thyroid	3.02	3.48	4.94	2.6	3.98	3.79	3.04	3.89
	% injected dose/gram tissue·[^{131}I]chloramine-T labeling method							
tumor	61.80	63.07	48.97	69.64	44.84	51.92	56.60	54.42
blood	12.03	13.20	9.77	12.10	4.71	9.67	5.77	5.42
liver	3.63	3.78	2.82	4.50	1.37	2.83	1.79	1.43
kidney	3.38	3.42	3.13	3.88	1.48	2.84	1.80	1.58
lung	5.70	4.57	5.15	4.79	2.36	4.07	3.41	2.92
spleen	3.88	2.50	1.74	2.08	1.04	1.91	1.29	1.13
heart	2.88	4.10	2.75	2.89	1.11	2.01	1.40	1.35
muscle	1.17	1.02	1.09	1.12	0.31	0.80	0.41	0.37
bone	1.67	1.04	1.27	1.34	0.58	1.28	0.81	0.54
carcass	2.22	1.99	1.54	1.98	0.82	1.69	1.07	0.87
thyroid	11.94	17.67	35.66	22.15	55.30	45.52	56.82	64.04

In Vivo Paired Labeling Studies

The paired labeling method[7] was used in these experiments to compare [^{125}I]iodophenethylamine-labeled MAb with [^{131}I]chloramine-T-labeled MAb. This direct comparison of the two labeling methods in the same individual mouse gives more reliable results than the testing of both labelings in different series of animals because the variable biological properties of each animal (such as blood flow, animal size, tumor size, tumor vascular permeability, and antibody secretion) are *identical* for both labelings. Validation of this premise is reflected by the fact that, *in each single animal*, tumor localization of the phenethylamine-labeled antibody is always higher than that of chloramine-T-labeled antibody. Indeed, this is the case regardless of tumor size and variations of antibody uptake into tumor in the individual animals (Tables III and IV). It is this fact which is analyzed statistically leading to the statement of significance. The reverse was found in thyroid where uptake of free iodine from chloramine-T-labeled antibody is always higher than that from phenethylamine-labeled antibody (Tables III and IV).

The biodistribution of [^{125}I]iodophenethylamine-labeled MAb 35 was obtained over a 7-day time period using nude mice (four per time point; two with small tumors and two with large tumors) bearing colon tumor T380 transplant. These data were compared to the biodistribution data for co-injected chloramine-T-labeled [^{131}I]MAb 35. The data established that biodistribution varies significantly as a

consequence of tumor size, so pooling of these results was inappropriate; rather, the results from this binding study are presented separately for large (Table III) and small (Table IV) tumors. Binding capacities for two batches of the [^{125}I]iodophenethylamine-labeled MAb were 88 and 90%, while binding capacities for two batches of the chloramine-T-labeled MAb were 84 and 92%; background bindings of all labeled MAb's on irrelevant protein were less than 0.5%. As stated above, the value of percent injected dose per gram tumor was always higher at the different time points of dissection for [^{125}I]iodophenethylamine-labeled MAb as compared to [^{131}I]chloramine-T-labeled MAb. This was the case both in mice bearing large tumors (0.6–1.9 g, mean 1.0 g; Table III) as well as in mice with small tumors (0.06–0.21 g, mean 0.12 g, Table IV). Inspection of Tables III and IV establishes that efficiency of tumor localization using phenethylamine versus chloramine-T-labeled MAb increases with time regardless of tumor size. These different localizations were statistically analyzed by a 2 factors-analysis of variance comparing all mice in relation to time. Increased tumor localization for phenethylamine as compared to chloramine-T labeling was significant with values of $p < 0.001$ and $p < 0.05$ for series of mice bearing small tumors and large tumors, respectively.

In most normal organs, the percent injected dose per gram was very similar for both labeling methods. The higher percent tumor localization for [^{125}I]iodophenethylamine-labeled MAb together with a retention in normal tissues that is similar for both labeling methods resulted in tumor to normal tissue ratios which are in favor of the phenethylamine labeled MAb as compared to the MAb conventionally labeled with chloramine-T.

A moderately increased accumulation of phenethylamine-labeled MAb relative to chloramine-T labeling was found in liver and spleen. While this difference was minimal in mice bearing small tumors (Table IV), the contrast was more pronounced in mice bearing large tumors (Table III).

The enhanced release of free ^{131}I after injection of chloramine-T-labeled MAb was reflected by the higher uptake of this isotope in the thyroid (Tables III and IV). In this organ, ^{131}I from chloramine-T-labeled MAb steadily increased from 15% injected dose per gram at day 1, to 60% at day 7 in mice bearing small tumors. The increase was even more pronounced (from 18% at day 1, to 270% at day 7) in mice bearing large tumors. In contrast, ^{125}I accumulation in thyroid associated with phenethylamine labeling was much less as compared to chloramine-T-labeled MAb in mice with small tumors (by factors of 4.5 to 17, Table IV) and was even less in mice with large tumors (by factors of 11.5–25, Table III). Clearly, formation of free iodine was by far slower with phenethylamine labeling as compared to chloramine-T labeling. Statistical analysis of decreased radioactivity accumulation after phenethylamine labeling gave values of $p < 0.0001$ and of $p < 0.03$ for the series of mice bearing small tumors and large tumors, respectively.

Finally, whole body retention was calculated for both isotopes. Calculation of biological half-lives gave values of 82 and 150 h after injection of phenethylamine-labeled MAb compared with 61 and 144 h after injection of choramine-T-labeled MAb in the groups of mice bearing large (Table III) and small tumors (Table IV), respectively.

As a control, biodistribution was measured in three mice bearing melanoma tumors weighing between 0.02 and 0.09 g. The percent injected dose nonspecifically accumulated in the melanoma at 3 days after injection of phenethylamine and chloramine-T-labeled MAb 35 were 4 ± 1.5% and 4.5 ± 1.6%, respectively, while thyroid radioactivity retention was 5.2 and 34.1% injected dose per gram, respectively. Percent injected dose per gram of other normal tissues was comparable to that obtained in mice with small colon tumors.

Two other control mice bearing colon carcinoma T380 tumors of 0.07 and 0.19 g were injected with irrelevant ^{131}I-labeled control MAb using chloramine-T, and with [^{125}I]iodophenethylamine-labeled MAb 35. At 3 days after injection, percent injected dose per gram tumor was 3.8 and 6.8% for ^{131}I control MAb while that of [^{125}I]-iodophenethylamine-labeled anti-CEA MAb 35 was 76 and 104% (data not shown).

Results and Discussion

Both the Bolton–Hunter [*N*-succinimidyl-3-(4-hydroxyphenyl)propionate][19] and Wood's (methyl 4-hydroxybenzimidate)[20] reagents are important, well-known conjugation labeling substrates for radioiodination of proteins and present alternatives to the most commonly used methods of protein iodination on tyrosine residues. However, both these reagents contain an electron-rich phenolic ring which is labeled by the chloramine-T[21] or Iodogen[22] method. Thus, they are subject to the same electronic instability and deiodinase enzyme catabolism as tyrosine, the amino acid residue primarily radioiodinated in direct protein-labeling protocols. As stated in the introduction, recent studies by Zalutsky[9] and Wilbur[10] have examined the feasibility of overcoming these limitations by exploiting conjugation radiolabeling strategies which do not require hydroxyl activation of the aromatic ring.

The conjugation radiolabeling strategy reported here extends this important area of study in two significant ways. It allows site-specific incorporation of the label at a point distant from the variable region of the antibody, thus avoiding the consequences of conjugation near the binding site. Second, our reagent has an unactivated aromatic ring; one that is neither electron rich as in the Bolton–Hunter or Wood strategies nor electron deficient as in the Zalutsky or Wilbur strategies (and is consequently less subject to loss of radioiodine by electrophilic or nucleophilic aromatic substitution).

The oxime strategy used here for the protein conjugation step enjoys two important intrinsic advantages over the standard strategy for conjugating to aldehydic proteins (i.e., formation and subsequent reduction of a Schiff base). First, because of its low pK_a, the aminooxy group is reactive at pH 4 whereas the numerous primary amino groups of the protein are for the most part protonated at this pH and are therefore much less reactive toward the aldehyde moiety. Second, while the resulting oxime is thermodynamically stable in an aqueous medium at pH 4, an imine (Schiff base) is not. We can therefore avoid exposing the conjugated protein to the sodium cyanoborohydride reduction which is normally required to stabilize the Schiff base. There is thus no danger of stabilizing unwanted intra- and intermolecular cross-links that might well be temporarily formed between the aldehydes and primary amino groups but which, without sodium cyanoborohydride reduction, will just as readily revert to the amino/aldehyde moieties.

In the course of this investigation, we identified and overcame four experimental problems that arose because of the considerable reactivity of the aminooxy group and the extremely low concentration of the reagent inevitable in radiochemical work at high specific activity. First, at the very low absolute concentration dictated by the use of nominally carrier-free isotopes, we found that even clean glass surfaces contained sufficient reactive sites to bind the aminooxy reagent. In order to block these sites, the latter was preincubated with *O*-methylhydroxylamine hydrochloride. Presumably incubation with this small aminooxy compound blocks all reactive sites on the glass surface, sites which otherwise irreversibly bind (([^{125}I]-iodoaryl)amino)oxy **6**. Second, it was found essential to preincubate the methanol used in this reaction with adipic acid dihydrazide–agarose to remove trace aldehyde impurities which otherwise consumed the ((iodoaryl)amino)-oxy. Third, the trifluoroacetic acid used in the deprotection step was diluted with water (to 10% water content) in order to minimize acylation of the aminooxy moiety by traces of trifluoroacetic anhydride. Fourth, for reasons that are not apparent, we found it necessary to treat the radiolabeling reaction mixture containing **5** + Na[^{125}I]I + chloramine-T with excess stable iodide (K[^{127}I]I) prior to sodium metabisulfite quench; if this was not done, the reaction of **6** with [Ox]MAb 35 was far less effective (i.e., ^{125}I incorporation in the protein containing fractions from Sephadex G-25 column purification of **5** → **7** decreased from ≈25% with K[^{127}I]I treatment to ≈8% without K[^{127}I]I treatment).

Evaluation of this new labeling technique included both in vitro and in vivo analyses of the antibody. After optimization of different parameters evaluated during this study, reproducible labeling of anti-CEA MAb 35 was obtained with complete retention of immunoreactivity as compared to chloramine-T labeling of the same MAb.

The animal experiments presented here are an essential step in evaluating this new labeling procedure as they allow us to analyze the novel radiolabeled MAb's in vivo stability, its tumor localization capacity, and its biodistribution in normal tissues. The double-labeling method used gives highly reliable results for the comparison of two labeling methods even when using small groups of mice per dissection time. Moreover, radioactivity measurements in organs of the reticuloendothelial system (such as liver and spleen) show that in mice bearing small tumors there is only minimally increased uptake of phenethylamine-labeled MAb as compared to chloramine-T-labeled MAb. Clearly our new bioconjugation/labeling method does not denature the antibody. In mice bearing larger tumors (see Table III), however, the new method does result in slightly higher radioactivity in liver and spleen. This phenomenon can most probably be explained by the higher amount of circulating CEA in mice bearing large tumors.[28] Circulating CEA leads to the formation of antibody–antigen complexes which, in turn, are taken up by the reticuloendothelial system (RES). While the conventionally labeled MAb is rapidly deiodinized by the deiodinase enzymes in the liver and RES, deiodination of phenethylamine-labeled MAb is probably a slower process leading to prolonged retention of this iodine.[23]

The increased percentage of tumor localization of phenethylamine-labeled MAb as compared to chloramine-T-labeled antibody reflects the better stability of the new bioconjugate and represents a real gain of radiolabel in tumor. Finally, the low thyroid uptake of iodine released from the phenethylamine-labeled antibody confirms the increased stability of our new radiolabeling method and might allow more precise thyroid tumor immunoscintigraphy or reduce thyroid irradiation under radioimmunotherapy.

Conclusion

Given the fact that tumor lines and MAbs were different, it is probably inappropriate to compare our results in a detailed manner with those obtained for the somewhat analogous reagents of Zalutsky[9,11] and Wilbur et al.[10] Indeed, the anti-CEA MAbs, labeled both conventionally and even more when labeled using phenethylamine, give much higher values in percent injected dose per gram tumor at any time after injection in our xenograft system than those obtained in the references cited above. Similarly, the half-life of our antibody in blood is 2–3 times longer than that of the MAb labeled by the ATE method of Zalutsky et al.[11] It is, however, impossible to compare the thyroid uptake results because Zalutsky's results are reported as percent injected dose per organ instead of percent injected dose per gram tissue. Thus, we confine ourselves to pointing out that our results fulfill the theoretical expectations which prompted our study. We conclude that our reagent and bioconjugation strategy represents a convenient means of introducing a biologically stable iodine label into monoclonal antibodies. In addition, given that this study constitutes a second successful example of the in vivo use of antibody labeling through an aminooxy compound,[12] application of this aminooxy strategy is recommended for other clinically useful conjugates.

Experimental Section

^{1}H NMR and ^{13}C NMR spectra were obtained in $CDCl_3$ at 300 and 75.5 MHz, respectively, and chemical shifts are reported in ppm (δ) relative to tetramethylsilane at 0.00. Thin-layer chromatography (TLC) was performed on plastic precoated analytical plates. Radioactivity was measured using a Packard dual-channel well-type scintillation counter. Aqueous solutions (pH 7–11, "NaOH") of Na[^{125}I]I and Na[^{131}I]I were purchased were purchased from Amersham (Buckinghamshire, England).

4-(Tri-*n*-butylstannyl)phenethylamine (2). A mixture consisting of 4-bromophenethylamine (**1**; 64 mg, 0.32 mmol), hexabutyldistannane (934 mg, 1.61 mmol), tetrakis(triphenylphosphine)palladium (4 mg, 3.4×10^{-3} mmol), and toluene were placed in a sealable tube, and the system was purged with dry nitrogen. The sealed tube was then heated at 95 °C for 48 h. Upon cooling to room temperature, the crude reaction mixture was placed directly on a gravity silica gel column (2.5- × 10-cm bed) and the excess hexabutyldistannane removed with $CHCl_3$ elution. The eluting solvent was then changed to 1:1 $CHCl_3$–MeOH, giving **2** (102 mg, 0.25 mmol, 78%) as a pale yellow oil: TLC R_f ($CHCl_3$) 0.77; ^{1}H NMR δ 0.89 (t, J = 7 Hz, 9 H), 1.02 (t, J = 9.3 Hz, 6 H), 1.33 (m, 6 H), 1.54 (m, 6 H), 2.81 (br t, J = 7.4 Hz, 2 H), 3.02 (br t, J = 7.4 Hz, 2 H), 4.82 (br s, 2 H), 7.17 (d, J = 8.3 Hz, 2 H), 7.39 (d, J = 8.3 Hz, 2 H); ^{13}C NMR δ 9.6, 13.6, 27.3, 29.0, 38.0, 42.4, 128.4, 136.7, 138.4, 139.6; IR (CCl_4) 2924, 2853, 1593, 1464, 1377, 1070, 756 cm^{-1}; MS (FAB) 411 (M + H). Anal. ($C_{20}H_{37}NSn$) C, H, N.

[[[(1,1-Dimethylethoxy)carbonyl]amino]oxy]acetic Acid (3).[24] *O*-(Carboxymethyl)hydroxylamine hemihydrochloride (1.09 g, 10 mmol) was dissolved (15 mL) in solution A [prepared from KOH (1.12 g, 20 mmol), MeOH (20 mL), and H_2O (30 mL)], and the pH was adjusted to 9 by incremental addition of A. Next, di-*tert*-butyl dicarbonate (4.36 g, 20 mmol) was added in one portion, and the mixture was stirred at room temperature while maintaining a pH value of 9 with further dropwise additions of solution A. After 16 h, evaporation (Buchi Rotavapor) to dryness

gave a white solid which was dissolved in H_2O (10 mL). This solution was cooled to 0 °C and vigorously stirred as the pH was lowered to 3 by dropwise addition of 6 N HCl. The resulting precipitate was collected by filtration, washed with water, and dried by lyophylization to give **3** (1.0 g, 5.2 mmol, 52%) [FAB-m/z 190 (M − H)].

***N*-[4-(Tri-*n*-butylstannyl)phenethyl][[[(1,1-Dimethylethoxy)carbonyl]amino]oxy]acetamide (5).** Compound **3** (224 mg, 1.17 mmol), *N*-hydroxysuccinimide (135 mg, 1.17 mmol), and *N,N'*-dicyclohexylcarbodiimide (240 mg, 1.17 mmol) were dissolved in EtOAc (35 mL), and the heterogeneous mixture was allowed to stir at room temperature overnight. An EtOAc (4 mL) solution of 4-(tri-*n*-butylstannyl)phenethylamine (**2**; 240 mg, 0.59 mmol) was added to the resulting EtOAc (35 mL) solution of pyrrolidinedione **4** (prepared in situ; 337 mg, 1.17 mmol). A pH of 8 was maintained for this heterogeneous reaction by admixture of triethylamine [dropwise addition of 200 μL of Et_3N in EtOAc (1 mL) during the first 10 min of reaction]. The resulting mixture was allowed to stir at room temperature overnight by which time some of the dicyclohexylurea formed in the previous step had precipitated. This precipitate was removed by centrifugation/decantation, and the supernatant was washed with 2.5% aqueous HCl (2 × 30 mL), 2.5% aqueous NaOH (2 × 30 mL), and brine. Drying (K_2SO_4), concentration, and purification by medium-pressure liquid chromatography [$CHCl_3$, 20–40 psi through an EM Lobar column packed with SiO_2 (40–60 μm)] gave **5** (300 mg, 0.51 mmol, 87%): TLC R_f ($CHCl_3$) = 0.69; ^1H NMR δ 0.91 (t, J = 7 Hz, 9 H), 1.04 (t, J = 9.2 Hz, 6 H), 1.35 (m, 6 H), 1.52 (m, 15 H), 2.84 (br t, J = 7.1 Hz, 2 H), 3.58 (m, 2 H), 4.34 (s, 2 H), 7.21 (d, J = 8.2 Hz, 2 H), 7.41 (d, J = 8.2 Hz, 2 H), 7.70 (s, 1 H), 8.15 (br s, 1 H); ^{13}C NMR δ 9.7, 13.7, 27.4, 28.1, 29.2, 35.7, 40.5, 76.2, 83.1, 128.5, 136.7, 136.8, 138.7, 139.6, 157.6, 168.6; IR (CCl_4) 3334, 2959, 2930, 1745, 1687, 1562, 1464, 1370, 1250, 1164, 1114 cm^{-1}; MS (FAB) 584 (M + H). Anal. ($C_{27}H_{48}N_2O_4Sn$): C, H, N.

[^{125}I]-*N*-(4-Iodophenethyl)(aminooxy)acetamide (6). An aqueous solution of *O*-methylhydroxylamine hydrochloride (250 μM) was placed in a 6-mm × 15-mm glass test tube. After being allowed to stand at room temperature for 1 h, the aqueous *O*-methylhydroxylamine hydrochloride solution was removed by aspiration and the vessel thoroughly rinsed with deionized/distilled H_2O. A methanolic solution of **5** (20 μL of a 0.23 mM solution; 4.6 nmol) was introduced into the air-dried vessel followed sequentially by Na[^{125}I]I [10 μL of an aqueous NaOH solution; 1 mCi, 0.92 nmol] and chloramine-T (10 μL of a 4.6 mM 1:1 MeOH–H_2O solution; 46 nmol). After 30 min at room temperature, an excess of K[^{127}I]I (10 μL of a 2.8 mM 1:1 MeOH–H_2O solution; 28 nmol) was added to consume the remaining **5** and the reaction mixture incubated an additional 15 min at room temperature at which time the iodination reaction was quenched by addition of $Na_2S_2O_5$ (6 μL of a 10 mM aqueous solution; 60 nmol). TLC (1:1 hexane–EtOAc) with autoradiographic analysis of the resulting mixture indicated 50–70% ^{125}I incorporation.

Next, the solvents were evaporated with a gentle stream of N_2, and 50 μL of a solution of trifluoroacetic acid containing 10% water was added. After 7 min at room temperature, the aqueous TFA was evaporated (over ≤7 min) with a gentle stream of N_2 giving ^{125}I-labeled **6** which was used without purification in the MAb coupling reaction. For both evaporation steps, the volatile [^{125}I]I_2 was captured by passing the N_2 efluent stream through an activated charcoal filter.

[Ox]MAb 35 Preparation. MAb 35, a monoclonal antibody (mouse, IgG1) directed against a specific epitope of carcinoembryonic antigen (epitope Gold 3),[25] was obtained as described[17] and stored frozen in a 0.1 M NaCl/0.05 M phosphate buffer pH 6.0. An aqueous solution of sodium periodate ($NaIO_4$; 100 mM, 8.1 μL) was added to 200 μg of MAb 35 in 154 μL (1.3 mg/mL) of phosphate-buffered saline mentioned above at 4 °C, giving a final $NaIO_4$ concentration of 5 mM. The resulting solution was incubated in the dark at 4 °C for 40 min and then dialyzed sequentially against 0.1 M NaCl/0.05 M phosphate buffer pH 6.0 (1 × 20 mL; 40 min at 4 °C in the dark) and 0.15 M HOAc/NaOAc pH 4.0 (3 × 20 mL; 30 min each at 4 °C in the dark) buffers. The final dialyzate was used directly in the subsequent coupling reaction (i.e., **6** + [Ox]MAb → **7**). In other experiments, 650 μg of MAb 35 in 100 μL (6.5 mg/mL in 0.1 M NaCl/0.05 M phosphate buffer pH 6.0 solution) was also oxidized in this manner.

^{125}I-Labeled [Ox]MAb 35 (7). [Ox]MAb 35 (1.3 mg/mL in 0.15 M HOAc/NaOAc pH 4.0; 200 μg, 154 μL) was added to the N_2-dried reaction vessel containing ^{125}I-labeled reagent **6**. The homogeneous mixture was stored for 3 h at 4 °C and then placed on a Sephadex G-25 column (10 mL), previously equilibrated with PBS (phosphate buffered saline; NaCl (0.15 M) and phosphate (0.01 M) pH 7.4), and eluted with the same buffer by collecting 700-μL fractions. Total radioactivity in the protein-containing fractions (generally fractions 6 and 7) established an overall ^{125}I incorporation (i.e., **5** → **7**) ranging from 25 to 28%. Subsequent Sephadex G-200 size fractionation established that about 95% of radioactivity was eluted in the 7S peak corresponding to IgG. With no other modifications, 650 μg of [Ox]-MAb 35, in 100 μL of HOAc/NaOAc (0.15 M) pH 4.0, was also conjugated in this manner, giving an overall ^{125}I incorporation (i.e., **5** → **7**) ranging from 39 to 43% with comparable results on Sephadex G-200.

Chloramine-T Labeling of MAb 35. A 400-μg portion of purified MAb 35 was labeled with 1 mCi ^{131}I by the chloramine-T method to a specific activity of about 2 μCi/μg antibody (80% incorporation of iodine onto protein). Then 50 μg of chloramine-T in 20 μL of 0.1 M NaCl/0.15 M Tris-HCl buffer pH 7.0 was added to 400 μg of MAb 35 in 50 μL of the same buffer, followed by 1 mCi of Na[^{131}I]I in a 10-μL NaOH solution. After incubation for 3 min at 4 °C, 50 μg of $Na_2S_2O_5$ in a volume of 500 μL of the same Tris-NaCl solution were added. The labeled antibody was separated from free iodine by filtration on Sephadex G-25 after addition of 100 μL of normal mouse serum as carrier protein. Subsequent Sephadex G-200 size fractionation established that >95% of radioactivity was eluted in the 7S peak corresponding to IgG.

In Vitro Testing of [^{125}I]Iodophenethylamine and [^{131}I]-Chloramine-T-Labeled Antibodies. The immunological reactivity was measured in a binding assay using CEA (carcinoembryonic antigen) immobilized on CNBr-activated Sepharose 4B (Pharmacia Uppsala, Sweden);[20] 20–40 ng (about 50 nCi) of labeled proteins were incubated for 16 h at 25 °C with 10 μL of packed CEA-Sepharose or control Sepharose (containing either 10 μg of CEA or 10 μg of irrelevant control protein) in 200 μL of PBS buffer pH 7.4 (containing additionally 1 mg/mL of bovine serum albumin and 1% normal mouse serum). After washing of the gels, bound radioactivity was counted and expressed as the percentage of the total radioactivity taken for the experiment.

Nude Mouse Tumor Model. The nude mouse tumor model for testing the in vivo localization of labeled antibodies has been previously described by Mach et al.[26] The human colon carcinoma T380[27] was serially transplanted subcutaneously into nude mice (7–10 weeks of age, male, "Swiss", homozygous nu/nu mice, from Iffa Credo, L'Arbresle, France) as described.[28] High amounts of CEA are produced in these tumor transplants (about 40 μg per gram tumor) and partially released into blood (10–18 ng/h for a 1-g tumor).[27] A high degree of vascularization allows the tumor to grow to a weight of more than 1 gram without necrosis.

In Vivo Biodistribution Studies. The [^{125}I]iodophenethylamine-labeled MAb 35 (2 × 10^6 CPM; i.e., 1.5 μCi on about 1.1 μg of MAb) was mixed with 2 × 10^6 CPM [^{131}I]MAb 35 (2 μCi on about 1 μg of MAb) labeled conventionally by the chloramine-T method. Radiolabeled MAbs were supplemented with unlabeled MAb to a total of 10 μg of antibody and co-injected into the tail vein of two groups of nude mice bearing either small or large T380 tumors. At 1, 3, 5, and 7 days after injection of radiolabeled MAbs, two mice per group were killed (by insufflation of CO_2 into the cage), 0.5 mL of blood was taken, tumors (weighing 0.06–0.21 g in one group and 0.6–1.9 g in the other group) and normal organs including thyroid as well as the whole carcass were dissected and weighed, and both isotopes were counted in a dual-channel γ counter. Crossover radioactivity counts of ^{131}I to the ^{125}I channel (16.6%) were corrected in the computer program that calculated radioactivity concentration per gram of tumor and of all organs including blood and expressed it as percent of injected dose after correction for physical half-life of the isotopes. As controls, mice bearing colon carcinoma were injected with a conventionally labeled, irrelevant [^{131}I]MAb and mice bearing a

melanoma tumor[29] were injected with [^{125}I]iodophenethylamine-labeled MAb 35.

Statistical Analysis. Tumor localization of [^{125}I]iodophenethylamine-labeled MAb was compared with that of [^{131}I]-chloramine-T-labeled MAb using "2-factor analysis of variance". The same comparison was done for the thyroid uptake of both free iodine isotopes released from the different labelings. By using this method, the results of all seven or eight animals used for each experiment were evaluated simultaneously in relation to time, and one significance factor was calculated per experiment for either tumor or thyroid radioactivity.

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