Radiohalogenation of a Monoclonal Antibody Using an N-Succinimidyl 3-(Tri-n-butylstannyl)benzoate Intermediate

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ABSTRACT

N-Succinimidyl 3-(tri-n-butylstannyl)benzoate (ATE) was evaluated for its utility in the radiohalogenation of monoclonal antibodies. The F(ab')2 fragment of monoclonal antibody OC 125 was labeled with 125I using the ATE reagent and with 131I using a conventional electrophilic iodination method (Iodogen). N-Succinimidyl 3-[125I]iodobenzoate was synthesized from ATE in >90% yield and purified using a disposable silica gel cartridge. About 60–65% of the radiiodinated product was coupled to the F(ab')2 fragment after a 30-min reaction. Two procedures were investigated, one involving exposure of antibody to 35 nmol of ATE and the other to 240 nmol of ATE. Using Scatchard analyses, affinity constants for binding to CA 125 antigens for OC 125 F(ab')2 labeled using the low ATE, Iodogen, and high ATE procedures were determined to be (5.2 ± 1.0) × 10^9, (2.5 ± 0.9) × 10^9, and (4.2 ± 2.4) × 10^9 M^-1, respectively. Paired-label studies in athymic mice bearing OVCAR-3 tumors treated with injections of antibody labeled via both ATE and Iodogen demonstrated that use of the ATE method (a) reduced thyroid uptake to <0.1% of the injected dose, more than 100 times less than that observed with Iodogen; (b) resulted in more rapid clearance of activity from normal tissues; and (c) with the low ATE preparations, increased the uptake of radioactivity in tumor from 27 to 49%. At 96 h, tumor:tissue ratios were generally at least 4-fold higher when antibody was labeled via ATE. These results suggest that the ATE method may be a valuable approach for the radiohalogenation of antibodies.

INTRODUCTION

A significant problem affecting the diagnostic and therapeutic utility of radionabeled monoclonal antibodies is the loss of nuclide from the antibody in vivo. Although the mechanisms involved and the labeled species which are formed are largely unknown, certain aspects of the pharmacokinetics of radioactivity following the administration of labeled antibodies appear to be related to separation of the nuclide from the protein in vivo.

When antibodies labeled with metallic nuclides such as 111In are administered to animals and patients, a large fraction of the injected dose is taken up by the liver (1–5). While to some degree, liver uptake of foreign proteins is not unexpected, there is evidence to suggest that liver accumulation of labeled catabolites, such as those resulting from the transchelation of nuclides such as 111In to ferroproteins, is also an important factor (6–9).

Catabolism of label from radiiodinated antibodies is reflected by uptake of 131I and 123I in the thyroid and the stomach (6, 10, 11). In addition, urinary excretion of high levels of non-protein-associated 131I activity has been reported in clinical studies with 131I-labeled antibodies (11, 12). A probable factor contributing to the dehalogenation of proteins in vivo is the recognition of labeled idophenyl groups on the protein by deiodinases known to be involved in the metabolism of thyroid hormones (13).

Because of the potential utility of the radiohalogens 131I and the α-emitter 211At for radioimmunodagnosis and radioimmuno-therapy, respectively, our laboratory has been investigating the development of alternative strategies for protein labeling which could decrease the loss of radiohalogen from an antibody in vivo. We have recently described a new approach for the iodination of proteins which differs from conventional methods in that it does not involve the substitution of the iodine ortho to a hydroxyl group on an aromatic ring (14). A two-step procedure, radioiodination of ATE followed by conjugation of this intermediate to the protein, resulted in decreased thyroid uptake of radioiodine in comparison to proteins labeled by a conventional method, Iodogen (15).

We report herein our initial investigations concerning the utility of the ATE method for the radiohalogenation of monoclonal antibodies. Studies were performed using the F(ab')2 fragment of OC 125, an antibody against a human ovarian carcinoma-associated antigen (16). OC 125 F(ab')2 was labeled with 125I via ATE and with 131I using Iodogen and the labeled proteins were compared in vitro for their affinity for CA 125 antigen and in paired-label biodistribution studies, for selective uptake in OVCAR-3 tumors implanted in athymic mice.

MATERIALS AND METHODS

Monoclonal Antibody. OC 125 F(ab')2, was obtained as a gift from Dr. Jeffrey Mattis (Centocor, Malvern, PA). It is an IgG1 murine monoclonal antibody which was derived from the immunization of BALB/c mice with the OVCA 433 cell line established from ascites fluid from a patient with papillary serous cystadenocarcinoma of the ovary (16). Purification of antibody from ascites was accomplished using a DE52 ion exchange column as described previously (17). The F(ab')2 fragment was generated by a 16-h digestion of the purified IgG with 1% pepsin.

Antibody Labeling Using Iodogen. OC 125 F(ab')2 (150 μg in 120 μl of 100 mM phosphate buffer, pH 7.4) and 131I (Dupont-New England Nuclear, Boston, MA) were added to a glass tube containing 10 μg of Iodogen (Pierce Chemical Co., Rockford, IL) and allowed to react for 10 min. The labeled antibody was isolated by chromatography over a Sephadex G-25 column (Pharmacia, Piscataway, NJ). Protein-associated activity in the void-volume peak was determined by precipitation with 10% trichloracetic acid. The protein concentration of the labeled product was determined using the Bio-Rad protein assay (Bio-Rad, Richmond, CA).

Antibody Labeling Using ATE. The synthesis of ATE as well as a detailed description of the radioiodination procedure have been described in a previous report (14). Briefly, to a 1-ml conical vial were added 125I (Dupont-New England Nuclear), 50 μl of 1 M acetic acid in CHCl3, 50 μl of 1 M t-butylhydroperoxide in CHCl3, and 10 μl of 0.1 M ATE in CHCl3. The reaction mixture was stirred for 30 min at room temperature and then purified by passage over a silica gel Sep-Pak column eluted sequentially with 40 ml of hexane, 25 ml of 8% ethyl acetate in hexane and 15 ml of 30% ethyl acetate in hexane. Iodine-125 labeled N-succinimidyl 3-iodobenzoate was eluted in the 30% ethyl

Received 9/14/87; revised 12/10/87; accepted 12/16/87.
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1 This work was supported in part by Grant CA 43234 from the National Cancer Institute.
2 To whom requests for reprints should be addressed, at Duke University Medical Center, Box 3808, Durham, NC 27710.

3 The abbreviations used are: ATE, N-succinimidyl 3-(tri-n-butylstannyl)benzoate; BSA, bovine serum albumin; PBS, phosphate-buffered saline; mIBA, m-[125I]iodobenzoate.

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acetate fractions. In the "high ATE" experiments, all 15 ml of the 30% ethyl acetate eluant were used; in the "low ATE" experiments, the first 3 ml of eluant were discarded. Using methods previously described (14), it was estimated that the low and the high ATE preparations contained approximately 35 and 240 nmol of ATE, respectively.

The 30% ethyl acetate eluate was pooled and evaporated to dryness in a 1-ml glass vial. Approximately 150 μg of OC 125 F(ab')2 in 50 μl of 100 mM borate buffer, pH 8.5, were added and the mixture incubated at 4°C for 30 min. The reaction was terminated by the addition of 0.3 ml of 0.2 M glycine in borate buffer. Purification of 125I-labeled OC 125 F(ab')2 and the determination of trichloroacetic acid-precipitable activity and protein concentration were performed as described above.

Affinity Constant Determination. The human ovarian carcinoma OVCA 433 cell line was obtained from the American Type Culture Collection, Rockville, MD. CA 125 antigen was purified from the OVCA 433/PCA/4B fraction using the method of Davis et al. (18). To each well of a 96-well polystyrene plate were added 100 units of CA 125 in 50 μl of 10 mM sodium bicarbonate buffer, pH 8.6, and this was allowed to dry overnight. Blocking of the wells was achieved by a 1-h incubation with 5% BSA in 100 mM PBS. Nonspecific binding was determined using additional plates coated with BSA. Serial dilutions (0.02 to 4 nM) of each labeled antibody preparation in 50 μl 1% BSA/PBS were added to the wells in triplicate and the plates were incubated overnight at 4°C. The plates were washed 3 times in 1% BSA/PBS and the bound or free activity in each well was determined using an LKB 1282 Compugamma automated gamma counter (LKB; Turku, Finland). Affinity constants were calculated from Scatchard plots of the binding data using the EBBDA/Ligand program developed by McPherson (19).

**RESULTS**

Labeling of Antibody Fragment. Using the iodogen method, 60–70% of the 131I activity was bound to the OC 125 F(ab')2 fragment. 125I labeling of ATE proceeded in greater than 95% yield; discarding the first 3 ml of 30% ethyl acetate eluate in order to reduce the concentration of unreacted ATE in the preparation decreased the effective yield to about 90%. Similar results have been obtained for radioiodination of ATE with 131I with a maximum specific activity of about 800 Ci/mmol being observed. Between 60 and 65% of the N-succinimidyl 3-[125I]iodobenzoate could be coupled to OC 125 F(ab')2, following a 30-min reaction. The ratio of iodine substitutions per antibody molecule for all preparations used in these experiments was less than 0.2:1. Greater than 97% of radioactivity in all antibody preparations was precipitable by trichloroacetic acid.

Assessment of Immunoreactivity. Following an overnight incubation of OC 125 F(ab')2, labeled using the iodogen or low ATE procedures, a maximum of 70–76% of input radioactivity bound to purified CA 125 antigen. Radioiodination of antibody using 125I-labeled ATE containing 240 nmol of unreacted ATE resulted in a product with lower and more variable maximal binding to CA 125 (35–55%). In all cases, binding to BSA-coated plates was less than 1%. Affinity constants for binding to purified CA 125 for OC 125 F(ab')2 labeled using the low ATE, iodogen, and high ATE procedures were (5.2 ± 1.0) x 10^10, (2.5 ± 0.9) x 10^10, and (4.2 ± 2.4) x 10^10 liters/mol, respectively.

Comparison of the Tissue Distribution of Iodide and mIBA. Paired-label experiments were performed in normal mice to evaluate the inertness of the mIBA structure to dehalogenation and to determine whether mIBA, a potential product formed in the catabolism of proteins labeled via ATE, cleared from the body more rapidly than iodide. Table 1 compares the thyroid uptake of 125I and 131I following the injection of m-[125I]IBA and [131I]iodide. In order to ensure that the thyroid gland was removed, some proximal tissue (150–200 mg of muscle and trachea) was included in the sample. The percentage of injected dose of 125I in the thyroid is less than 0.1% at all time points except 4 h, when a thyroid uptake of 0.15% was seen. These levels are significantly lower (P < 0.0001) than those observed for [131I]iodide (9.5–25.7%). If these data are corrected for the presence of extrathyroidal tissue in the sample (using the percentage of uptake per g muscle as a correction factor), the reduced thyroid uptake following injection of mIBA is even more apparent.

The pharmacokinetics of radioiodine activity in the whole body, gastrointestinal tract, liver, blood, lungs, and spleen following injection of iodide and mIBA are compared in Fig. 1. In the kidney, maximum accumulation occurred 1 h after injection of both m-[125I]IBA (0.63%) and [131I]iodide (2.1%). One h after injection of mIBA, whole body radioiodine activity had decreased to 10% of the injected dose, a value 6 times lower than that for iodide. Differences were most apparent in the tissues of the gastrointestinal tract where the levels of 125I were more than 20-fold lower than those of 131I. Counting of urine samples obtained at sacrifice demonstrated that the primary route of excretion for both nuclides was via the urinary system.

**Table 1 Thyroid uptake following injection of [131I]iodide and m-[125I]iodobenzoic acid**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>% of injected dose*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iodide</td>
<td>m-IBA</td>
</tr>
<tr>
<td>1</td>
<td>9.5 ± 3.8</td>
</tr>
<tr>
<td>2</td>
<td>16.6 ± 2.4</td>
</tr>
<tr>
<td>4</td>
<td>24.9 ± 7.8</td>
</tr>
<tr>
<td>6</td>
<td>20.2 ± 5.5</td>
</tr>
<tr>
<td>24</td>
<td>25.7 ± 2.1</td>
</tr>
<tr>
<td>48</td>
<td>19.0 ± 5.5</td>
</tr>
<tr>
<td>72</td>
<td>25.5 ± 5.6</td>
</tr>
<tr>
<td>96</td>
<td>17.3 ± 2.3</td>
</tr>
</tbody>
</table>

* Thyroid, including 150–200 mg of proximal muscle and trachea; 8–11 animals/group; mean ± SD.

* Significance of difference, P < 0.0001 at all time points.
RADIOHALOGENATION OF MONOCLONAL ANTIBODIES

Although the activity ranges observed in these samples were large, the average values of 57, 54, 6, and 2% for $^{125}$I and 18, 16, 20, and 12% for $^{131}$I at 1, 2, 4, and 6 h, respectively, were consistent with the whole body clearance data illustrated in Fig. 1.

Comparative Biodistribution of OC 125 F(ab')$_2$ Radioiodinated via ATE and Iodogen. Paired-label biodistribution measurements were performed to compare the extent of dehalogenation and tumor specificity of OC 125 F(ab')$_2$ labeled with $^{125}$I using the ATE reagent and with $^{131}$I using Iodogen. Since no statistically significant differences were observed in thyroid uptake between the low and the high ATE experiments, data from both sets of animals were combined and summarized in Fig. 2. Thyroid uptake of $^{131}$I ranged between 5.36% of the injected dose at 24 h and 5.91% at 96 h. Use of the ATE method for antibody radiohalogenation reduced the thyroid uptake of radioiodine by more than two orders of magnitude ($P < 0.0001$) to less than 0.1% of the injected dose.

The biodistribution of $^{125}$I activity after injection of $^{125}$I-labeled OC 125 F(ab')$_2$ prepared using the more stringent ATE purification procedure is summarized in Table 2. At 24 h, the highest concentration of activity was seen in the liver, OVCAR-3 tumor and spleen with less than 0.2% of the injected dose per g tissue noted in the heart, muscle, bone, stomach, and intestines. The half-life for clearance of $^{125}$I from tumor was about 3 days. With the exception of the spleen, clearance of $^{125}$I from normal tissues occurred with half-lives of about 6 h.

Tumor to normal tissue ratios of radioiodine following injection of F(ab')$_2$ labeled using the ATE (35 nmol preparation) and the Iodogen methods are compared in Fig. 3. For all tissues except the spleen, higher tumor:tissue ratios were obtained with antibodies labeled via the ATE method. At 96 h, tumor to blood, stomach, intestine, lung, and kidney ratios for F(ab')$_2$ labeled using ATE were more than 4 times higher than those for antibody labeled by the Iodogen method. Similar trends were observed in the paired-label experiments using OC 125.

Table 2. Tissue distribution of $^{125}$I in athymic mice bearing OVCAR-3 tumors following injection of OC 125 F(ab')$_2$ labeled using the ATE reagent.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>24 h % of injected dose/g</th>
<th>48 h % of injected dose/g</th>
<th>72 h % of injected dose/g</th>
<th>96 h % of injected dose/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>4.12 ± 0.88</td>
<td>0.88 ± 0.25</td>
<td>0.25 ± 0.09</td>
<td>0.05 ± 0.02</td>
</tr>
<tr>
<td>Spleen</td>
<td>1.77 ± 1.03</td>
<td>1.03 ± 0.31</td>
<td>0.34 ± 0.15</td>
<td>0.21 ± 0.07</td>
</tr>
<tr>
<td>Lung</td>
<td>0.53 ± 0.22</td>
<td>0.12 ± 0.04</td>
<td>0.06 ± 0.03</td>
<td>0.025 ± 0.011</td>
</tr>
<tr>
<td>Heart</td>
<td>0.08 ± 0.04</td>
<td>0.015 ± 0.003</td>
<td>0.023 ± 0.009</td>
<td>0.005 ± 0.001</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.86 ± 0.42</td>
<td>0.08 ± 0.01</td>
<td>0.045 ± 0.015</td>
<td>0.018 ± 0.004</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.19 ± 0.06</td>
<td>0.06 ± 0.01</td>
<td>0.035 ± 0.005</td>
<td>0.014 ± 0.008</td>
</tr>
<tr>
<td>Small intestine</td>
<td>0.17 ± 0.08</td>
<td>0.06 ± 0.01</td>
<td>0.04 ± 0.01</td>
<td>0.014 ± 0.006</td>
</tr>
<tr>
<td>Large intestine</td>
<td>0.16 ± 0.07</td>
<td>0.034 ± 0.009</td>
<td>0.024 ± 0.008</td>
<td>0.007 ± 0.003</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.07 ± 0.03</td>
<td>0.009 ± 0.003</td>
<td>0.008 ± 0.004</td>
<td>0.003 ± 0.002</td>
</tr>
<tr>
<td>Bone</td>
<td>0.10 ± 0.03</td>
<td>0.038 ± 0.008</td>
<td>0.03 ± 0.01</td>
<td>0.013 ± 0.007</td>
</tr>
<tr>
<td>Blood</td>
<td>0.22 ± 0.09</td>
<td>0.046 ± 0.007</td>
<td>0.013 ± 0.005</td>
<td>0.013 ± 0.003</td>
</tr>
<tr>
<td>Tumor</td>
<td>2.61 ± 0.79</td>
<td>2.18 ± 0.46</td>
<td>1.56 ± 0.19</td>
<td>1.27 ± 0.47</td>
</tr>
</tbody>
</table>

Fig. 2. Comparison of the percentage of injected dose of radioiodine taken up by the thyroid following injection in athymic mice of OC 125 F(ab')$_2$, radioiodinated using the Iodogen (lod.) and ATE methods.

Fig. 3. Tumor: normal tissue ratios observed in athymic mice bearing OVCAR-3 tumors. Mice given injections of 8 μg of OC 125 F(ab')$_2$, half labeled with $^{131}$I using Iodogen (O) and half labeled with N-succinimidyl 3-(125I)iodobenzoate (●) containing 35 nmol unreacted ATE.
F(ab')₂ exposed to a higher level of ATE; however, the magnitude of improvement in the specificity of tumor uptake was somewhat less.

In order to relate the more optimal tumor specificity of antibodies labeled via ATE to increased tumor uptake and/or decreased retention of nuclide in normal tissue, the ratio of ¹²⁵I activity to ¹³¹I activity in each tissue was calculated for both the low and the high ATE experiments (Table 3). In the low ATE experiment, the concentration of ¹²⁵I activity in the tumor was 27–49% greater than that of ¹³¹I. With the exception of the 96-h animals where a modest 10 ± 4% improvement was seen, antibody exposed to the higher level of ATE did not exhibit a statistically significant difference in tumor uptake when compared to antibody labeled via iodogen. The differences in the tumor ¹²⁵I to ¹³¹I uptake ratios between the high and the low ATE procedures were significant (P < 0.01–0.001).

With the exception of the spleen, and at early time points the liver, use of the ATE method for antibody labeling reduced the uptake of radioiodine in normal tissues by as much as a factor of 10. Generally, the decrease in normal tissue accumulation was similar for antibodies exposed to both levels of ATE; however, at later time points, it appears that use of the low ATE preparation may offer a slight advantage.

DISCUSSION

Radiolabeled monoclonal antibodies are a promising approach to the diagnosis and treatment of cancer; however, their impact on the clinical domain remains somewhat limited. If the potential of antibody-mediated targeting of nuclides is to be exploited more fully, a number of problems must be addressed, not the least of which is the development of improved methods for radiolabeling monoclonal antibodies.

While the ideal approach for labeling each antibody will probably be different and may depend on the nature of the intended clinical application, at least three criteria should be considered: (a) the in vivo stability of the bond between the nuclide and the antibody should be maximized; (b) the labeled species created in the catabolism of the antibody should be excreted rapidly; and (c) the labeling process should not decrease the affinity of the antibody for its antigenic target. With these factors in mind, we have evaluated the utility of a new reagent, N-succinimidyl-3-(tri-i-butylstannyl)benzoate, for the radiohalogenation of antibodies.

One approach to decreasing the retention of radioisotope in normal tissues following the administration of labeled antibodies is to couple the nuclide in a chemical form which will be excreted rapidly following antibody catabolism. Degradation of proteins labeled using the ATE method should result in the production of iodide and m-iodobenzoic acid as labeled metabolites. The lack of thyroid uptake observed in the tissue distribution studies suggests that iodide is not a major catabolic product. When mIBA was injected into mice, whole-body clearance of radioiodine was considerably faster than that of iodide. This is not surprising since benzoic acids are known to form glycosyl conjugates which are excreted rapidly via the urine as hippuric acid (24).

The importance of preserving the affinity of monoclonal antibodies for their antigenic targets after radiolabeling cannot be overemphasized. Badger et al. (25) and Herlyn et al. (26) have both shown that the concentration of radioactivity in a tissue is dependent upon the amount of unreacted ATE, the affinity constant of the labeled protein was statistically significant difference in tumor uptake when compared to antibody labeled via iodogen: The differences in the tumor ¹²⁵I to ¹³¹I uptake ratios between the high and the low ATE procedures were significant (P < 0.01–0.001).

With the exception of the spleen, and at early time points the liver, use of the ATE method for antibody labeling reduced the uptake of radioiodine in normal tissues by as much as a factor of 10. Generally, the decrease in normal tissue accumulation was similar for antibodies exposed to both levels of ATE; however, at later time points, it appears that use of the low ATE preparation may offer a slight advantage.
method. However, reaction of F(ab')₂ with preparations of N-succinimidyl 3-[¹²⁵I]iodobenzoate containing higher levels of ATE decreased the affinity constant of the ¹²⁵I-labeled antibody to about 5 times less than that measured for iodogen-labeled preparations. We speculate that the decrease in affinity may be due to coupling of the highly lipophilic ATE compound to amine sites on the protein or perhaps may be related to non-specific interactions of ATE with hydrophobic regions of the antibody. In addition to adversely affecting antibody affinity [at least for OC 125 F(ab')₂], we have previously reported that exposure of proteins to relatively high levels of the ATE reagent decreased protein radiiodination yields (14).

In the high ATE paired-label biodistribution experiments, tumor uptake following injection of antibody labeled using ATE and Iodogen was quite similar suggesting that the lower affinity of the ATE preparation may have been offset by its greater stability in vivo. When the low ATE preparation was used, the combination of higher affinity and decreased loss of label in vivo resulted in a significant improvement in tumor uptake compared to iodogen-labeled antibody.

Thus, it is reasonable to assume that the superior tumor:normal tissue ratios observed with OC 125 F(ab')₂ labeled using the ATE method (Fig. 3) may be due to a combination of improvements in inertness to deiodination in vivo, rate of clearance of labeled catabolites, and antibody affinity. It is interesting to note that in the spleen, and to some extent the liver, retention of radiiodine is greater with OC 125 F(ab')₂ labeled via ATE. Since CA 125 antigen is shed into the circulation in the mouse model used in these experiments (27), it is possible that these differences reflect a differential rate of dehalogenation of labeled immune complexes in these tissues. Similar differences have been reported for the liver and spleen uptake of conventionally radioiodinated and ¹¹¹In-labeled anti-carinoembryonic antigen antibodies in animal models (28).

In summary, the results reported in this investigation suggest that the ATE method holds great promise as an approach for the radiohalogenation of monoclonal antibodies. Experiments are in progress to investigate the utility of the ATE reagent for labeling other antibodies and their fragments.

ACKNOWLEDGMENTS

The authors wish to thank Cary Harrison and Susan Slade for excellent technical assistance.

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