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Reagents for Astatination of Biomolecules. 4. Comparison of Maleimido-*c*-closo-Decaborate(2-) and *meta*-[²¹¹At]Astatobenzoate Conjugates for Labeling anti-CD45 Antibodies with [²¹¹At]Astatine

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Abstract

An investigation was conducted to compare the in vivo tissue distribution of a rat anti-murine CD45 monoclonal antibody (30F11) and an irrelevant MAb (CA12.10C12) labeled with ²¹¹At using two different labeling methods. In the investigation, the MAbs were also labeled with ¹²⁵I to assess the in vivo stability of the labeling methods towards deastatination. One labeling method employed N-hydroxysuccinimidyl *meta*-[²¹¹At]astatobenzoate, [²¹¹At]**1c**, and N-hydroxysuccinimidyl *meta*-[¹²⁵I]iodobenzoate, [¹²⁵I]**1b**, in conjugation reactions to obtain the radiolabeled MAbs. The other labeling method involved conjugation of a maleimido-*c*-closo-decaborate(2-) derivative, **2**, with sulfhydryl groups on the MAbs, followed by labeling of the MAb-**2** conjugates using Na[²¹¹At]At or Na[¹²⁵I]I and chloramine-T. Concentrations of the ²¹¹At/¹²⁵I pair of radiolabeled MAbs in selected tissues were examined in BALB/c mice at 1, 4 and 24 h post injection (pi). The co-injected anti-CD45 MAb, 30F11, labeled with [¹²⁵I]**1b** and [²¹¹At]**1c** targeted the CD-45-bearing cells in the spleen with the percent injected dose (%ID) of ¹²⁵I in that tissue being 13.31 ± 0.78; 17.43 ± 2.56; 5.23 ± 0.50 and ²¹¹At being 6.56 ± 0.40; 10.14 ± 1.49; 7.52 ± 0.79 at 1, 4 and 24 h pi (respectively). However, better targeting (or retention) of the ¹²⁵I and ²¹¹At was obtained for 30F11 conjugated with the *c*-closo-decaborate(2-), **2**. The %ID in spleen of ¹²⁵I (i.e. [¹²⁵I]30F11-**2**) being 21.15 ± 1.33; 22.22 ± 1.95; 12.41 ± 0.75 and ²¹¹At (i.e. [²¹¹At]30F11-**2**) being 22.78 ± 1.29; 25.05 ± 2.35; 17.30 ± 1.20 at 1, 4 and 24 h pi (respectively). In contrast, the irrelevant MAb, CA12.10C12, labeled with ¹²⁵I or ²¹¹At by either method had less than 0.8% ID in the spleen at any time point, except for [²¹¹At]CA12.10C12-**1c**, which had 1.62 ± 0.14 and 1.21 ± 0.08 %ID at 1 and 4 h pi. The higher spleen concentrations in that conjugate appear to be due to in vivo deastatination. Differences in ¹²⁵I and ²¹¹At concentrations in lung, neck and stomach indicate that the *meta*-[²¹¹At]benzoyl conjugates

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Supporting Information Available: Tables (S1–S5) containing biodistribution data, either %ID/g or %ID, in selected tissues, and copies of UV- and radiochromatograms (Figures S1–S4) for [²¹¹At]**1c** and [¹²⁵I]**1b** are provided. This information is available free of charge via the Internet at <http://pubs.acs.org>.

underwent deastatination, whereas the ^{211}At -labeled *closo*-decaborate(2-) conjugates were very stable to in vivo deastatination. In summary, using the *closo*-decaborate(2-) ^{211}At labeling approach, resulted in higher concentrations of ^{211}At in target tissue (spleen) and higher stability to in vivo deastatination in this model. These findings, along with the simpler and higher yielding ^{211}At -labeling method, provide the basis for using the *closo*-decaborate(2-) labeling reagent, **2**, in our continued studies of the application of ^{211}At -labeled MABs for conditioning in hematopoietic cell transplantation.

Keywords

Radioiodination; Astatination; *closo*-Decaborate; Astatobenzoate Conjugation; Antibody Labeling

INTRODUCTION

Our laboratory is investigating the application of antibody-targeted α -particle emitting radionuclides as a replacement to total body irradiation (TBI) in conditioning for hematopoietic cell transplantation (1). In prior studies, we demonstrated that anti-CD45 and anti-TCR $\alpha\beta$ monoclonal antibodies (MABs) labeled with the α -emitting radionuclide [^{213}Bi]bismuth could replace TBI in a conditioning regimen to obtain stable hematopoietic cell engraftment in a dog model (2–4). Although the studies successfully demonstrated that stable chimera could be obtained by combining the targeted alpha therapy with immunosuppression, the cost and availability of the parent radionuclide [^{225}Ac]actinium required to generate the ^{213}Bi precluded translation into a clinical study.

The ability to produce the α -emitting radionuclide [^{211}At]astatine at our institution led us to consider that radionuclide as an alternative to ^{213}Bi in the conditioning regimen. In planning the transition to ^{211}At , we deliberated about the best method for coupling ^{211}At to the MAB. It has been well established that direct coupling of ^{211}At to MABs through electrophilic astatination results in proteins that are rapidly deastatinated in vivo (5). To circumvent the in vivo deastatination, our research group (6) and other research groups (7–11) have developed reagents for astatination using ^{211}At -labeled aryl-containing pendant groups for conjugation with proteins. The ^{211}At -labeled aryl conjugates are now widely used to radiolabel MABs with ^{211}At for preclinical studies. Importantly, one of the astatination reagents, N-hydroxysuccinimidyl *meta*-[^{211}At]astatobenzoate, [^{211}At]**1c**, has been used for labeling MABs in a clinical trial for therapy of malignant brain tumors (12).

Our studies have shown that ^{211}At -labeled benzoates can be relatively stable to in vivo deastatination on intact MABs, but are quite unstable when used with more rapidly metabolized MAB Fab' fragments (6,13) or smaller biomolecules such as biotin derivatives (14). To improve in vivo stability, we have evaluated protein conjugates that contain boron cage moieties as ^{211}At -labeling functionalities. From those studies, it was found that ^{211}At -labeled conjugates containing *closo*-decaborate(2-) are stable to in vivo deastatination (15). One conjugate in particular, a maleimido-*closo*-decaborate(2-) derivative, **2**, was found to have high stability to deastatination while having minimal effect on the conjugated protein.

Both of the ^{211}At labeling reagents, [^{211}At]**1c** and **2**, have features that make them attractive for use in labeling MABs for conditioning regimens. The fact that the astatinated benzoate [^{211}At]**1c** had previously been used in a clinical trial made that reagent particularly attractive. However, that fact did not override the issues of the potential for; (a) radiolysis in the preparation of [^{211}At]**1c** (16–18), (b) hydrolysis of the active ester during MAB conjugation of [^{211}At]**1c**, and (c) in vivo instability to deastatination on the ^{211}At -labeled MAB. The simplicity of direct labeling of proteins conjugated with the maleimide-

*closo*decaborate(2-) derivative, **2**, and the high in vivo stability observed for the ^{211}At -labeled proteins when using the reagent, made this novel reagent attractive for our studies. To choose between the two reagents, we conducted an investigation to compare the ^{211}At -labeling efficiency and in vivo stability of a rat anti-murine-CD45 MAb (30F11) and an irrelevant MAb (CA12.10C12) in mice. The results of that investigation are described herein.

MATERIALS AND METHODS

Reagents

All reagents obtained from commercial sources were used without further purification. Bismuth metal used as the target for preparation of ^{211}At was obtained from Alpha Aesar (Ward Hill, MA) as Puratronic 99.999%. Solvents for HPLC analysis were obtained as HPLC grade and were filtered (0.2 μm) prior to use. Chloramine-T (ChT) and phosphate buffered saline (PBS) were obtained from Sigma (St. Louis, MO). Sephadex G-25 desalting columns (PD-10 and NAP-10) were obtained from Amersham Biosciences (Piscataway, NJ). Vivaspin 20 ultrafiltration concentrators were obtained from Sartorius Stedim North America, Inc. (Edgewood, N.Y.). The reagents, **1a** and **2**, used in labeling the antibodies, 30F11 and CA12.10C12 were prepared prior to the study. The N-hydroxysuccinimidyl *meta*-(tri-*n*-butylstannyl) benzoate, **1a**, was prepared as previously described (19). The protein modification reagent, N-(15-(aminoacyl-*closo*-decaborate)-4,7,10-trioxatridecanyl)-3-maleimidopropionamide, **2**, was prepared as previously described (15).

Antibodies

The rat anti-murine CD45 MAb, 30F11, employed in the study is a rat IgG2b antibody that recognizes all murine CD45 isoforms (20). A 30F11 hybridoma cell line expressing the rat antimurine CD45 MAb was a gift from Dr. Irv Bernstein (FHCRC). The 30F11 was produced by injecting the hybridoma into pristane-primed mice to generate ascites. The 30F11 was purified from ascitic fluid by protein G immunoabsorption column chromatography. The irrelevant MAb, CA12.10C12 is an IgG1 anti-CD45 canine-specific antibody (21). A hybridoma producing monoclonal antibodies against canine CD45 was provided by Dr. Peter Moore (School of Veterinary Medicine, University of California, Davis). This MAb was produced and purified at the Biologics Production Facilities of the Fred Hutchinson Cancer Research Center (Seattle, WA) as described previously (22). A fluorescein isothiocyanate (FITC) labeled goat F(ab')₂ anti-rat was obtained from Biosource International (Camarillo, CA) for murine hematopoietic cell binding (serum) using flow cytometry. The 30F11 MAbs, both native and conjugated with **2**, were conjugated with fluorescein isothiocyanate according to manufacturer's standard protocols, and were also used in flow cytometry studies.

Radioactivity

All radioactive materials were handled according to approved protocols at the University of Washington. Na[^{125}I] was purchased from Perkin-Elmer Life and Analytical Sciences, Inc. (Waltham, MA) as a high concentration solution in 0.1 N NaOH. ^{211}At was produced by irradiation of a thin layer of bismuth metal with a 28 MeV α -particle beam on a Scandatronix MC-50 cyclotron (University of Washington). Isolation of Na[^{211}At]At was conducted in a charcoal-filtered glove box (Innovative Technologies, Inc., radioisotope glove box) by distillation from the irradiated target using the conditions previously described (13,15). Radiohalogenations were conducted within a charcoal-filtered Plexiglas enclosure (Biodex Medical Systems Inc., Shirley, N.Y.) housed in a radiochemical fume hood. Radiohalogenation reactions were carried out in vials capped with Teflon-coated septa vented through a 10 mL charcoal-filled syringe.

Measurement of ^{125}I and ^{211}At quantities was accomplished on the Capintec CRC-15R Radioisotope Calibrator using the manufacturer's settings for those radionuclides. Tissue samples were counted in a measured with a γ -counter (PACKARD® COBRA™ GMI, Inc., Minnesota, USA), using the open window setting: channels 20–2000. In experiments, $^{211}\text{At}/^{125}\text{I}$ counting was conducted twice, soon after animal sacrifice to obtain total counts and after samples had decayed for 3–5 days to obtain the ^{125}I counts. ^{211}At counts were obtained by subtracting ^{125}I counts from the total counts. All ^{211}At counts were corrected for decay during the counting process using 1 μL of injectate as standards.

Analytical Chromatography

HPLC separations of the non-radioactive compounds, **1a** and **2** (and their precursors) were obtained using a system that contained a Hewlett-Packard quaternary 1050 gradient pump, a variable wavelength UV detector (254 nm), and an ELSD 2000 evaporative light-scattering detector (Alltech, Deerfield, IL). Analysis of the HPLC data were conducted on Hewlett-Packard HPLC ChemStation software. Reversed-phase HPLC chromatography was carried out on an Alltech Altima C-18 column (5 μm , 250 $\times$ 4.5 mm) using a gradient solvent system at a flow rate of 1 mL/min. The gradient mixture was composed of MeOH (eluant A) and 0.05 M, pH 5.5 aqueous triethylammonium acetate (eluant B). The gradient started with 40% MeOH and 60% eluant B for two minutes; then the % MeOH was increased linearly to 100% over a 13 min period; following this the elution continued with 100% MeOH for an additional 5 min.

Radio-HPLC

The product mixtures from the preparation of [^{125}I]**1b** and [^{211}At]**1c** were analyzed by HPLC using a 5 μm C-18 column (PartiSphere C-18, Whatman) eluting at a flow rate of 1 mL/min with a gradient beginning with 40% MeOH / 60% of an aqueous Et_3NHOAc solution. The HPLC equipment used in the analyses consisted of a HP 1050 quaternary pump, a Waters 481 UV detector (254 nm), a 1050 A/D converter, and a Beckman model 170 radioisotope detector.

Isoelectric Focusing

IEF were obtained on a Novex PowerEase 500 instrument with the XCell II chamber using Invitrogen (Novex) precast gels, pH 3–10 (1.0 mm, 12 well) or pH 3–7 (1.0 mm, 12 well) running under the standard IEF Program. The protein was stained with GelCode Blue Stain (Pierce). IEF standards used were from Serva Electrophoresis GmbH (Heidelberg, Germany): (pI) 10.7 - cytochrome C; 9.5 - ribonuclease A; 8.3, 8.0, 7.8 - lectin; 7.4, 6.9 - myoglobin; 6.0 - carbonic anhydrase; 5.3, 5.2 - β -lactoglobulin; 4.5 - trypsin inhibitor; 4.2 - glucose oxidase; 3.5 - amyloglucosidase.

Fluorescein Isothiocyanate (FITC) Conjugation

One milligram of fluorescein isothiocyanate (Sigma-Aldrich, Wisconsin, MI) was dissolved in 0.05 mL of DMSO and further diluted to a concentration of 1 mg/mL with 1M sodium bicarbonate, pH 9.4. Then 0.25 mL of this solution is added to 1 mg/mL of the antibody in 1x phosphate buffered saline (PBS). After incubation at 37°C, CO_2 humidified incubator, the antibody is transferred to a dialysis cassette, 10MW cut off (Pierce Biotechnology, Rockford, IL) and dialyzed against 1x PBS to remove unbound FITC. Antibody concentration is measured using a BioMate spectrophotometer (Thermo Electron Corporation, Madison WI).

Flow Cytometry

Flow cytometry was used to assess antigen binding after modification of the anti-CD45 MAb, 30F11, with the maleimido-*closo*-decaborate(2-) reagent, **2**. SL2 cells, a mouse cell line expressing the CD45 antigen, was grown in 10% fetal bovine serum - RPMI until ready for use. Two hundred thousand SL2 cells in 50 μ L of 15% horse serum (HS)-1x PBS were incubated with 50 μ g/mL of the 30F11 or 30F11-**2** conjugate on ice for 20 minutes. After washing, cells were resuspended in 50 μ L of 15% HS-PBS and added 10 μ g/mL of the secondary antibody, goat F(ab')₂ anti-rat FITC (Biosource, Camarillo, CA), and incubated on ice for 20 min. To test the blocking ability of the antibody, FITC conjugated 30F11 was added to cells incubated with 30F11 or 30F11-**2**, respectively. After incubation, cells were washed once with 2% HS-PBS, then once with 1x PBS and resuspended in 200 μ L of 1% paraformaldehyde. Cells were analyzed on a FACScan Flow cytometer (Becton Dickinson, San Jose, CA).

Radioiodination of 30F11 using [¹²⁵I]1b

To 100 μ L of a solution of **1a** (1 mg/mL in MeOH/5% HOAc) was added 3.5 μ L (1.2 mCi) of Na[¹²⁵I]I in 0.1 N NaOH followed by 20 μ L of a N-chlorosuccinimide solution (1 mg/mL in MeOH). After 10 min at room temperature, 20 μ L of a sodium metabisulfite solution (1 mg/mL in H₂O) was added to quench the reaction. A small amount of the mixture was injected on the HPLC to check the reaction yield (require at least 90%). The mixture was then reduced to dryness under a stream of argon and a mixture containing 500 μ L of a 0.5 M sodium borate solution, pH 8.0, and 100 μ L of 30F11 solution (6.7 mg/mL in PBS) was added. The conjugation reaction was allowed to run for 15 min at room temperature before eluting on a PD-10 column with PBS, pH 7.2. The protein fractions were pooled to give [¹²⁵I]30F11-**1b** with a specific activity of 0.8 mCi/mg and a radiochemical yield of 61%.

Radioiodination of CA12.10C12 with [¹²⁵I]1b

This reaction was conducted using the same conditions as described for preparation of [¹²⁵I]30F11-**1b** with the following differences. 1.5 μ L of Na[¹²⁵I]I (920 μ Ci) in 0.1 N NaOH and 147 μ L of 2.7 mg/mL CA12.10C12 was used. [¹²⁵I]CA12.10C12-**1b** was obtained with a specific activity of 0.92 mCi/mg and a radiochemical yield of 63%.

Astatisation of 30F11 with [²¹¹At]1c

To a 200 μ L solution of **1a** (1 mg/mL in MeOH/5% HOAc) was added 20 μ L (780 μ Ci) of Na[²¹¹At]At (in 0.05 N NaOH) followed by 20 μ L of a N-chlorosuccinimide solution (1 mg/mL in MeOH). After 10 min at room temperature, 20 μ L of a sodium metabisulfite solution (1 mg/mL in H₂O) was added to quench the reaction. A small amount of the mixture was injected on the HPLC to check the reaction yield (90% [²¹¹At]1c required). The mixture was then reduced to dryness under a stream of argon and a mixture containing 500 μ L of a 0.5 M sodium borate solution, pH 8.0, and 90 μ L of 30F11 solution (6.7 mg/mL in PBS). The conjugation reaction was allowed to run for 15 min at room temperature before eluting on a PD-10 column with PBS. The protein fractions were pooled to give [²¹¹At]30F11-**1c** with a specific activity of 0.03 mCi/mg and a radiochemical yield of 4.4%.

Astatisation of CA12.10C12 with [²¹¹At]1c

This reaction was conducted under the same conditions as those used to prepare [²¹¹At]30F11-**1c**, with the following changes. A 100 μ L quantity of **1c**, 150 μ L of Na[²¹¹At]At (1.2 mCi), 30 μ L of 1 mg/mL N-chlorosuccinimide, and 147 μ L of a CA12.10C12 solution (2.7 mg/mL in PBS) were used. [²¹¹At]CA12.10C12-**1c** was obtained with a specific activity of 0.03 mCi/mg and a radiochemical yield of 4.8%.

Conjugation of 30F11 with 2

To a 2 mL solution containing 5.3 mg/mL 30F11 in PBS, pH 7.2, was added 270 μ L of 100 mM dithiothreitol. After 1 h of gentle mixing at room temperature, the reduced protein was eluted over a PD-10 column with PBS (pH 6.5 containing 1 mM EDTA). The protein-containing fractions were pooled to give 7 mL at a concentration of 1.5 mg/mL (UV 280 nm). To that solution was added 49.3 μ L (10 equivalents) of a 10 mg/mL solution of **2** in DMF. The reaction solution was gently mixed for 30 min at room temperature before quenching with 2.4 mg of sodium tetrathionate. The protein was concentrated in a Vivaspinn 30K MWCO concentrator and washed 5x with 10 mL each of PBS pH 7.2, concentrating between each wash. The isolated 30F11-**2** was obtained in 4 mL solution at a concentration of 2.4 mg/mL (90% protein recovery).

Conjugation of CA12.10C12 with 2

The same procedure as described for conjugation with 30F11 (above) was used, with the following difference in reagent quantities. To 1.5 mL solution containing 6.1 mg/mL CA12.10C12 in PBS was added 200 μ L of 100 mM dithiothreitol. The CA12.10C12-**2** was obtained in 6.5 mL at a concentration of 1.2 mg/mL (85% protein recovery).

Radioiodination of 30F11-2

To 25 μ L of a 0.5M solution of sodium phosphate, pH 7.4, was added 1.5 μ L (274 μ Ci) of Na[¹²⁵I]I solution (0.1N NaOH), followed by 163 μ L (400 μ g) 30F11-**2** and 20 μ L of a chloramine-T solution (1 mg/mL in H₂O). The reaction was allowed to run for 30 s at room temperature before quenching with 20 μ L of a sodium metabisulfite solution (1 mg/mL in H₂O). The mixture was eluted over a NAP-10 column using PBS, pH 7.2. The protein fractions were pooled to give [¹²⁵I]30F11-**2** with a specific activity of 0.63 mCi/mg and a radiochemical yield of 81%.

Radioiodination of CA12.10C12-2

The radioiodination was conducted using the same conditions as used for preparation of [¹²⁵I]30F11-**2**, with the following changes. One μ L (484 μ Ci) of Na[¹²⁵I]I in 0.1N NaOH was added followed by 250 μ L (300 μ g) CA12.10C12-**2** and 30 μ L of chloramine-T solution (1 mg/mL in H₂O). [¹²⁵I]CA12.10C12-**2** was obtained with a specific activity of 1.5 mCi/mg and a radiochemical yield of 62%.

Astatination of 30F11-2

To 100 μ L of a 0.5M aqueous sodium phosphate solution, pH 7.4, was added 400 μ L (580 μ Ci) of Na[²¹¹At]At solution (0.05N NaOH) followed by 163 μ L (400 μ g) 30F11-**2** and 80 μ L of a chloramine-T solution (1 mg/mL in H₂O). The reaction was allowed to run for 30 s at room temperature before quenching with 80 μ L of sodium metabisulfite solution (1 mg/mL). The mixture was eluted over a NAP-10 column using PBS. The protein fractions were pooled to give [²¹¹At]30F11-**2** with a specific activity of 0.9 mCi/mg and a radiochemical yield of 65%.

Astatination of CA12.10C12-2

The astatination was conducted using the same conditions as described for preparation of [²¹¹At]30F11-**2**, with the following changes. One hundred μ L (577 μ Ci) of Na[²¹¹At]At in ~0.05N NaOH, followed by 250 μ L (300 μ g) CA12.10C12-**2** and 50 μ L of a chloramine-T solution (1 mg/mL in H₂O). [²¹¹At]CA12.10C12-**2** was obtained with a specific activity of 1.6 mCi/mg and a radiochemical yield of 81%.

Biodistribution Studies

Biodistribution studies were conducted under a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the Fred Hutchinson Cancer Research Center. Female BALB/c mice were obtained from the Jackson Laboratory (Bar Harbor, Maine USA). In each biodistribution experiment, the radioactive MABs isolated from size-exclusion columns (Sephadex G25; PD-10) were mixed to prepare an admixture. The admixture was diluted with phosphate buffered saline (PBS) to prepare injection quantities of ~200 μ L. This quantity was injected into each of 15 mice via the lateral tail vein. The actual amount of injectate each animal received was determined by weighing the administering syringe before and after injection. Groups of 5 mice were sacrificed at 1, 4 and 24 h post injection. Selected tissues were excised, blotted free of blood, weighed, and counted. Blood weight was estimated to be 6% of the total body weight (23). Calculation of the percent injected dose per gram (%ID/g) and percent dose (%ID) in the tissues was accomplished using internal standards for the ^{211}At and ^{125}I counts. The tissues examined, quantities injected and biodistribution data obtained in the studies are provided in Tables S1 – S4 (Supporting Information).

RESULTS

MAB modification and labeling

The rat anti-murine CD45 MAB, 30F11, and canine-specific (irrelevant) MAB, CA12.10C12, were each radiolabeled with ^{211}At and ^{125}I by two different methods. One labeling method involved preparation of N-hydroxysuccinimidyl *meta*-[^{211}At]astatobenzoate, [^{211}At]**1c**, and N-hydroxysuccinimidyl *meta*-[^{125}I]iodobenzoate, [^{125}I]**1b** from N-hydroxysuccinimidyl *meta*-tri-*n*-butylstannylbenzoate **1a** (Figure 1). Radioiodination and astatination of **1a** was accomplished by reaction with N-chlorosuccinimide (NCS) in MeOH/5% HOAc at room temperature for 10 min. Preparation of the radiolabeled intermediates [^{125}I]**1b** and [^{211}At]**1c** was accomplished in >90% purity by HPLC analyses (see Figures S1–S4 in Supporting Information). Once prepared, [^{125}I]**1b** was reacted with 670 μ g of 30F11 or 397 μ g of CA12.10C12 in borate buffer, pH 8.0 for 15 min at room temperature to give conjugation yields of 61% and 63% respectively. In contrast to the radioiodinations using [^{125}I]**1b**, astatinations under the same conditions using [^{211}At]**1c** produced very low yields, providing only 4.4% of [^{211}At]30F11-**1c** and 4.8% of [^{211}At]CA12.10C12-**1c**. The reason for the difference in radiochemical (conjugation) yields between the astatinated benzoates, [^{211}At]**1c** and the radioiodinated benzoates, [^{125}I]**1b** is unknown. Since the quantities of MAB used (603 μ g 30F11 and 397 μ g CA12.10C12) in the conjugation reactions using [^{211}At]**1c** were similar to those used in the conjugations of [^{125}I]**1b**, differences in protein concentrations does not appear to be the reason for the lower yields. Although the radiolabeled intermediates in each reaction were >90% pure by HPLC analyses prior to removal of the solvents, it is possible that impurities or differences in pH of the astatinated preparations led to a more rapid hydrolysis of [^{211}At]**1c** on removal of the solvents. The volumes of aqueous Na[^{125}I] solution used (1.5 or 3.5 μ L) in the radiolabeling reactions were considerably lower than the volumes of aqueous Na[^{211}At]At solution used (i.e. 20 μ L and 150 μ L), making this possibility plausible. Irrespective of the low conjugation yields for the astatinated proteins, enough labeled protein was obtained after purification in each case to conduct the animal studies.

The second method of labeling MABs involved conjugation of the maleimide-*closo*-decaborate(2-) reagent, **2** (Figure 1), followed by direct radiolabeling of the MAB conjugates. In the conjugation, 30F11 or CA12.10C12 was treated with dithiothreitol (DTT) for 1 h at room temperature, then 10 molar equivalents of **2** in DMF was added and the reaction mixture was stirred for 30 min at room temperature. After that time sodium

tetrathionate was added to stop the reaction by reformation of disulfide bonds (24). The resulting MAb conjugates, 30F11-2 and CA12.10C12-2, were purified by Sephadex G-25 size exclusion chromatography (PD-10). Although the number of *closo*-decaborate(2-) conjugates per modified MAb was not readily obtained, the extent of modification was assessed by the change in protein isoelectric point (pI) observed with isoelectric focusing (IEF) gel electrophoresis. Each conjugation of **2** changes the overall protein charge by -2. Scanned IEF gels are shown as Figure 2. In Figure 2 it is apparent that the two unmodified IgG have very different pI values (30F11: ~7.5–7.9; CA12.10C12: ~6.2–6.6) and that the proteins are modified in the conjugation reaction. While not quantifiable, it appears that at least one *closo*-decaborate derivative, **2**, was conjugated to each protein molecule, as all of the bands (lanes C and G) of the native protein are shifted to a more negative pI in the conjugation reaction. Further characterization was conducted to determine if the CD45 cell binding was affected in the 30F11. Cell binding for the 30F11-2 and non-modified 30F11 as measured by a FITC-labeled goat anti-rat F(ab')₂ MAb are shown in Figure 3. The graphed data indicate that there is similar binding of 30F11-2 relative to the non-modified 30F11. Running the samples in the presence of excess FITC-labeled 30F11 shows that the cell bound 30F11-2 (and 30F11) is CD45 specific binding as it can be displaced.

Direct labeling of 30F11-2 and CA12.10C12-2 was accomplished by reaction of Na[²¹¹At]At or Na[¹²⁵I]I with chloramine-T in sodium phosphate buffer. The reactions were quenched after 30 seconds to provide high yields of the labeled anti-CD45 MAb, [²¹¹At]30F11-2 or [¹²⁵I]30F11-2 (65 and 81% respectively), and the labeled irrelevant MAb, [²¹¹At]CA12.10C12-2 or [¹²⁵I]CA12.10C12-2 (81% and 62% respectively) after purification by size exclusion chromatography.

Biodistribution Studies

Four biodistribution studies were conducted in this investigation. Two biodistributions were conducted with the rat anti-murine CD45 MAb, 30F11, and two biodistributions were conducted with a canine-specific irrelevant MAb, CA12.10C12. In all biodistribution studies 10 µg quantities of ¹²⁵I-labeled (1–5 µCi) and ²¹¹At-labeled (1–10 µCi) MABs were mixed (20 µg total protein) and were injected into 15 BALB/c mice. Sacrifices of groups of 5 mice were conducted at 1, 4 and 24 h post injection (pi). The data obtained are shown graphically as panels A–D in Figure 4, and are provided in Tables S1–S4 (Supporting Information). Note that the concentrations in spleen shown in Figure 4, which can be very high (as high as 546 %ID/g in panel A; 1409 %ID/g in panel B), are graphed off-scale so that the concentrations in the other tissues can be compared visually. There was a large animal-to-animal variance in the concentration of radionuclides in the groups. The %ID/g variation in spleen from individual mice appeared to correlate with the spleen size. Indeed, the variance was much smaller when the size of spleen was not factored in, thus, the percent total injected dose (%ID) of each radionuclide in the spleen was used for comparison of CD45 targeting. Presumably, some of the spleen size variance was due to a decreased weight after ²¹¹At was localized to the spleen. Because of the large variation in radionuclide concentrations in spleens of mice from the same group, discussions of localization or retention in that organ in the following paragraphs are limited to the percent of injected dose rather than injected dose per gram. The %ID in spleens for the ²¹¹At and ¹²⁵I-labeled MABs in the four biodistributions are shown graphically in Figure 5, and is provided in Table S5 (Supporting Information).

In an initial biodistribution study, [¹²⁵I]30F11-1b and [²¹¹At]30F11-1c were coinjected and their tissue concentrations were evaluated. The concentrations (%ID/g) of ¹²⁵I and ²¹¹At in selected tissues is shown graphically as Figure 4, panel A (Table S1, Supporting Information). Specific targeting of the CD45-containing cells in spleen was obtained, as the average (± std dev) percent injected dose (%ID) of [¹²⁵I]30F11-1b in that tissue was 13.31 ±

0.78; 17.43 ± 2.56 ; 5.23 ± 0.50 and [^{211}At]30F11-**1c** was 6.56 ± 0.40 ; 10.14 ± 1.49 ; 7.52 ± 0.79 at 1, 4 and 24 h sacrifice times (respectively). The concentrations of ^{211}At and ^{125}I in blood taken from mice at 1 h pi were found to be significantly different (Student's t test, $P < 0.05$), as were their concentrations in lung, liver, neck and stomach. The fact that the concentrations of ^{211}At are higher than ^{125}I in lung, neck and stomach is an indication that deastatination has occurred (5).

In a second biodistribution study, [^{125}I]30F11-**2** and [^{211}At]30F11-**2** were coinjected and their tissue concentrations were evaluated. The concentrations (%ID/g) of ^{125}I and ^{211}At in selected tissues is shown graphically as Figure 4, panel B (Table S2, Supporting Information). Specific targeting of the CD45-containing cells in spleen was obtained, as the average ($\pm$ std dev) of %ID in spleens of mice injected with [^{125}I]30F11-**2** was 21.15 ± 1.33 ; 22.22 ± 1.95 ; 12.41 ± 0.75 and [^{211}At]30F11-**2** was 22.78 ± 1.29 ; 25.05 ± 2.35 ; 17.30 ± 1.20 at 1, 4 and 24 h pi (respectively). It should be noted that the %ID values found in spleen for the radiolabeled 30F11 modified with a *closo*-decaborate(2-), **2**, is higher than that obtained for the same MAb modified using the *meta*-halobenzoate, **1b/1c**, labeling approach. This difference may be due to retention of the dianionic radiolabeled *closo*-decaborate(2-) MAb or metabolites rather than a difference in the labeled MAb targeting. Indeed, the fact that the liver and kidney concentrations of the radiolabeled *closo*-decaborate(2-) conjugate, 30F11-**2**, do not appear to decrease as quickly as the blood or lung concentrations seems to support retention in the metabolizing/excretory tissue. It is important to note that the only significant differences in distributions at 1 and 4 h pi of the two radionuclides, when using 30F11-**2**, was noted in the neck (1h) and liver (4 h). The significant difference in neck concentrations at 1 h was not brought about by release of ^{211}At (it is lower), but rather by release of ^{125}I . Our studies have shown that the ^{211}At only reacts with the *closo*-decaborate(2-) (15). However, the ^{125}I appears to react with the *closo*-decaborate(2-) moiety (primarily), but also with tyrosine residues. Reaction with tyrosine residues permits release of [^{125}I]iodide after degradation of the protein (25). The low ^{211}At quantities in lung, stomach and neck support our belief that there is a high stability of ^{211}At towards deastatination when attached to the *closo*-decaborate(2-) moiety.

In a third biodistribution, [^{125}I]CA12.10C12-**1b** and [^{211}At]CA12.10C12-**1c** were coinjected and their tissue concentrations were evaluated. The concentrations (%ID/g) of ^{125}I and ^{211}At in selected tissues is shown graphically as Figure 4, panel C (Table S3, Supporting Information). As expected, the canine-specific irrelevant antibody did not target the spleen. Significant differences (Student's t test; $P < 0.05$) between the ^{125}I and ^{211}At were seen in most tissues at all three time points. The differences in radionuclide concentrations in lung, spleen, neck and stomach are visible on the graph. The higher concentrations of ^{211}At in those tissues indicate that deastatination has occurred.

In a fourth biodistribution, [^{125}I]CA12.10C12-**2** and [^{211}At]CA12.10C12-**2** were coinjected and their tissue concentrations were evaluated. The concentrations (%ID/g) of ^{125}I and ^{211}At in selected tissues is shown graphically as Figure 4, panel D (Table S4, Supporting Information). Interestingly, the blood concentrations for the irrelevant MAb are what might be expected for an intact MAb that does not localize in any tissue and does not release the ^{211}At . There were no significant differences (Student's t; $P < 0.05$) in ^{211}At or ^{125}I concentrations for most tissues. Although there are significant differences in intestine and stomach at 1 h pi, and lung, kidney and liver at 4 h pi, the differences are quite small. Deastatination does not appear to occur, and again, the significant difference between the radionuclides at 1 h pi appears to be due to deiodination rather than deastatination.

DISCUSSION

Our interest in switching from ^{213}Bi -labeled anti-CD45 MAb to ^{211}At -labeled anti-CD45 MAb as a replacement for TBI in a conditioning regimen required additional preclinical studies to be conducted. Prior to evaluation of ^{211}At -labeled anti-CD45 MAb in the dog model, we felt it was important to gain a better understanding of the differences between ^{211}At - and ^{213}Bi -labeled anti-CD45 MABs in the mouse model. The mouse represents a better model to observe the differences that were expected as larger numbers of animals could be assessed to gain statistical significance in the data (26). Before initiating that study, a decision on the method to be used for labeling the MAb with ^{211}At was required.

Labeling of proteins with ^{211}At is very different from labeling with its nearest halogen neighbor, radioiodine, as directly astatinated proteins are readily deastatinated in vivo (5). To circumvent this inherent instability, indirect (2-step) protein labeling approaches employing protein reactive reagents that have ^{211}At -aryl bonds were developed (27,28). One of those reagents, N-hydroxysuccinimidyl *meta*-[^{211}At]astatobenzoate, [^{211}At]**1c**, has been used in preparation of an astatinated antibody for clinical evaluation (12). That fact made the [^{211}At]**1c** labeling reagent of particular interest, as its prior use in a clinical study may make it easier to conduct another clinical study employing that labeling approach from a regulatory standpoint. Further, literature examples demonstrating that intact MABs labeled with aryl conjugates can be relatively stable to in vivo deastatination (6,29,30) offered hope that would be the case for our anti-CD45 MABs. However, the two-step procedure for labeling with the benzoate NHS ester can be quite problematic. While labeling the stannylbenzoate, **1a**, provides high yields, issues with hydrolysis of the active ester in the second step often decrease the yields substantially (maximum yields 40–60% being obtained). Recent reports indicate this is particularly true when scaling up to clinical levels of ^{211}At . Although the initial report on using the [^{211}At]**1c** in clinical preparations indicated that radiolysis was not a factor in labeling (31), subsequent studies indicated that there was a problem with radiolysis in higher level clinical preparations (16–18). This difficulty in obtaining high labeling yields, along with the fact that aryl- ^{211}At conjugates are not always stable towards in vivo deastatination made it prudent to evaluate another labeling approach as well.

Results from our studies of boron cage moieties as reactive groups for labeling with ^{211}At also made that approach to labeling of interest for astatination of the anti-CD 45 MAb. In prior studies it was demonstrated that MAb Fab' fragments conjugated with a halogen-reactive *closo*-decaborate(2-), **2**, and astatinated had a high in vivo stability toward deastatination (15). The same MAb Fab' labeled with N-hydroxysuccinimidyl *para*-[^{211}At]benzoate, was quite unstable to in vivo deastatination, making it apparent that ^{211}At -labeled MAb conjugates containing the *closo*-decaborate(2-) moiety had higher in vivo stability to deastatination. In addition to the high stability, the simplicity of direct labeling of the protein (same as radioiodination reactions) and high yields obtained (70–80%) in the prior studies made this new labeling approach very attractive for the anti-CD45 study. Further, direct labeling of MAb-decaborate(2-) conjugates with ^{211}At eliminates the issues of hydrolysis and/or radiolysis of the labeling reagents used in the 2-step approach. The direct labeling approach should also minimize radiolysis of the labeled protein as the reactions are conducted in aqueous medium and the fast labeling kinetics (i.e. 30 sec) allows rapid introduction of a radioprotective reagent such as citrate or ascorbate.

The differences in ^{211}At labeling yields for the two approaches were surprising. While the high labeling yields for the MABs conjugated with the *closo*-decaborate(2-) were expected, the low yields obtained with [^{211}At]**1c** were unexpected. Our prior experiences with labeling

MAbs using the astatinated benzoate esters suggest that the conjugation yields should be in the range of 40–60%. Based on the purity of the ^{211}At -labeled benzoate NHS ester (see Figures S1 & S3, Supplemental Information), it seems likely that hydrolysis of the NHS ester occurred during isolation or conjugation, resulting in the low yields. Importantly, the results point out the potential for difficulties in labeling using the ^{211}At -labeled benzoate approach.

The in vivo distribution might be considered the most important measure of the value of each ^{211}At -labeling approach. Specific targeting of radiolabeled 30F11 to CD45-containing cells within the spleen was achieved irrespective of the method of labeling, however, higher quantities appeared to be attained with radiolabeled 30F11-**2** (Figure 5). The difference seen may be reflective of a higher retention of the radiohalogenated *closo*-decaborate(2-) metabolites rather than lower amounts accumulated with the radiohalogenated benzoate conjugates. While the amounts of ^{125}I and ^{211}At in spleen are similar for radiolabeled 30F11-**2**, they are very different for 30F11- ^{211}At 1c and 30F11- ^{125}I 1b. That difference is likely due to deastatination of the benzoate, but that does not occur unless the protein is metabolized. We did not expect to observe deastatination of MAbs labeled with ^{211}At 1c in these studies as the CD45 antigen is not internalized. However, this example is consistent with other examples in the literature that suggest stability towards in vivo deastatination may be dependent on the antibody / cell targeting system involved (9,10,32).

The deastatination decreases the amount of ^{211}At in the target organ (spleen) and increases it in the non-target tissues, e.g. lung. On the other hand, the ^{211}At -labeled *closo*-decaborate(2-) conjugate, ^{211}At 30F11-**2** has a higher uptake in liver than the *meta*-benzoate conjugate, ^{211}At 30F11-1c. This might be perceived as a problem of non-specific localization (or retention) in the liver. However, after comparison of the liver ^{211}At concentrations in Figure 1, panels B, C, and D it appears that the higher concentrations in liver may be due to specific binding with CD45 antigens in that tissue. This statement is based on the fact that the amount of radioiodinated irrelevant MAb, CA12.10C12, in the liver is the same irrespective the method of labeling.

CONCLUSIONS

This investigation was conducted to compare ^{211}At labeling of an anti-CD45 MAb, 30F11, and an irrelevant MAb, CA12.10C12, using a conventional astatination approach, i.e. conjugation of N-hydroxysuccinimidyl *meta*- ^{211}At astatobenzoate, and a new labeling approach where the MAbs were labeled after conjugation with a *closo*-decaborate(2-) derivative. The studies demonstrated the utility of *closo*-decaborate(2-) MAb conjugates over the *meta*- ^{211}At benzoate conjugates for labeling with ^{211}At . The studies further confirmed that the ^{211}At -labeled MAb conjugates had a high in vivo stability to deastatination. Additionally, it was demonstrated that CD45 specific targeting was not adversely affected by the protein modification step. These factors, along with the simplicity of labeling using this method and the potential benefit for scale up to clinical levels of ^{211}At labeled MAbs, make the MAb-*closo*-decaborate(2-) conjugates our preferred choice for continuing studies with ^{211}At -labeled anti-CD45 MAbs as a replacement of TBI in conditioning for hematopoietic cell transplantation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Conjugation reagents used to radiolabel MAb with ²¹¹At. The stannylbenzoate ester **1a** is reacted with ²¹¹At or ¹²⁵I to prepare [²¹¹At]**1c** or [¹²⁵I]**1b** prior to conjugation with the MAb. The maleimide-*closo*-decaborate(2-) reagent, **2** is conjugated with the MAb, and that conjugate is subsequently labeled with ²¹¹At or ¹²⁵I. (Note: the open circles in the *closo*-decaborate(2-) cage structure in compound **2** represent B or B-H atoms.)

Figure 2.

Isoelectric focusing gels showing protein bands of 30F11 (left gel; Novex 3–10) and CA12.10C12 (right gel; Novex 3–7) before and after the proteins were conjugated with maleimide-*closo*-decaborate(2-), **2**. Lanes are: (A) IEF markers, (B) unmodified 30F11, (C) 30F11-**2**, (D) unmodified 30F11, (E) IEF markers, (F) unmodified CA12.10C12, and (G) CA12.10C12-**2**. IEF markers (Serva) are: (pI) 10.7 - cytochrome C; 9.5 - ribonuclease A; 8.3, 8.0, 7.8 - lectin; 7.4, 6.9 - myoglobin; 6.0 - carbonic anhydrase; 5.3, 5.2 - β -lactoglobulin; 4.5 - trypsin inhibitor; 4.2 - glucose oxidase; 3.5 - amyloglucosidase.

Figure 3. Histograms from flow cytometry showing cell binding of unmodified 30F11 (red) and *closo*-decaborate(2-) modified 30F11 (green). Competitive binding of 30F11-FITC with 30F11 (purple) and 30F11-2 (blue) is also shown.

Figure 4.

Graphs in panels A–D show concentrations of ^{211}At and ^{125}I in selected tissues of BALB/c mice. The four graphs are plots of data for the two MAbs labeled using two different methods. Black, brown and red bars represent ^{211}At concentrations (%ID/g) at 1, 4, and 24 h pi (respectively) and green, light blue and dark blue bars represent ^{125}I concentrations at 1, 4, and 24 h pi (respectively). The graphed data are from 5 mice per time point $\pm$ standard deviation (error bars). The highest concentration value has been limited to 70% ID/g so that the tissues with lower concentrations than spleen can be compared (see Tables S1–S4 in Supporting Information for complete data, including spleen values).

Figure 5.

Graphed percent injected dose (%ID) of radiolabeled MAb conjugates in spleens of BALB/c mice. Each set of 3 bars represents the amount of ^{211}At - or ^{125}I -labeled MAb in spleen at 1h (black), 4h (brown) and 24h (green) post injection. The conjugate on the antibody and radionuclide attached (At = ^{211}At ; I = ^{125}I) are listed below each set of bars. The antibodies used are shown above the graph, with a red line separating the CD45 specific MAb (30F11) and the irrelevant MAb (CA12.10C12). Data were obtained from 5 mice per time period (per bar) and are plotted as the average %ID/g $\pm$ standard deviation (error bars).