Radiopharmaceutical Preparation of a Monoclonal Antibody, Lym-1, and its $F(ab')_2$ Fragment for Imaging Lymphoma with In-111

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SUMMARY

(S)-4-[2,3 bis[bis(carboxymethylamino)]propyl]phenyl isothio-cyanate (CITC) was used to attach In-111 to an intact monoclonal antibody (Lym-1) or its fragment $F(ab')_2$ to produce two new radiopharmaceuticals. Immunoreactivity of the Lym-1, specific for B cell lymphoma, was not affected by the coupling reaction for 1.0 and 4.3 CITC molecules per whole antibody and 3.0 CITC molecules per $F(ab')_2$ fragment. Both retained in excess of 90% immunoreactivity even at the level of 25 mCi of In-111 per mg of Lym-1 and 15 mCi per mg $F(Ab')_2$. The labeling reaction produced yields in excess of 98% eliminating the requirement for routine purification of the final radiopharmaceutical.

Key Words: Monoclonal Antibodies, Radioimmunodiagnosis, Imaging, Lymphoma, Radiopharmaceutical

INTRODUCTION

The physical characteristics of In-111 are suited for radioimmunodiagnosis because a 2.8 day half-life is sufficiently long to permit localization, blood clearance, and imaging with the 173 and 249 keV gamma photons. Various bifunctional chelates have been developed to attach radiometals to monoclonal antibodies (MAB). The derivatives of diethylenetriamine-penta acetic acid (DTPA) have received the greatest attention. Both DTPA mixed anhydride (1) and DTPA cyclic anhydride (2) have been reported to be effective for the attachment of indium to macromolecules. In vitro plasma stability studies of monoclonal antibodies-

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DTPA-In-111 chelates demonstrate that (5-10)% of the In-111 is transfered to the transferrin (3). Imaging studies of MoAb-DTPA-In-111 radiopharmaceuticals have demonstrated prolonged hepatic uptake with little radionuclide excretion from the liver or whole body. However, benzyl derivatives of ethylenediaminepentaacetic acid (EDTA) appear to be more stable in tracer concentration in living organisms with (1-2)% transfered in plasma (3,4). We have previously reported the labeling of microgram amounts of antibodies with I-123 (5). Although I-123 labeled antibody provides a useful tool for evaluating the pharmacokinetics and distribution of a new MoAb in patients, pure I-123 in labeling grade is more difficult to obtain, and tumor-blood ratios have been reported to be less than its In-111 DTPA analog (6).

The variations in the distribution patterns of radioiodinated and radiometal chelated to MAB are well documented (7-9). These variations appear to be related to the processing of the radiolabel after metabolism of the MAB. The bifunctional chelate used to attach the radiometal to the MAB should remain attached to the radiometal rather than dissociate during catabolism of the MAB. In this manner transferrin binding is decreased, urinary elimination is increased, and radiation to nontarget tissues is decreased. The purpose of this publication is to describe the preparation and characterization of radiopharmaceutical quality In-111 labeled MAB and its $F(Ab')_2$ fragment chelated with (S)-4-{2,3 bis[bis-(carboxymethyl)amino]propyl]phenylisothiocyanate (CITC) for imaging patients with B cell lymphoma or leukemia.

MATERIALS AND METHODS

Preparation of the (S)-4-[2,3 bis[bis(carboxymethylamino)]propyl]phenyl isothiocyanate (CITC)

To 50 ml of cold, metal-free H_2O was added (507 mg, 1.2 mmol) of p-Nitrobenzyl-EDTA and the material dissolved by the dropwise addition of NaOH. The pH was adjusted to 11.5 with additional 0.1 M NaOH and 10% Pd/charcoal (92 mg) was slowly added to the cold solution. The reaction was stirred in an ice bath for 3-5 hours under 1 atmosphere of H_2 and neutralized with 1.0 N HCl after cessation of H_2 uptake. A 0.45 um nitrocellulose filter was used to remove the catalyst prior to the reaction liquid being removed by lyophilization. The intermediate product, (S)-p-aminobenzyl-EDTA (405 mg) was dissolved in 3 N HCl (10 ml) and thiophosgene (1.0 ml, 85% in CCl_4) was added while the mixture was

stirring in a fume hood. The reaction mixture was stirred at room temperature until the aqueous layer was negative to fluorescamine (approximately 6 hours). Ethyl ether extraction of the organic layer removed thiophosgene and CCl_4 leaving the acidic aqueous layer to be removed again by lyophilization. The residue that contained 418 mg of CITC was stored in acid washed 1.5 ml plastic centrifuge tubes at -70^o C (10).

Lot Preparation of MoAb-CITC

A 15x125 mm pyrex test tube was fitted with a sterile 0.5 ml polypropylene micro test tube and the test tube capped to preserve sterility. One ml sterile plastic syringes were soaked overnight in a metal-free, sterile 3 M HCl solution prepared by passing chlorine gas through sterile water. The sterile metal-free syringes were fitted with porous polyethylene disks cut from a sheet (1/16-in. thick, 70-um pore size, Bolab, Inc., Cat. No. BB2062-70L) using a No. 1 cork borer. The disks were sterilized and soaked in the same metal free sterile 3 M HCl as the syringes and assembled in a laminar air flow hood. To the syringes was added sephadex G-50 in sterile metal free 0.1 M phosphate buffer or sephadex G-50 in sterile metal free 0.1 M ammonium citrate buffer, labeled, and centrifuged at 100 g for 2.0 minutes. The volume of the sephadex G-50 in the syringe was maintained at 0.7 ml following centrifugation.

The whole antibody (49 mg/ml,30 mg) or F(ab')$_2$ fragment (22.5 mg/ml, 5 mg) was purified of metal contaminants and the conjugation buffer changed by adding the material to a previously prepared centrifuge column containing sephadex G-50 in 0.1 M sterile metal free phosphate buffer (11). The columns containing 0.1-0.2 ml of antibody solution each were centrifuged at 100 g for two minutes. The eluents were combined, final volume noted and the protein concentration determined at 280 nm using a Beckman UV spectrometer. A fresh 23 mM solution of CITC was prepared from a lyophilized powder using sterile metal free water and added to the whole antibody or F(ab')$_2$ fragment to give a final concentration of one mM of CITC in the reaction mixture. A saturated solution of trisodium phosphate was used to adjust the pH of the reaction mixture to between 8.0 and 9.0 and this solution was incubate at 37^o C for two hours. Following incubation the reaction mixture was added to the previously prepared centrifuge columns containing sephadex G-50 in 0.1 M sterile metal free ammonium citrate buffer and centrifuged as before. The eluents were combined and frozen at -70^o C for later use.

Quantitation of the Chelates per Antibody

Following attachment of the CITC to the protein, the concentration of the conjugated protein was evaluated at 280 nm using a Beckman UV spectrometer previously calibrated with known standards. To 10 ul of 0.1 M ammonium citrate buffer, pH 6.5, was added 10 ul of a one mM solution of cobalt-57 chloride, and 10 ul of the purified product. The mixture was incubated for 30 minutes and 10 ul of a 10 mM solution of EDTA, pH 6.5 was added and incubation continued for an additional 5 minutes. At the end of incubation 100 ul of buffer was added and the entire reaction mixture was added to a centrifuge column and centrifuged for 2 minutes at 100 g. The Co-57 attached to the protein was collected in the microcentrifuge tube while the Co-57 not bound to protein remained on the column. The ratio of bound to free was multiplied by the molar concentration of Co-57 (1.0 mM) to give the molar concentration of chelates bound to protein. The ratio of chelates per antibody molecule was determined by dividing the molar concentration of chelates bound to protein by the molar concentration of protein (10).

Purification and Preparation of In-111 Chloride

A stock slurry of AG1-X4 anion-exchange resin (200-400 mesh) in 2.0 M HCl was prepared and allowed to equilibrate overnight. Following equilibration the 2.0 M HCl was decanted and replaced with additional HCl until no color change was observed in the 2.0 M HCl. The equilibrated AG1-X4 was loaded onto a one cc centrifuge column and eluded with 0.2 M HCl, then metal free sterile water, and finally 2.0 M HCl. The Indium-111 chloride (50 uCi/ul) was loaded onto the column and the column eluted with 2.0 M HCl equal to twice the bed volume. The Indium-111 chloride was then eluted from the AG1-X4 column in 0.2 M HCl. The Indium-111 chloride in 0.2 M HCl eluent was divided into acid washed (1:1, sulfuric:nitric acid) sterile 2.0 ml borosilicate glass vials at 5.0 mCi/vial. A heater block was used to warm the vials under a sterile nitrogen gas stream to reduce the volume to dryness. Once the Indium-111 chloride was reduced to dryness the heating and nitrogen gas stream was continued for 30 minutes at which time the vials were sealed and stored for later use.

Antibody Labeling with In-111 Chloride

The whole antibody or $F(ab')_2$ (1.0 mg/30 ul) was removed from the -70^{O} C freezer and allowed to come to room temperature. The dry purified In-111 chloride was transferred

from the refrigerator to the sterile hood behind lead shielding. When the antibody solution reached room temperature it was transferred to the vial containing In-111 chloride and incubated at room temperature for 30 minutes. Following incubation the volume of the reaction mixture was increased to one ml with 0.1 M sterile citrate buffer, 10 ul of a 10mM sterile EDTA solution was added, and the mixture incubated for two minutes at room temperature. If quality testing indicated greater than 95% labeling efficiency the radiopharmaceutical was further diluted to 20ml with normal saline solution before use.

Purification of In-111 labeled Antibodies

1) A sterile slurry of Sephadex G-25 in pH 6.5, 0.1 molar phosphate buffer with 0.4 ml of commercial 25% human serum albumin was aseptically added to a 1x30 cm column to within 5 cm of the top. After the column was eluted with normal saline for injection equal to twice the bed volume, the reaction mixture (one ml) was loaded onto the column and the column eluent collected in one to two ml fractions for a total of 20 ml. The fractions comprising the first peak eluted from the column were combined, diluted to 20 ml and immediately stored at 2 to 8^{o} C until administered.

2) A sterile slurry of Sephadex G-50 in pH 5.5, 0.1 molar phosphate buffer was add to a one ml sterile syringe fitted with a polyethylene disk, 70 um pore size. The slurry was added until one ml of slurry remained in the syringe after allowing the slurry to settle. The column was centrifuged for two minutes at 100 g resulting in a bed volume of 0.7 ml. The reaction mixture was added (maximum volume of 200 ul per column) to the column and centrifuged for two minutes at 100 g. The eluents were collected in sterile 0.5 ml centrifuge tubes, combined, diluted to 20 ml with normal saline for injection, and stored at 2 to 8^{o} C until administered.

Analysis of Radiolabeled Antibodies

1) Cellulose acetate electrophoresis (CAE) was performed at 340 volts, 2-3 milliamps per strip in 0.05 molar pH 6.8 sodium barbital buffer. After equilibration with the buffer the CAE strips were blotted, labeled with sample, and mounted in the electrophoresis chamber. One microliter sample of the carrier-free In-111 chloride was transferred to the cathode end of the CAE strip and electrophoresed for 11 minutes. A two microliter sample of the whole antibody or F(ab')$_2$ reaction mixture following incubation was transferred to the cathode end of two CAE strips. The first was electrophoresed for 11 minutes and the

second for 45 minutes. Following electrophoresis, each strip was analyzed by a radiochromatogram scanner.

2) Molecular sieving high pressure liquid chromatography (HPLC) was performed on the whole antibody and $F(ab')_2$ fragment before and after labeling with the In-111 chloride. A Beckman model 110A pump was attached to a model 421 controller to produce a flow rate of 1 ml/min. through a TSK 3000 (1x30 cm) column. The column eluent was passed through a Beckman model 160 absorbance monitor set at 280 nm and a model 170 radioisotope detector, the output from both recorded with a duel pen recorder.

3) The in vitro immunoreactivity of the In-111 labeled Lym-1 CITC and $F(ab')_2$ fragment was evaluated by a solid phase radioimmunoassay that had been standardized against a conventional in vitro live cell binding assay (12) at 10^6 Raji cells/well. For this assay, purified membrane fragments were prepared from Raji cells (13) and 0.2 mg of protein per well was dried overnight in microliter plate wells (polyvinylchloride) at 37^{o} C. Two hundred microliters of 5% (W/V) bovine serum albumin in phosphate buffered saline (0.15 molar, pH 7.4) was added to each well and removed by inversion to minimize nonspecific protein binding prior to analysis of the samples. Aliquots (1 and 100 nanograms) of the test and reference materials were then added to the wells in triplicate and incubated for one hour at 37^{o} C. The unbound radiolabeled antibody was removed by suction and the wells were washed four times with 1% bovine serum albumin. Identical aliquots (1 and 100 nanograms) were also added in triplicate to counting tubes for determination of total activity added to wells for both test and reference materials.

Spleen cell membrane fragments from Balb/C mice were prepared from the same protocol and were treated identically in order to serve as a negative control for the In-111 Lym-1 CITC and $F(ab')_2$ CITC. Lym-1 radioiodinated at one I-125 per 40 antibodies was used as a reference standard; 80-90% of this reference standard was cell bound in the in vitro live cell assay (10). Following incubation, wells were cut individually and counted in a scintillation well counter. The relative immunoreactivity was expressed as: (percent binding of sample to the Raji cell membranes minus percent binding to the spleen cell membranes) divided by (percent binding of the reference Lym-1 to Raji cell membranes minus percent binding to the spleen cell membranes) x 100.

4) Pyrogen testing was performed on the stock solution of Lym-1 and $F(ab')_2$ following purification and after the attachment of the CITC bifunctional chelator using the LAL test

(Cape-Cod Associates). Each of the final labeled products was again tested for pyrogens prior to injection.

5) Sterility testing was performed on the Lym-1 and F(ab')2 following purification and after the CITC conjugation with standard USP sterility testing media; soybean casein and fluid thioglycolate.

RESULTS

The In-111 labeled whole Lym-1 or F(ab')$_2$ fragment of Lym-1 contained less than two percent radiochemical impurities and less than one percent radiolabeled molecules with a molecular weight greater than 150,000. The 11 minute electrophoresis of the labeled product demonstrated greater than 98% of the radioactivity migrating as a single peak 1.5 or 3.0 cm from the origin, for In-111 Lym-1 CITC or In-111 F(ab')$_2$ CITC, respectively. Unbound In-111 chloride following radiolabeling was indicated by a radioactive peak at 3.2 cm from the origin on the 11 minute CAE indicating chelation of the In-111 by the EDTA chase. The 45 minute CAE of the labeled product demonstrated greater than 98% of the radioactivity migrating as a single peak 3.0 cm and 4.5 cm from the origin, for In-111 Lym-1 CITC or In-111 F(ab')$_2$, respectively. EDTA chelation of any free In-111 was seen as a single peak migrating 7.0 cm from the origin on the 45 minute CAE (**Table. 1**). Evidence for denatured protein was noted in one labeling where residual HCl was present and was characterized by failure of the radioactivity to migrate on either the 11 minute or 45 minute CAE and reduced immunoreactivity.

Table 1. Results of electrophoresis of In-111 labeled products on cellulose acetate under conditions defined in the text.

11 minutes	Cm from Origin
Lym-1 CITC	1.5
F(ab')$_2$ fragment	1.5
EDTA	3.5
45 minutes	
Lym-1 CITC	3.0
F(ab')$_2$ fragment	4.0
EDTA	7.0

HPLC evaluation of both the In-111 CITC Lym-1 and the In-111 CITC F(ab')$_2$ demonstrated a purity similar to that observed on CAE; 98% of the radioactivity eluted from the column

as a single peak corresponding to labeled CITC Lym-1 (8.5 cm) or labeled CITC F()ab')$_2$ (10.5 cm) and less than 2% eluted at 14 ml corresponding to non-protein bound In-111 with a molecular weight of less than 2kd (**Table. 2**).

Quality assurance testing of the In-111 labeled Lym-1 CITC and In-111 labeled F(ab')$_2$ using a fixed quantity of purified membrane fragments (0.2 mg/well) and two different concentrations of labeled product, 1 ng and 100 ng produced similar results. The bound/free ratio of the labeled product was consistently greater than 90% of that of Lym-1 or F(ab')$_2$ fragment when these molecules were iodinated at one iodine to 40 MAB molecules and tested using the same solid phase assay. Binding of this magnitude corresponded to 80% binding using a conventional live cell assay technique (14).

Table 2. Results of HPLC-DEAE chromatography of In-111 labeled products on under conditions defined in the text.

Material eluded	Time to elution
Lym-1 CITC	9.5
F(ab')$_2$ fragment	10.5
EDTA	15.0

The ratio of chelate to MAB was determined following centrifuge column purification of the material prior to storage and reaction results ranged from 1.0 to 4.3 CITC/Lym-1 molecule and 1.0 to 3.2 CITC/(Fab')$_2$ molecules. Variations in the number of chelates/MAB was dependent upon the pH of the reaction mixture. The reaction is optimum between pH 8-9 with fewer chelates/MAB at pH 8 increase as the reaction pH approaches pH 9. No variations in the immunoreactivity was observed between these ratios even when 1.0 mg of Lym-1 CITC was labeled with 25 mCi of In-111 chloride.

Storage at -70 degrees C for five months did not alter the chemical quality of either product, the labeling yields, or the immunoreactivity of the final products. All materials were determined to be sterile and pyrogen free both at the intermediate level and as a final radiopharmaceutical product.

DISCUSSION

The development of radiopharmaceuticals based upon monoclonal antibodies for the diagnosis and therapy of disease is dependent on the ability to firmly attach radionuclides while maintaining the unique specificity of the MAB. The potential exists to produce radio-

pharmaceuticals to image diseases based upon specific differences between the diseased and healthy cells. Radiochemical procedures for the attachment of radionuclides to MAB usually must be adapted to produce radiopharmaceuticals because of the additional requirements placed upon preparations for human use.

To be practical for clinical studies the production of MAB radiopharmaceuticals must be simple. A very high radiochemical yield eliminates the need for purification prior to administration. Most methods of purification require sterile technique and equipment that is not available in many nuclear medicine departments. In addition, some purification procedures can reduce the integrity of the protein product. Post labeling purification increases radiation exposure to personnel preparing the radiopharmaceutical, and increases costs and time for preparation of the radiopharmaceutical. Regulatory standards generally require radiopharmaceuticals to contain less than 10% radiochemical contaminants. However, very pure products are preferable for evaluating the pharmacokinetics of a new radiopharmaceutical and to reduce radiation absorbed dose to nontarget tissue. The radiopharmaceutical labeling procedure described in this publication consistently produced radiolabeling yields of at least 98% when the chelated MAB and the stock In-111 chloride were meticulously purified before radiolabeling. The radiolabeling procedure also produced radiopharmaceutical grade In-111 labeled MAB, which consistently had immunoreactivity greater than 90% of that of the unlabeled MAB. Current labeling procedures for the attachment of radiometals to MAB have not yielded specific activity approaching the theoretical limit. With one chelate group per antibody and one indium atom per chelate it is theoretically possible to achieve specific activities on the order of 324 uCi/ug (15). Radiochemical methods yielding specific activities of 1 to 6 uCi/ug have been described and commercial preparations at 5 uCi/ug are being evaluated. In the absence of non-specific binding of MAB, high specific activities may improve target to non-target ratios. The labeling procedure described here produced specific activities in excess of 25 uCi/ug. At this level it is possible to administer 5 mCi on less than 200 ug of protein.

In conclusion, this labeling procedure for attaching In-111 to monoclonal antibody by using the bifunctional chelate (CITC) produces radiopharmaceuticals with specific activities in excess of 25 uCi per ug, radiolabeling yields greater than 98%, while maintaining immunoreactivity in excess of 90%. Early studies with this radiopharmaceutical has demonstrated effective imaging in Lymphoma (16).

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