Preparation of α-Emitting ^{213}Bi-Labeled Antibody Constructs for Clinical Use

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Preclinical evaluation of α particle-emitting ^{213}Bi-labeled antibody constructs have demonstrated the specificity and potency of these agents in a variety of cancer systems. The transition of a ^{213}Bi-radiolabeled antibody from a preclinical construct to a clinical drug represented a difficult task that involved development of reliable and validated methods to provide multiple MBq quantities of a pure, immunoreactive agent that met pharmaceutical standards to treat patients. **Methods:** The methods used for the preparation of (^{213}Bi)CHX-A-diethylenetriamine pentaacetic acid (DTPA)-HuM195, an α particle-emitting anti-CD33 antibody construct for therapy of myeloid leukemias, is used as a specific example. This article describes methods for reagent purification, drug labeling, radioprotection and chromatographic purification. Quality of the drug is evaluated using radiochemical incorporation and purity assays with instant thin-layer chromatography (ITLC) and high-performance liquid chromatography (HPLC), determination of cell-based antibody total immunoreactivity, small animal safety, pyrogen level, sterility and radionuclidic purity. **Results:** Sixty-seven doses were prepared. Individual doses ranged from 148 to 814 MBq. Specific activities ranged from 329 to 766 MBq/mg. The radiolabeling efficiency (median ± SD) of CHX-A-DTPA-HuM195 with ^{213}Bi was 81% ± 9% (n = 67) after 9 min. The construct was purified by size-exclusion chromatography and was found to be 99% ± 2% pure (n = 67) by either ITLC or HPLC methods. The immunoreactivity of (^{213}Bi)CHX-A-DTPA-HuM195 was 89% ± 9% (n = 44) and was independent of the specific activity. The formulated pharmaceutical was found to contain ≤4 ± 1 EU/mL pyrogens (n = 66); all samples examined were sterile. An ^{225}Ac radionuclidic impurity was present at a level of 0.04 ± 0.03 × 10^{-6}/mL (n = 10) in a product volume of 7.4 ± 0.5 mL (n = 67). Each of the 67 doses was injected intravenously into patients without complication as part of a phase I clinical trial. **Conclusion:** These data show that ^{213}Bi-labeled antibody constructs can be prepared and administered safely to humans at a wide range of therapeutic levels.

Key Words: α particles; ^{213}Bi; HuM195; monoclonal antibodies; therapy

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Bismuth-213 is an α particle-emitting radionuclide that has been demonstrated in several preclinical investigations (*1–10*) to be an extremely potent cytotoxic agent and specific when attached to a monoclonal antibody (mAb) construct. These studies have examined the targeting and cytotoxicity of ^{213}Bi-labeled antibody constructs directed against leukemia, lymphoma, prostate and other carcinoma cells and normal vasculature in vitro and in vivo. Until recently, there has been some doubt that such targeted α particle emitters could be feasibly used in humans.

^{213}Bi possesses physical characteristics that make it attractive for radioimmunotherapy: (a) α particles have high linear energy transfer (100 keV/μm) and are thus up to 100 times as potent as β^- particles; (b) the nonspecific irradiation of normal tissue around the target cell is greatly reduced or absent, because the path lengths of typical α particles are on the order of 60 μm; and (c) a short half-life ($t_{1/2}$ = 46 min) allows rapid dose delivery. However, the short half-life also imposes the requirements that the generator-produced ^{213}Bi be rapidly attached to the carrier molecule and processed for administration and that the drug be rapidly targeted.

The ability to scale-up and use the ^{213}Bi-radiolabeled antibody constructs in a clinical setting depended on several key advancements. These were (a) the development of a robust ^{225}Ac/^{213}Bi generator (*11,12*) that was capable of reliably producing large quantities (925–2405 MBq) of ^{213}Bi activity for the preparation of therapeutic doses; (b) the establishment of a reliable supply of ^{225}Ac (*13–15*) of a quality that could be used for therapeutic purposes; (c) the synthesis of a nonimmunogenic and stable antibody construct, prepared from a stable metal-chelating moiety (*16*) attached to a highly specific mAb (*17–21*); and (d) the development of a rapid, reliable procedure for radiolabeling, purification, formulation and quality control of the desired product, described herein.

Integration of all of these developments has yielded (^{213}Bi)CHX-A-diethylenetriamine pentaacetic acid (DTPA)-HuM195, which was used in the first phase I clinical trial involving targeted α particles (*22*). This article will describe the methods developed for the production of (^{213}Bi)CHX-A-DTPA-HuM195, an α particle-emitting anti-CD33 antibody construct, for therapy of myeloid leukemias. The production of 67 doses of this specific α particle-emitting antileukemic agent has been used to treat 15 patients at Memorial Sloan-Kettering Cancer Center.

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MATERIALS AND METHODS

HuM195 Construct

HuM195 (Protein Design Labs, Inc., Mountain View, CA) is a recombinant IgG1 mAb that was constructed by combining the CDR regions of the murine M195 antibody with human framework and constant regions. Both HuM195 and M195 mAbs bind with high affinity to the CD33 antigen (*17–21*). The mAb HuM195 was manufactured under GMP conditions.

The bifunctional chelating agent, 2-(4-isothiocyanatobenzyl)diethylenetriamine pentaacetic acid (SCN-CHX-A-DTPA), is a backbone-substituted derivative of DTPA obtained from Brechbiel and Gansow (*16*). The CHX-A-DTPA-HuM195 agent was constructed at TSI Washington (Bethesda, MD) using an Amicon (Beverly, MA) continuous buffer exchange/dialysis apparatus similar to the system in Nikula et al. (*23*) for clinical use. The average number of chelates per antibody was approximately 10, as determined by the yttrium arsenazo spectrophotometric method (*24*).

Reagents

Chemicals used in the radiolabeling and purification steps were American Chemical Society (ACS) reagent grade or better. Metal-free water was obtained from a MilliRO+10 reverse osmosis water purification system (Millipore, Bedford, MA) and was sterile filtered. The 3 mol/L ammonium acetate (Fisher Scientific, Pittsburgh, PA) and 150 g/L l-ascorbic acid (Aldrich Chemical Co., Inc., Milwaukee, WI) solutions were prepared, rendered metal free with Chelex 100 resin, 200–400 mesh, sodium form (BioRad Laboratories, Inc., Hercules, CA) and sterile filtered through a 0.22- or 0.45-μm filter device. Hydrochloric acid solutions were prepared using Optima grade HCl (Fisher Scientific). Solutions of 0.2 mol/L NaI (Fisher Scientific) and 10 mmol/L ethylenediaminetetraacetic acid (EDTA; Fisher Scientific) were sterile filtered as aforementioned. Human serum albumin (HSA) (Swiss Red Cross, Bern, Switzerland) and 0.9% NaCl (Abbott Laboratories, North Chicago, IL) were used as received.

An $^{225}Ac/^{213}Bi$ generator was constructed using the methods described (*11,12*). The ^{225}Ac used to construct the generator was obtained from the Institute for Transuranium Elements (Karlsruhe, Germany) or from Oak Ridge National Laboratory (Oak Ridge, TN). ^{213}Bi was eluted every 3 h from the generator and was used for radiolabeling.

Sterile and pyrogen-free plasticware (Corning Inc., Corning, NY) was used for the reaction vessels and for reagent storage. Metal-free pipette tips (BioRad Laboratories, Inc.) were used to aliquot solutions, and various size plastic syringes (Becton Dickinson & Co., Franklin Lakes, NJ) were used as received.

Radiolabeling and Purification

The generator was eluted with 3 mL of 0.1 mol/L HCl/ 0.1 mol/L NaI to elute ^{213}Bi, expected to be a $(BiI_5)^{2-}$ anion species (*25*). The acidic generator eluate was brought to pH 4–4.5 with the addition of 0.25 mL of 3 mol/L ammonium acetate, and enough 150 g/L l-ascorbic acid solution was added to yield a final 5 g/L reaction mixture concentration. This solution was used immediately to radiolabel 0.5 mg of the CHX-A-DTPA-HuM195 mAb construct (10.6 g/L). Reactions reached approximately 80% completion in approximately 8–10 min and were then quenched with 0.020 mL 10 mmol/L EDTA. Quenched reaction mixtures were purified by passage over a sterilized 10 DG size-exclusion resin and column (BioRad Laboratories, Inc.) using 1% HSA as the mobile phase. The entire radiolabeled antibody product fraction was collected in the first 7.4 ± 0.5 mL (n = 67) eluted. Additional antibody was added to the product to adjust the specific activity as prescribed. This fraction was drawn up into a sterile syringe that was then fitted with a sterile filtering disk for patient administration. A small amount (0.1 mL) of residual product was used for all of the quality control analyses.

Instant Thin-Layer Chromatography

To determine the labeling efficiency of the reaction mixture and the purity of the final product, a 0.001-mL aliquot was removed for instant thin-layer chromatography (ITLC) using silica gel-impregnated paper (Gelman Science Inc., Ann Arbor, MI) (*23*). The paper strips were developed using two different mobile phases. Mobile phase I was 10 mmol/L EDTA, and mobile phase II was 9% NaCl/10 mmol/L NaOH. The R_f of the radiolabeled antibody was 0, and both the free metal species and metal chelates were characterized by an R_f of 1.0 in mobile phase I. In mobile phase II, the radiolabeled antibody and free metal species were characterized by an R_f of 0, and the R_f of the metal chelates was 1.0. The strips were cut at R_f = 0.5 and were counted in a Packard Cobra gamma counter (Packard Instrument Co., Inc., Meriden, CT) using a 340- to 540-KeV window, or they were counted intact using a System 400 Imaging Scanner (Bioscan Inc., Washington, DC).

High-Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) analyses were performed using a Rainen HPLX system (Rainen, Woburn, MA) equipped with a Bioscan Flowcount (Bioscan Inc.). The stationary phase was a 300 × 7.8 mm TSK 3000SWXL size-exclusion column (Supelco, Bellefonte, PA) and the mobile phase was 0.15 mol/L sodium chloride/0.02 mol/L sodium acetate, pH 6.5. Fractions were collected using a Pharmacia fraction collector, model FRAC-100 (Pharmacia Biotech Inc., Piscataway, NJ) and were counted with a Packard Cobra gamma counter (or equivalent) using a 340- to 540-KeV window.

Radionuclide Detection and Quantification

^{213}Bi activity was measured with a Squibb CRC-17 radioisotope calibrator (or equivalent model) (E.R. Squibb and Sons, Inc., Princeton, NJ) set at 775 and the displayed activity value multiplied by 10. The ^{213}Bi activity measured by the CRC-17 was verified by counting an aliquot of the measured sample as a point source with pulse height multichannel analysis (MCA) using an HPGe detector (Canberra Industries, Meriden, CT). Pulse height MCA using an HPGe detector and gas ionization detection (Ambis 4000; Ambis Inc., San Diego, CA) were used to determine the radionuclidic purity of aliquots (0.005–0.020 mL) of the purified radiolabeled antibody construct, (^{213}Bi)CHX-A-DTPA-HuM195, by looking for ^{225}Ac impurity. Samples were allowed to stand 48–96 h until all the ^{213}Bi and its daughter ^{209}Pb decayed; ^{225}Ac in secular equilibrium with its daughters remained. The ^{225}Ac impurity was expressed as the Bq of ^{225}Ac per mL of the final product per MBq of ^{225}Ac on the generator. Selected samples containing ^{225}Ac impurity were held and were counted repeatedly over a several-week period and the activity decay values were analyzed by curve fitting to determine the identity of the radionuclidic impurity. The detection and quantification methods were previously described in detail (*12*).

Immunoreactivity

The immunoreactivity of the (^{213}Bi)CHX-A-DTPA-HuM195 construct was determined by incubating 2 ng of radiolabeled mAb in 0.030 mL total volume with a 500-fold excess of antigen

(approximately 10×10^6 CD33 positive AL67 cells). These cells express approximately 400,000 CD33 positive-binding sites per cell and were in antigen excess to added HuM195. After 30-min incubation at 0°C, the cells were collected by centrifugation and the supernatant was removed; the cells were washed once with phosphate-buffered saline, and this wash was removed. The three components (cell pellet, supernatant and wash) were then counted. The percent immunoreactivity was calculated as equal to [(^{213}Bi)CHX-A-DTPA-HuM195 bound to cells)/(total bound plus unbound activity (supernatant and wash)] × 100. Specific binding in these assays was confirmed by lack of binding of the (^{213}Bi)CHX-A-DTPA-HuM195 construct to CD33 negative RAJI or MOLT4 cell lines. To avoid nonspecific and Fc receptor binding, the assays were performed in the presence of 2% human serum. The immunoreactivity can be corrected for radiochemical purity by dividing the value for the percent immunoreactive fraction by the fraction of pure radiolabeled antibody.

Independent Safety, Sterility and Pyrogen Testing

Three separate (^{213}Bi)CHX-A-DTPA-HuM195 labeling/purification runs were performed under typical labeling circumstances using typical reagents, and the three products were analyzed by Leberco Testing (Roselle Park, NJ) for the following: general safety (per CFR-21–610.11), sterility (per USP/CFR-21) and biological assay for pyrogenic substances (per USP XXIII).

Pyrogen Level Determination on Site

The level of pyrogen that was present in the final formulated product was determined using an aliquot of the residual product diluted 1:8 or 1:16 with the *Limulus* amoebocyte lysate (LAL) testing kit (Associates of Cape Cod, Inc., Woods Hole, MA). Samples containing protein were placed in an ultrasonic bath for 0.5 h to avoid the possibility of a false-positive pyrogen determination (no gel formation).

Sterility Testing On Site

The final formulated product was terminally filtered through a conventional filter disk before patient injection.

A 14-d sterility assay was carried out on selected residual samples of final products. Fluid thioglycollate medium and soybean-casein digest medium (Becton Dickinson & Co.) were inoculated with residual product and were held for 2 wk at 32°C and 22°C, respectively. The absence or presence of growth in the media during this period was the indication for sterility pass or fail, respectively.

RESULTS

The radiolabeling reaction was allowed to proceed for 8–10 min at ambient temperature with gentle mixing. The ^{213}Bi radiolabeling reaction efficiency was 81% ± 9% (n = 67) as determined by ITLC. Purification of the reaction mixture after 8–10 min through size-exclusion chromatography yielded a final product of 99% ± 2% purity (n = 67) as determined by both ITLC and HPLC (Fig. 1). The duration of time from the end of generator elution to formulation of product for patient injection was 23 ± 3 min (n = 66).

A variety of different specific activities and dose sizes were prepared depending on the required dose level and patient weight. Specific activities were adjusted by varying either generator eluate size or unlabeled antibody dose. The specific activity ranged from 403 to 1040 MBq/mg at the end of processing the drug; at the time of injection, the specific activity ranged from 329 to 766 MBq/mg. The fraction (purity corrected) of (^{213}Bi)CHX-A-DTPA-HuM195 that was determined by cell-based assay to be immunoreactive was 89% ± 9% (n = 44).

The addition of l-ascorbic acid (reaction concentration of approximately 5 g/L) to the reaction mixture prevented significant loss of radiolabeled antibody during the purification over the 10 DG resin. In the absence of l-ascorbic acid, the radiochemical recovery yield of ^{213}Bi in the form (^{213}Bi)CHX-A-DTPA-HuM195 after passage over a 10 DG desalting column containing P6 gel was 54% ± 25% (n = 13). There was a significant amount (15% ± 17%; n = 13)

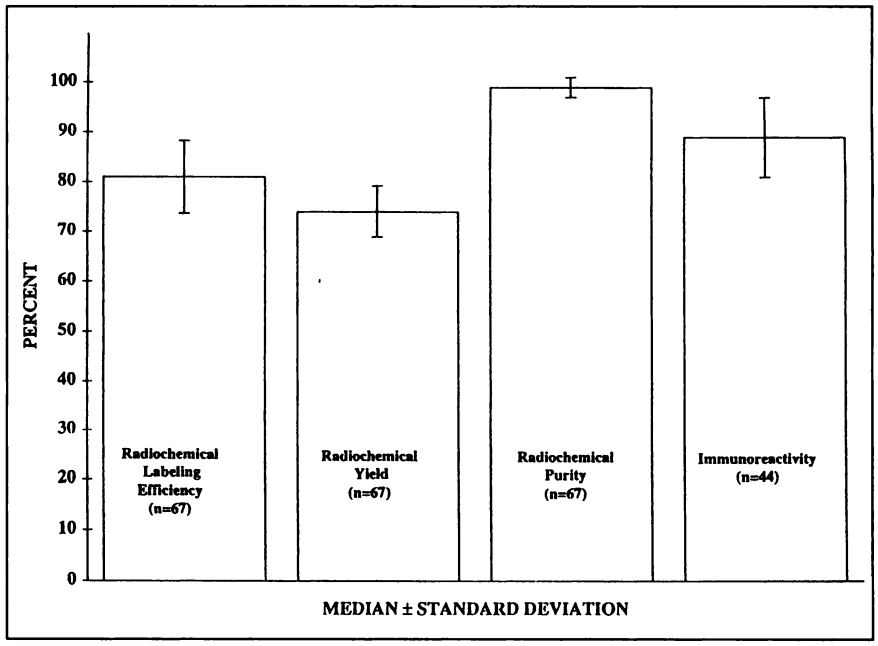

FIGURE 1. Summary of critical data for radiolabeling of (^{213}Bi)CHX-A-DTPA-HuM195.

of radioactivity remaining on the column after sufficient washing. This loss was presumably a consequence of protein denaturation due to the high radiation dose from the α and β particle emissions in solution as evidenced by the disparity between radiochemical reaction yields and radiochemical recovery yields. The addition of l-ascorbic acid to the reaction mixture (n = 67) resulted in a 74% ± 7% radiochemical recovery yield of ^{213}Bi radioactivity in the form (^{213}Bi)CHX-A-DTPA-HuM195. There was only a small amount (2% ± 1%; n = 28) of ^{213}Bi radioactivity remaining on the column after washing. This radioprotecting agent significantly improved both the yield and reproducibility of the antibody construct radiolabeling and purification processes.

Aliquots (0.020 mL) from residual samples of final products (7–8 mL) prepared for the first 57 doses were analyzed for ^{225}Ac impurity using pulse height MCAs with an HPGe detector (740 Bq [20 × 10^{-9} Ci] limit of detection). These analyses did not reveal the presence of any ^{225}Ac in the final products. Gas ionization detection of the β and γ emissions in the ^{225}Ac decay cascade was found to be 400-fold more sensitive than MCA using an HPGe detector, so that the effect of counting an aliquot of the total sample (approximately a 375-fold dilution) was overcome. The gas ionization detector (1.9 Bq [50 × 10^{-12} Ci] limit of detection) proved a more powerful technique to quantify the actual level of ^{225}Ac radionuclidic impurity in the injected dose. The level of ^{225}Ac per product dose was 0.04 ± 0.03 × 10^{-6}/mL (n = 10), which was 518 ± 518 Bq (n = 10) per product volume of 7.4 ± 0.5 mL (n = 67); the ^{225}Ac impurity was 3.1 × 10^{-5}% ± 2.8 × 10^{-5}% (n = 10) of the total activity injected. The generator impurity values decreased as a function of increasing column volumes of 0.1 mol/L HCl/NaI eluent. Furthermore, it appears that the ^{225}Ac present in the final product was associated with the antibody construct.

It was confirmed that ^{225}Ac was the radionuclide impurity present in the sample aliquots. Selected samples containing radionuclide impurity were held and were counted repeatedly over a several-week period. The activity decay values were analyzed by curve-fitting routines, and these data fit the expected decay curve for ^{225}Ac.

The pyrogen level in the final formulated product was ≤4 ± 1 EU/mL (n = 66), which amounted to approximately 29 ±10 EU/injection (n = 66). All 27 samples tested using a 14-d assay were found to be sterile. Before initiating patient studies, we performed three separate (^{213}Bi)CHX-A-DTPA-HuM195 labeling/purification runs under typical labeling circumstances using typical reagents and the three products analyzed by an independent testing laboratory for the following: general safety (per CFR-21–610.11), sterility (per USP/CFR-21) and biological assay for pyrogenic substances (per USP XXIII). All three samples were sterile, met the requirements for absence of pyrogens and met the requirements for general safety.

DISCUSSION

The development of a clinically useful therapeutic α particle-emitting ^{213}Bi-labeled antibody product requires stringent specifications including: (a) a dose of activity sufficient to treat the patient (148–925 MBq); (b) an immunoreactive radiolabeled construct; (c) a highly pure final agent; and (d) a drug that is safe, sterile and contains low pyrogen levels. In addition to these requirements, the methods for obtaining this product must also yield a reproducible quality of product, the total processing time must be short (^{213}Bi $t_{1/2}$ = 46 min) and the radionuclide must be capable of being handled safely by the staff preparing and administering the agent. In this article, we describe methods for accomplishing these goals and describe the results of 67 consecutive preparations for human clinical use. These procedures can be used in other situations to treat cancers similarly with ^{213}Bi-labeled mAb constructs.

Patients with relapsed leukemia, in particular, may require many megabecquerels of ^{213}Bi to treat a tumor burden of a kilogram of disease or more (one trillion or more cells). The tumor burden in patients with other cancers or therapeutic situations may be far less, and, in these cases, the importance of this specification is minimized. The methods and procedures we used to prepare (^{213}Bi)CHX-A-DTPA-HuM195 were thus constrained by the requirement that the final product contain enough activity to treat patients with leukemia. Therefore, the level of activity eluted from the generator, the rapid processing time and the labeling reaction yield were critical. With the exception of the ITLC determination of the radiolabeling incorporation yield, the majority of the quality control steps (i.e., ITLC of final purity, LAL, immunoreactivity and HPLC) were actually completed during or after the dose was administered. The sterility assay required 14 d. The radionuclidic impurity determination could not be made for 2–4 d until all of the ^{213}Bi and daughter ^{209}Pb decayed, leaving behind only generator breakthrough radionuclides.

The successful production of the final labeled product depends to a great extent on careful attention to key details and preparations. The elimination and exclusion of metal ion contaminants is perhaps most critical, because it can have a significant detrimental effect on the radiolabeling yield. Furthermore, all the reagents and apparatus must be sterile and free of pyrogen. This can be ascertained by testing reagents for sterility and pyrogen levels during practice radiolabeling of drug (following the same methods to be used for patient doses) before patient dose preparation. These samples can be sent to an independent testing laboratory and assayed for sterility, safety and pyrogen level.

Radioprotection agents such as l-ascorbic acid were found to be essential in the reproducible production and recovery of immunoreactive (^{213}Bi)CHX-A-DTPA-HuM195. In the absence of a radioprotecting agent, losses during purification were high and varied widely, presumably due to protein denaturation in the radiation field generated by the high activity levels of ^{213}Bi. The low-molecular-weight l-ascorbic

acid functioned as a sacrificial redox reagent until the size-exclusion column purification step, in which most of the l-ascorbic acid was removed. The 1% HSA, which was used as the mobile phase in the chromatography step and as the carrier formulation, also functioned as a radioprotecting agent.

The level of nonbismuth radionuclide impurities in the final product was very low and was determined to be ^{225}Ac. This extremely high level of radionuclidic purity was in part due to the generator design (*11,12*) and in part due to the purification by size-exclusion methods (*26*).

The therapeutic doses used here (n = 67) have been prepared in quantities up to 888 MBq from 2479 MBq generators. The γ and β emissions were readily shielded and worker exposure was minimal. The work station was set up in a standard chemical fume hood, with a lead/lead glass shield and small lead pigs that were used to hold multiple megabecquerel levels of ^{213}Bi.

α particle-emitting radionuclide-conjugated mAbs for the radioimmunotherapy of cancer have now been introduced into patients (*22*; M. Zalutsky, *personal communication* June 1998) 15 y after proposals (*27,28*) for the development of such agents. Several major obstacles had to be overcome to achieve this goal. Among these were (a) the establishment of a sufficient supply of ^{225}Ac (*13–15*) that could be used to produce ^{213}Bi for therapeutic purposes; (b) design and construction of a robust, clinically relevant ^{225}Ac/^{213}Bi generator that was able to yield high levels of clean, reactive ^{213}Bi (*12*); and (c) synthesis of a stable antibody construct containing a metal-chelating moiety (*16–21*). Finally, the methods and components had to be integrated logistically to allow clinical dose preparation in a hospital environment. This entailed defining the standard radiolabeling and purification conditions and incorporating sufficient quality control steps to ensure that the product was safe and reliable.

As part of a phase I dose-escalation clinical protocol, each of the 67 doses prepared as aforementioned was administered to patients intravenously. A total of 15 patients were treated. There were no toxicities associated with the 67 infusions. There were no intermediate clinical or laboratory documented toxicities or adverse events from these infusions.

Moreover, the patient images (*29,30*) showed distribution within 5–10 min of the injected (^{213}Bi)CHX-A-DTPA-HuM195 to the sites where leukemia target cells were found: the liver, spleen and bone marrow (*31;* G. Sgouros, *unpublished data* 1999). Over 2–3 h (three ^{213}Bi half-lives), there was little to no activity found in the kidney, gastrointestinal tract or elsewhere, reflecting the targeting specificity of this agent, the relative purity and stability of the radiolabeled antibody construct and the lack of significant loss of the agent after metabolism or catabolism in vivo.

CONCLUSION

Methods used to reproducibly prepare, purify and analyze the α particle-emitting anti-CD33 antibody construct, (^{213}Bi)CHX-A-DTPA-HuM195, for therapy of myeloid leukemias are described (Fig. 2). Thus far, 15 patients have had administration of a total of 67 doses of (^{213}Bi)CHX-A-DTPA-HuM195.

Similar procedures have also been applied to several other antibody constructs that could be used to treat other cancers, including ^{213}Bi-radiolabeled anti-CD19 for lymphomas and anti-Lewis-y, anti-TAG-72 and anti-PSMA$_{ext}$ (external binding domain) for ovarian and breast, colon and prostate carcinomas, respectively. Other studies are ongoing to

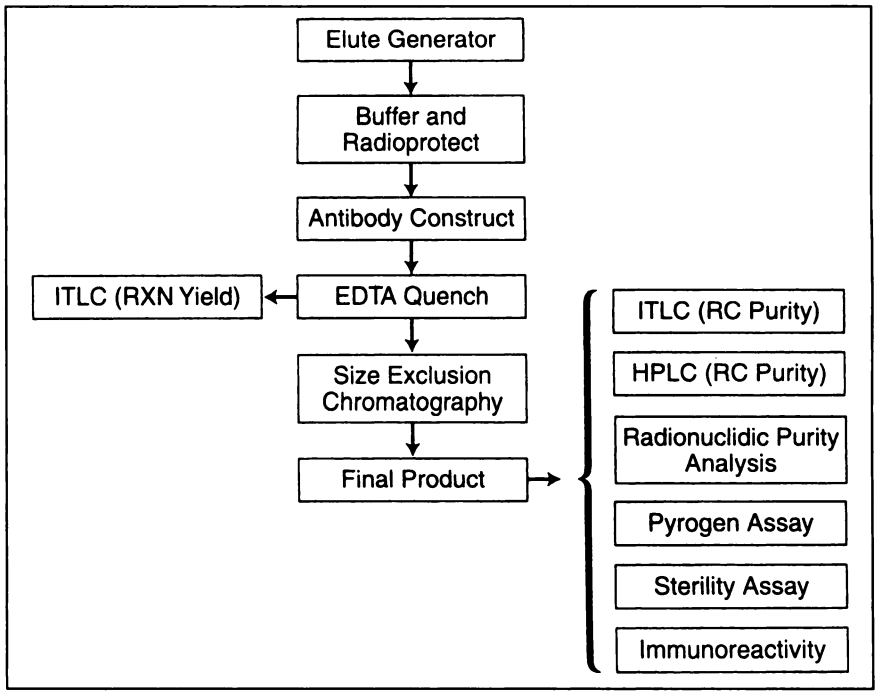

FIGURE 2. Flow diagram for ^{213}Bi radiolabeling of the antibody construct and quality control process. ITLC = instant thin-layer chromatography; RXN = reaction; EDTA = ethylenediaminetetraacetic acid; RC = radiochemical; HPLC = high-performance liquid chromatography.

increase the radiochemical reaction yield, further minimize the processing time and modify the procedure to allow for more widespread utilization of ^{213}Bi-labeled therapeutic agents against a wide variety of disorders.

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