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Development and Characterization of Anti-Renal Cell Carcinoma × Antichelate Bispecific Monoclonal Antibodies for Two-Phase Targeting of Renal Cell Carcinoma[1]

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Abstract

To test a two-step approach for radioimmunotargeting of renal cell cancer, quadroma cells secreting antichelate × anti-renal cell carcinoma bispecific antibodies were obtained by somatic cell fusion. Five monoclonal antibodies against the chelate 1,4,7-triazaheptane-*N,N′,N′′*-pentaacetic acid (DTPA) were produced and characterized. Competitive binding assays indicated that the anti-DTPA antibodies reacted with DTPA chelated with indium, yttrium, chromium, iron, or zinc. The affinity constants of the anti-DTPA antibodies for ^{111}In-DTPA ranged from 0.19 to 0.23 nM^{-1}. Using different chelates, a remarkable chelate specificity of the anti-DTPA antibodies was demonstrated. The chelates recognized by the antibodies DTIn1, DTIn2, and DTIn4 share a *N*(*N′′*)-diacetic acid group, whereas the chelates recognized by DTIn3 share a *N′*-acetic acid group, suggesting the presence of different essential structures within the DTPA molecule that determine the reactivity of the antibodies.

Five anti-DTPA antibody-producing hybridomas were used for somatic cell fusion with hybridoma G250 directed against renal cell carcinoma, resulting in three bispecific antibody-producing quadroma cell lines. The bispecific monoclonal antibodies were purified from ascites fluid using protein A affinity chromatography followed by hydroxylapatite chromatography and/or cation exchange chromatography. Of the total IgG amount present in the ascites fluid, 10–15% represented the bispecific antibodies. These bispecific antibodies will allow testing and optimization of a two-step approach for radioimmunotargeting of chelated radionuclides.

Introduction

Although radiolabeled antitumor antibodies can target tumors selectively *in vivo*, optimal visualization is delayed for several days, because the antibody has to clear from the blood pool and normal tissues. In addition to obscuring visualization of tumors, sustained levels of radiolabeled antibody in the blood and normal tissues increase toxicity to nontarget tissues and limit the amount of radioactivity that can be administered in radioimmunotherapy.

The efficacy of radioimmunodetection or radioimmunotherapy might be optimized by: (*a*) enhancing tumor uptake; or (*b*) reducing background activity. The use of antibody fragments with faster blood clearance (1–3) or antiantibodies to reduce serum levels of circulating antibody (4–6) has resulted in reduction of background activity.

Alternatively, reduction of background activity might be accomplished by pretargeting protocols. In these, the antitumor antibody is administered and allowed to clear from the circulation, after which the radionuclide is given as a low-molecular-weight ligand. The main advantage of these protocols is the very rapid excretion of the small ligand when not targeted to the tumor. The avid interaction between biotin and streptavidin has been exploited in such a multistep approach (7–10). Analogously, pretargeting protocols may make use of bispecific antibodies and radiometal-labeled chelates. In this approach, the tumor is pretargeted with bispecific antibodies reactive with a tumor-associated antigen and with a radiolabeled chelate. The feasibility of this approach has been shown in studies in nude mouse models (11, 12) and in immunoscintigraphy studies in colon carcinoma (13, 14) and medullary thyroid cancer patients (15). The marked reduction of the background activity and the good retention of the radionuclide in the tumors observed in these studies suggest that this approach also could be used for radioimmunotherapeutic purposes.

Bispecific antibodies can be produced either by: (*a*) chemical coupling of two antibody fragments; or (*b*) fusion of two hybridoma cell lines, resulting in quadromas (16). Chemical production of bispecific antibodies suffers from batch-to-batch variation, whereas quadromas are infinite sources of bispecific antibodies. In addition, the fusion technique results in quadromas that synthesize, assemble, and secrete bispecific antibodies by the same process as native immunoglobulins.

To investigate the possibilities of a two-phase radioimmunotargeting protocol, we have produced hybridomas secreting MAbs[3] specific for the chelate DTPA and fused these to a hybridoma producing a MAb recognizing a renal cell carcinoma-associated antigen. In this report, we describe the isolation and characterization of anti-DTPA MAbs, the production of quadromas, and the purification of bsMAbs.

Materials and Methods

Antibodies and Chelates

Production of Protein-Chelate Conjugates. KLH (Pierce, Rockford, IL) and BSA (Sigma Chemical Co., St. Louis, MO) were conjugated with the cyclic anhydride of DTPA (Sigma) according to the method of Hnatowich *et al.* (17). After PD10 (Pharmacia, Uppsala, Sweden) chromatography to remove unreacted DTPA, excess $InCl_3$ (Merck, Darmstadt, Germany) or YCl_3 (Aldrich Chemical Co., Milwaukee,WI) was added. Free In^{3+} or Y^{3+} was removed by PD10 column chromatography.

BSA, fibrinogen (ICN Biochemicals, Costa Mesa, CA), and calf lens protein (a kind gift from Dr. W. de Jong, University of Nijmegen, Nijmegen, the Netherlands) were conjugated with ITC-Bz-DTPA (kindly provided by Dr. G. Griffiths, Immunomedics, Morris Plains, NJ) according to the method of Ruegg *et al.* (18). BSA and calf lens protein were conjugated with ITC-Bz-DOTA (kindly provided by Dr. O. A. Gansow, National Cancer Institute, Bethesda, MD) according to the method of Ruegg *et al.* (18).

Preparation of ^{111}In-DTPA. Five μl $^{111}InCl_3$ (Mallinckrodt, Petten, the Netherlands) and 2 μl $InCl_3$ (2.5 ng/μl) were diluted in 50 μl 40 mM HCl and mixed with 850 μl aqueous DTPA solution (0.05 μg/ml) and 100 μl 0.1 M sodium acetate buffer (pH 6), resulting in a final pH of 3.0. Following 30 min of incubation at room temperature, the solution was neutralized with 17 μl 0.1 M NaOH. Complexing of In^{3+} was assessed by paper chromatography on Whatman 1 paper with methanol:water (55:45) as an eluent. The specific activity of the ^{111}In-DTPA was 700–800 Ci/mmol.

Anti-DTPA Antibodies. BALB/c mice were immunized with an emulsion of 50 μg In-DTPA-KLH or Y-DTPA-KLH in complete Freund's adjuvant

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[3] The abbreviations used are: MAb, monoclonal antibody; DTPA, 1,4,7-triazaheptane-*N,N′,N′′*-pentaacetic acid; bs, bispecific; KLH, keyhole limpet hemocyanin; ITC-Bz, isothiocyanato-benzyl; DOTA, 1,4,7,10-tetraazadodecane-*N,N′,N′′,N‴*-tetraacetic acid.

(Sigma). Animals received boosters of antigen mixed with incomplete Freund's adjuvant. Three days after an i.v. boost of antigen, spleen cells were harvested and fused with Sp2/0-Ag14 myeloma cells (19) as described by Lane (20). Culture supernatants of resulting clones were analyzed for anti-DTPA reactivity using a direct antigen-binding ELISA. Cells of clones reactive with In- or Y-DTPA-BSA were subcloned at least twice in soft agarose (30 g/l; agarose type VII; Sigma) containing RPMI 1640 medium with hypoxanthine-thymidine supplement (GIBCO-BRL, Gaithersburg, MD).

Production of bsMAbs. Enzyme-deficient hybridomas were isolated to enable selection of fused cells (21). Hypoxanthine guanine phosphoribosyl transferase deficiency was introduced in the anti-DTPA MAb-producing hybridomas by 8-azaguanidine (Sigma) selection. The anti-renal cell carcinoma MAb G250 was used as the antitumor antibody in these studies. MAb G250 and its subclass variants are directed against the renal cell carcinoma-associated antigen G250 that is expressed homogeneously in 75% of renal cell carcinomas, whereas expression in normal tissues is restricted to gastric mucosal cells and cells of the larger bile ducts (22). MAb G250 has shown excellent targeting of renal cell carcinoma in patients (23). Thymidine kinase-deficient hybridoma G250 variants were isolated by bromodeoxyuridine (Boehringer Mannheim, Mannheim, Germany) selection. Anti-DTPA hybridomas were fused with hybridoma G250 at a 1:1 ratio and seeded in soft agarose containing low-protein hybridoma medium (GIBCO-BRL) with hypoxanthine-aminopterin-thymidine supplement (GIBCO-BRL). Growing colonies were picked, grown in suspension, and tested for the production of bsMAb by a double-specificity ELISA. Positive clones were recloned at least twice.

ELISAs

In all the assays described in this section, the following conditions were used, unless indicated otherwise. Wells of 96-well microtiter plates (Costar, Cambridge, MA) were coated overnight at 4°C, and remaining protein binding sites were blocked for 1 h with 50 mM phosphate buffer (pH 7.4)/150 mM NaCl/BSA (5 g/liter). Wells were rinsed with the same buffer. Peroxidase activity was revealed using 3,3′,5,5′-tetramethylbenzidine (Merck)/H_2O_2 for 10–20 min, and *A* was determined at 450 nm.

Direct Antigen-binding Assay. Wells coated with 400 ng DTPA-BSA, 600 ng ITC-Bz-DTPA-fibrinogen, or 300 ng ITC-Bz-DOTA-calf lens protein were incubated with the culture supernatant, washed, and incubated with peroxidase-conjugated rabbit antimouse immunoglobulins (Dakopatts, Glostrup, Denmark).

Competitive Antigen-binding Assay. Wells were coated with In-DTPA-BSA. Serially diluted Cr-DTPA, Fe-DTPA, Zn-DTPA, Y-DTPA, or In-DTPA was added simultaneously with anti-DTPA tissue culture supernatant. After 3 h of incubation, wells were washed and incubated with peroxidase-conjugated rabbit antimouse immunoglobulins.

Double-Specificity ELISA. Wells coated with 400 ng In-DTPA-BSA were incubated with serially diluted quadroma tissue culture supernatant, washed, and incubated with biotinylated NUH82. NUH82 (IgG1) is an internal-image, anti-idiotype MAb resembling MAb G250 antigen with an affinity-binding constant of 8.2 nM^{-1} for MAb G250 (24). Wells were incubated with a streptavidin-biotinylated horseradish peroxidase complex (Amersham, Little Chalfont, England). This assay assured measurement of bispecific antibodies with functional DTPA- and G250-binding pockets only. The detection limit was determined to be 100 ng bsMAb/ml.

Determination of Isotypes. The isotypes of the anti-DTPA MAbs were determined in an ELISA with mouse subclass IgG-specific antisera (Southern Biotechnology Associates, Inc., Birmingham, AL).

Determination of Affinity Constants. Affinity constants for ^{111}In-DTPA were determined by Scatchard analysis (25). Microtiter wells coated with rabbit antimouse immunoglobulins (Dakopatts) were incubated with excess anti-DTPA MAb or bsMAb. Increasing amounts of ^{111}In-DTPA were added, and after 1 h at room temperature, wells were washed and counted.

Purification of MAbs

Ascites fluids were cleared by centrifugation and diluted 1:1 in 3 M NaCl/1.5 M glycine (pH 8.9) and subjected to protein A affinity chromatography. Immunoglobulins were eluted by sequential application of 0.1 M sodium citrate buffers (pH 6, 5, 4, and 3).

Cation exchange chromatography was performed on a mono-S HR 5/5 column (Pharmacia). Protein A-purified material was dialyzed against 40 mM NaAc (pH 5.2) and loaded on the cation exchange column. Material was eluted with a 0–400 mM linear LiCl gradient.

For hydroxylapatite (Bio-Rad Laboratories, Richmond, CA) purification, protein A-purified material was dialyzed against 10 mM sodium phosphate (pH 6.8). The bound immunoglobulins were eluted with a linear ionic gradient ranging from 10 to 300 mM sodium phosphate (pH 6.8).

Results

Anti-DTPA Antibodies. Fusion of spleen cells from mice immunized with In- or Y-DTPA-derived KLH with Sp2/0-Ag14 myeloma cells resulted in hybridoma cells secreting immunoglobulins reactive with In-DTPA-BSA and Y-DTPA-BSA. No reactivity was observed with BSA, indicating recognition of DTPA. After subcloning, five hybridomas were selected: DTIn1 (IgG2a), DTIn2 (IgG1), DTIn3 (IgG1), DTIn4 (IgG1), and DTY4 (IgG2a).

Metal Selectivity of the Anti-DTPA Antibodies. To study whether the binding of DTPA was influenced by chelation of different metals, competitive antigen-binding ELISAs were performed. For all anti-DTPA MAbs, with the exception of DTIn3, In-DTPA was the best competitor for In-DTPA-BSA binding, irrespective of the origin of the anti-DTPA MAb; *i.e.*, even DTY4, isolated after immunization with Y-DTPA-KLH, showed superior binding of In-DTPA. However, all metal-DTPA chelates, including Y-DTPA, competed for In-DTPA-BSA binding. For DTIn3, Cr-DTPA was the best competitor for In-DTPA-BSA binding, but all chelates competed for In-DTPA-BSA binding. As an example, the competitive antigen-binding assay of DTIn2 is shown in Fig. 1.

Chelate Specificity of Anti-DTPA Antibodies. The fine specificity of the anti-DTPA MAbs was determined in direct antigen-binding assays. All antibodies reacted with protein-conjugated In-DTPA. In addition, DTIn1, DTIn2, DTIn4, and DTY4 reacted with protein-conjugated-ITC-Bz-DOTA. DTIn3 and DTY4 reacted with protein-conjugated ITC-Bz-DTPA. DTPA-BSA binding of DTIn3 and DTY4 could be inhibited completely with EDTA.

Affinity Constants of Anti-DTPA Antibodies. Based on Scatchard analysis the affinity constants of the anti-DTPA MAbs for binding ^{111}In-DTPA ranged from 0.19 to 0.23 nM^{-1}. The affinity constants of bsMAb G250 × DTY4 and bsMAb G250 × DTIn1 were comparable to the parental antibodies DTY4 and DTIn1, respectively (Table 1).

Production of bsMAbs. Fusion of hypoxanthine guanidine phosphoribosyl transferase-deficient anti-DTPA hybridoma cells with thy-

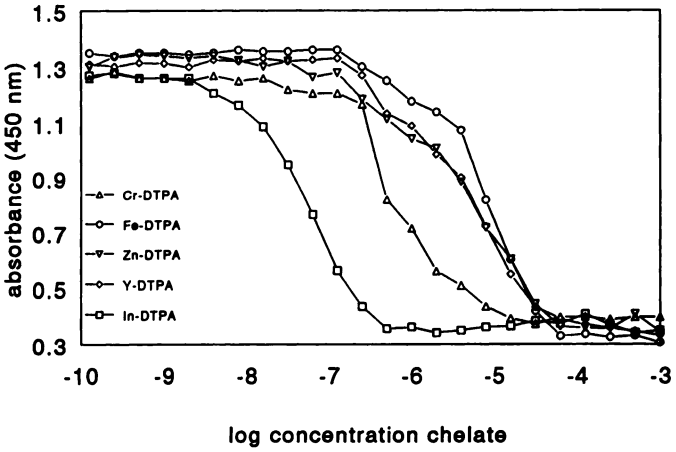

Fig. 1. Competitive antigen-binding assay of DTIn2. The amount of DTIn2 bound to the solid-phase-coated In-DTPA-BSA is plotted *versus* the concentration of the competing chelate added.

Table 1 *Affinity constants for binding ^{111}In-DTPA*

MAb	Affinity constant (nM^{-1})
DTIn1	0.23
DTIn2	0.23
DTIn3	ND[a]
DTIn4	0.19
DTY4	0.22
G250 × DTY4	0.28
G250 × DTIn2	ND[a]
G250 × DTIn1	0.24

[a] ND, not determined.

midine kinase-deficient hybridoma G250 resulted in viable quadroma colonies. Quadromas G250 (IgG1) × DTY4, G250 (IgG1) × DTIn2, and G250 (IgG1) × DTIn1 produced bsMAbs, as evidenced by reactivity in the double-specificity ELISA. Fusion of G250 (IgG2b) with DTIn3 and G250 (IgG1) with DTIn3 resulted in quadroma cells that did not produce detectable levels of bsMAbs, as judged by the double-specificity assay. The IgG molecules produced by the quadroma G250 (IgG2b) × DTIn3 were partly characterized. IgG molecules with functional G250-binding pockets and nonfunctional DTIn3-binding pockets were produced, as well as IgG molecules with intact DTIn3 sites and nonfunctional G250 sites, as determined in ELISAs determining solid-phase binding to either NUH82 or BSA-DTPA using anti-IgG1 or anti-IgG2b as the tracer antibody, respectively.

Purification of Antibodies. BsMAb G250 × DTY4 was purified from ascites fluid using protein A chromatography followed by cation exchange chromatography (Fig. 2). Protein A-bound bsIgG was eluted at pH 5 (Fig. 2*A*) and subjected to cation exchange chromatography. Application of a LiCl gradient resulted in four protein peaks (Fig. 2*B*), which were analyzed for G250 × DTY4 activity. bsG250 × DTY4 antibody eluted at 223 mM LiCl. The cation exchange-purified material represented 10–15% of the IgG eluted from the protein A column. The protein peak eluting at 206 mM LiCl contained immunoglobulins with intact IgG1 × IgG2a heavy-chain pairing, as shown by a mixed-isotype ELISA, but incorrect heavy-light-chain pairing, as shown by the negative reactivity in the double-specificity assay, resulting in nonfunctional binding pockets.

bsMAb G250 × DTIn2 was purified from ascites using protein A chromatography followed by hydroxylapatite chromatography and cation exchange chromatography. After protein A chromatography, the material eluting at pH 6 contained bsMAb. This material separated into four peaks on hydroxylapatite chromatography, with the bsMAb eluting at 133 mM phosphate. Additional separation of bsMAb from contaminants was achieved by cation exchange chromatography, with G250 × DTIn2 activity eluting at 165 mM LiCl. The cation exchange chromatography-purified material represented 10–15% of the initial amount of protein A-bound IgG.

BsMAb G250 × DTIn1 was purified from ascites fluid using protein A affinity chromatography followed by cation exchange chromatography. Protein A-bound bsIgG eluted at pH 5 and was subjected to cation exchange chromatography. This material separated into seven peaks, with the bsMAb eluting at 276 mM LiCl. The cation exchange chromatography-purified material represented 10–15% of the protein A-bound IgG.

Discussion

Two-step strategies using bsMAbs may optimize radioimmunotargeting of tumors *in vivo*. The main advantage of this approach is the very rapid excretion of the low-molecular-weight radiolabeled ligand when not targeted to the tumor. Several preclinical and clinical studies have shown that two-phase targeting results in high tumor:nontumor ratios shortly after injection of the radiolabel (11–15). To investigate the possibilities of two-phase radioimmunotargeting, we have developed five hybridomas producing MAbs reactive with DTPA and fused these with the hybridoma G250, producing a MAb reactive with renal cell carcinoma, to obtain bsMAbs.

The affinity constants of the anti-DTPA MAbs for binding ^{111}In-DTPA were in the same order of magnitude (0.19–0.23 nM^{-1}) as reported for other antichelate MAbs (12, 26).

All anti-DTPA MAbs reacted also with DTPA loaded with different metals. For all anti-DTPA MAbs, with the exception of DTIn3, In-DTPA was bound most efficiently, irrespective of the immunogen used (In-DTPA-KLH or Y-DTPA-KLH). In the competitive binding assays, the affinity for binding In-DTPA was 10–100-fold higher than the affinity for binding DTPA loaded with other metals. Similar results have been obtained by Reardan *et al.* (26) and Bosslet *et al.* (27). They showed that the affinity of their antichelate antibodies for chelates labeled with indium was at least 100-fold higher than for chelates labeled with other metals. Involvement of In^{3+} in the antibody-antigen interaction may cause the higher affinity of the MAbs for the In^{3+}-labeled chelates (28).

The anti-In-DTPA MAbs displayed a remarkable chelate specificity. The chelates recognized by DTIn1, DTIn2, and DTIn4 (DTPA and

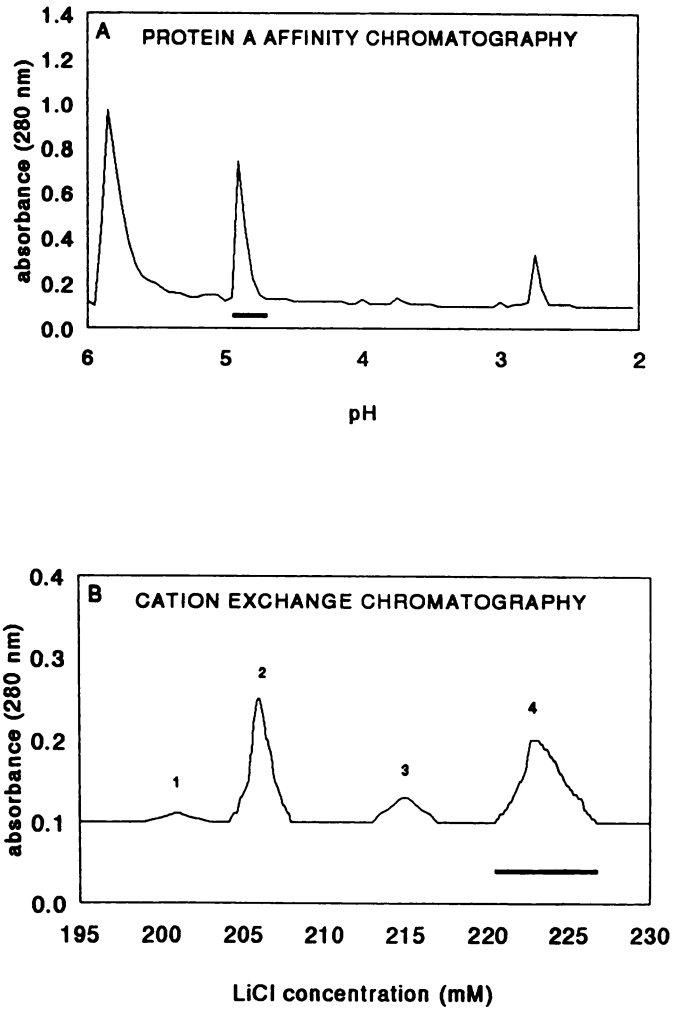

Fig. 2. Purification of bsMAb G250 × DTY4 using protein A affinity chromatography (*A*) followed by cation exchange chromatography (*B*). *A*, protein A-bound material was eluted by sequential application of 0.1 M sodium citrate buffers (pH 6, 5, 4, and 3). *B*, cation exchange-bound, protein A-purified material was eluted using a 0–400 mM LiCl gradient in 40 mM NaAc (pH 5.2). The peaks containing bsMAb are indicated by the *solid bar*.

ITC-Bz-DOTA) share an $N(N')$-diacetic acid group, whereas the chelates recognized by DTIn3 (DTPA, ITC-Bz-DTPA, and EDTA) share an N'-acetic acid group, suggesting the presence of different essential structures within the DTPA molecule that determine the reactivity of the anti-In-DTPA MAbs. For the anti-Y-DTPA MAb DTY4 (reacting with DTPA, ITC-Bz-DTPA, ITC-Bz-DOTA, and EDTA) the recognized structure is unclear. The broad reactivity of the MAbs makes them suitable for imaging (using ^{111}In-DTPA or $^{99\,m}$Tc-DTPA) as well as therapy (with ^{90}Y-DTPA or ^{90}Y-DOTA).

Because of these characteristics, all anti-DTPA MAbs were used for production of bsMAbs. Three of five quadroma fusions resulted in quadromas actually secreting bsMAbs. Fusion of isotype-mismatched or isotype-matched hybridomas G250 and DTIn3 did not result in the production of functional bsMAb. Therefore, it seems that for successful assembly of bsMAb, factors other than the isotype of the parental antibodies are important.

Individual purification protocols were developed for every bsMAb. bsMAb G250 × DTY4 was purified using protein A affinity chromatography followed by cation exchange chromatography. The amount of bsMAb obtained after cation exchange chromatography was 10–15% of the total amount of IgG present in the ascites fluid. bsMAb G250 × DTIn2 was purified by protein A affinity chromatography followed by hydroxylapatite chromatography and cation exchange chromatography. Similarly, bsMAb G250 × DTIn2 also represented 10–15% of the total amount of IgG present in the ascites fluid. After protein A affinity chromatography and cation exchange chromatography, bsMAb G250 × DTIn1 was shown to be 10–15% of the amount of IgG present in the ascites fluid. This is in concordance with total random association of the two heavy and light chains (provided that the heavy and light chains of both parents are expressed codominantly) as suggested by Suresh *et al.* (29).

We have developed five anti-DTPA MAbs reactive with a broad range of chelated metals. Fusion of the anti-DTPA-producing hybridomas with the G250 hybridoma resulted in three quadroma cell lines producing bsMAbs. These purified bsMAbs will allow testing and optimization of a two-phase targeting protocol in nude mice models.

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