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

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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-triazahexapeptane-N,N',N''-pentanoic 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 111In-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 DTIna, DTInb, and DTIn4 share a N(N')-acetic 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.

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 multi step 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 MAbs3 specific for the chelate DTPA and fused these to a hybridoma producing a MA 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 InCl₃ (Merck, Darmstadt, Germany) or YCl₃ (Aldrich Chemical Co., Milwaukee, WI) was added. Free In³⁺ or Y³⁺ 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. Ganow, National Cancer Institute, Bethesda, MD) according to the method of Ruegg et al. (18).

Preparation of 111In-DTPA. Five µl 111InCl₃ (Mallinkrodt, Petten, the Netherlands) and 2 µl InCl₃ (2.3 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 In³⁺ was assessed by paper chromatography on Whatman 1 paper with methanol-water (55:45) as an eluent. The specific activity of the 111In-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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3The abbreviations used are: MAb, monoclonal antibody; DTPA, 1,4,7-triazahexapeptane-N,N',N''-pentanoic acid; bs, bispecific; KLH, keyhole limpet hemocyanin; ITC-Bz, isothiocyanato-benzyl; DOTA, 1,4,7,10-tetraazadodecane-N,N',N''-tetraacetic acid.
deficient hybridomas G250 variants were isolated by bromodeoxyuridine (Boehringer Mannheim, Mannheim, Germany) selection. Anti-DTPA hybridomas were fused with hybridomas G250 at a 1:1 ratio and seeded in soft agar containing low-protein hybridoma medium (GIBCO-BRL) with hypoxanthine-aminopterin-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 S-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 and after a 1:1 ratio and seeded in soft agar 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 30 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)/H2O2 for 10–20 min, and it 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- or Y-DTPA-derived KLH with Sp2/0-Agl4 myeloma cells. Wells were incubated with the supernatant, washed, and incubated with the concentration of the competing chelate added.

**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 NUHS2. NUH82 (IgGl), DTIn4 (IgGl), and DTIn4 (IgG2a).

**Metal Selectivity of the Anti-DTPA Antibodies.** To study 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-BSA, 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. DTY4 in DTPA was reconstituted 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 111In-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 guanine phosphoribosyl transferase-deficient anti-DTPA hybridoma cells with thy-
midline kinase-deficient hybridoma G250 resulted in viable quadroma colonies. Quadromas G250 (IgG1) × DTY4, G250 (IgG1) × DTIn2, and G250 (IgG1) × DTIn1 produced bsM Abs, 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 bsM Abs, as judged by the double-specificity assay. The IgG molecules produced by the quadroma G250 (IgG2b) × DTIn3 and G250 (IgG1) with DTIn3 resulted in quadroma cells with intact DTIn3 sites and functional 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.** BsM Ab 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. 2A) and subjected to cation exchange chromatography. Application of a LiCl gradient resulted in four protein peaks (Fig. 2B), 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.

bsM Ab G250 × DTIn2 was purified from ascites fluid using protein A chromatography followed by hydroxylapatite chromatography and cation exchange chromatography. After protein A chromatography, the material eluting at pH 6 contained bsM Ab. This material separated into four peaks on hydroxylapatite chromatography, with the bsM Ab eluting at 133 mM phosphate. Additional separation of bsM Ab 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.

BsM Ab 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 bsM Ab 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 bsM Abs 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:non-tumor ratios shortly after injection of the radiolabel (11–15). To investigate the possibilities of two-phase radioimmunotargeting, we have developed five hybridomas producing M Abs reactive with DTPA and fused these with the hybridoma G250, producing a M Ab reactive with renal cell carcinoma, to obtain bsM Abs.

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

All anti-DTPA M Abs reacted also with DTPA loaded with different metals. For all anti-DTPA M Abs, 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 Reardon 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 In³⁺ in the antibody-antigen interaction may cause the higher affinity of the M Abs for the In³⁺-labeled chelates (28).

The anti-In-DTPA M Abs displayed a remarkable chelate specificity. The chelates recognized by DTIn1, DTIn2, and DTIn4 (DTPA and DTPA were in the same order of magnitude (0.19–0.23 nM⁻¹) as reported for other antichelate M Abs (12, 26).

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ful assembly of bsMAb, factors other than the isotype of the parental antibodies are important.

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 DTn3 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× DTn4 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× DTn2 was purified by protein A affinity chromatography followed by hydroxylapatite chromatography and cation exchange chromatography. Similarly, bsMAb G250× DTn2 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× DTn1 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 columinantly) 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.

References