Establishment and Characterization of Monoclonal Antibodies Against an Octahedral Gallium Chelate Suitable for Immunoscintigraphy with PET

Margot Zöller, Jochen Schuhmacher, Jennifer Reed, Wolfgang Maier-Borst, and Siegfried Matzku

Institute of Radiology and Pathophysiology and Institute of Experimental Pathology, German Cancer Research Center, Heidelberg, Germany

As a prerequisite for preparing bispecific antibody conjugates containing anti-tumor and anti-metal chelate binding sites that can be used for pretargeted immunoscintigraphy, monoclonal antibodies (Mabs) have been raised against an octahedral metal chelate synthetized from gallium (Ga) and the hexadentate ligand N,N′*bis*[2-hydroxy 5-(ethylene β carboxy) benzyl] ethylenediamine N,N′ diacetic acid (Ga-HBED-CC). With use of the Farr assay, binding studies with the ^{67}Ga-labeled chelate and three clones of anti-chelate Mabs showed that none of the Mabs were able to precipitate more than 50% of the Ga-chelate, suggesting an enatiomerism of the Ga-chelate and a sensitivity of the Mabs to either one or the other chelate enantiomer. This could be confirmed by comparing the circular dichroism spectra of the Ga-chelate fractions that passed affinity columns containing the Mabs immobilized on sepharose without retention. With use of a Ga-HBED-CC enantiomer, whole-body retention in mice, preinjected with the corresponding anti-metal chelate Mab of ca. 70% ID, was measured compared to 2.1% retention in mice not preinjected with the Mab. Due to the high affinity of chelate-to-Mab binding in vivo, bispecific antibody conjugates prepared from the fragments of the anti-Ga-chelate Mab might be suitable for pretargeted immunoscintigraphy with the short-lived positron-emitter ^{68}Ga.

J Nucl Med 1992; 33:1366–1372

Contrary to the initially expected selective tumor accumulation of monoclonal antibodies (Mabs), clinical studies with radiolabeled Mabs demonstrated a reduced sensitivity of tumor detection ranging from 60% to 80% (*1–3*). Low tumor-to-nontumor tissue ratios are the main reason for this limited success. The cross-reactivity of murine Mabs with human nontumor tissues (*4*) or the presence of tumor antigen in the circulation (*5*) have been discussed as factors reducing tumor uptake or increasing accumulation of Mabs in nontarget tissues. What is crucial, however, seems to be the slow clearance of radiolabeled Mabs from the blood and an elevated interstitial pressure of tumors acting as a barrier for the extravasation of high molecular weight Mabs (*6*).

Following the suggestion of Reardan et al. (*7*), who first reported the production of anti-chelate Mabs, bispecific antibody conjugates (BACs) containing anti-tumor and anti-chelate binding sites were proposed for a novel method of pretargeted immunoscintigraphy by Goodwin et al. (*8*). Unlabeled BACs are allowed to localize for several days in the tumor, followed by the administration of a nonradioactive chelate coupled to a high molecular weight carrier protein to trap and eliminate the excess BACs from the circulation. Subsequently, a radiolabeled low molecular weight chelate is given, which rapidly penetrates into the tumor tissue and binds to the prelocalized BAC, while excess chelate is rapidly cleared from the blood via the kidneys. The theoretical advantage of pretargeted immunoscintigraphy with bispecific antibodies should be high tumor-to-nontumor tissue contrast and the possible use of short-lived radionuclides for labeling chelates. Studies done so far have used ^{111}In-labeled EDTA or DTPA complexes for targeting the prelocalized BACs (*8–10*).

Since a Ga-chelate can be readily labeled with the short-lived positron-emitter ^{68}Ga, which would enable the use of PET in immunoscintigraphy, Mabs against a Ga-chelate were established and the potential of the Ga-chelate for serving as a radioactive hapten in pretargeted immunoscintigraphy was evaluated. HBED-CC (Fig. 1), a two-fold carboxylic acid substituted HBED, was used as the ligand because the stability constant of the basic chelator HBED for complexing Ga^{3+} at physiological pH was demonstrated to be $10^{30.8}$, which is 9.6 and 10.9 log units higher than that of Ga-DTPA and Ga-EDTA (*11*) and ensures a high in-vivo stability of the Ga-HBED-CC chelate. The substitution of HBED with carboxylic acid groups was necessary to increase solubility and renal clearance of the chelate, or in the case of immunization of mice to provide

Received Jun. 14, 1991; revision accepted Mar. 10, 1992.

For reprints contact: J. Schuhmacher, Institute of Radiology and Pathophysiology, German Cancer Research Center, Im Neuenheimer Feld 280, 6900 Heidelberg, FRG.

an additional functional group for coupling of the chelate to a carrier protein.

MATERIALS AND METHODS

Synthesis and Characterization of the Ligand

HBED-CC was synthesized by modifying a method of Kroll et al. (*12*). The reaction scheme is presented in Figure 1. One hundred millimols of 3-(o-hydroxyphenyl)propionic acid (Aldrich, Steinheim, FRG) were dissolved in 75 ml methanol, 20 ml H_2O and 10 ml 4.0 N NaOH. To this solution, 50 mmol of N,N' ethylenediamine diacetic acid (Aldrich) in 25 ml 2.0 N NaOH were added with continual stirring. The pH of this mixture was adjusted to 6.7 and the solution was cooled in an ice bath. Subsequently, 100 mmol of formaldehyde (aqueous solution 37%) were added in several portions while maintaining the pH at 6.7 by intermittent addition of 4.0 N NaOH. After refluxing the mixture for 3.5 hr, methanol was removed on a rotary evaporator. The remaining aqueous solution was diluted with H_2O to 150 ml, acidified with HCl to pH 5.5 and twice extracted with diethylether to remove unreacted hydroxyphenylpropionic acid. The aqueous phase was then adjusted to pH 1.5 with 6 N HCl and the HBED-CC was allowed to crystallize for 4 days at 4°C. The crude product was extracted with hot methanol and recrystallized in the form of white needles from NaOH/HCl. The yield was 2.2 g (8% of theory) and the melting point was 208-210°C. The compound migrated as a single spot in thin-layer chromatography (TLC), with Rf = 0.30 and Rf = 0.59, using silica gel plates (Kieselgel 60 F_{254} 0.25 mm Merck, Darmstadt, FRG) developed with ethylacetate, methanol, acetic acid (6:3:1) and n-propanol, water, acetic acid (10:2.5:1), respectively. Microanalysis showed C = 57.4%, H = 6.11% and N = 5.02%, which fits best with the formula $C_{26}H_{32}N_2O_{10}\times 1H_2O$ (calculated C = 56.7%, H = 6.23%; N = 5.09%). For evaluation of the chemical structure, ^{1}H-NMR spectra were measured at 500 MHz on a Bruker AM 500 magnetic resonance spectrometer. Perdeuterated dimethylsulfoxide was used as a solvent and tetramethylsilane as the internal standard.

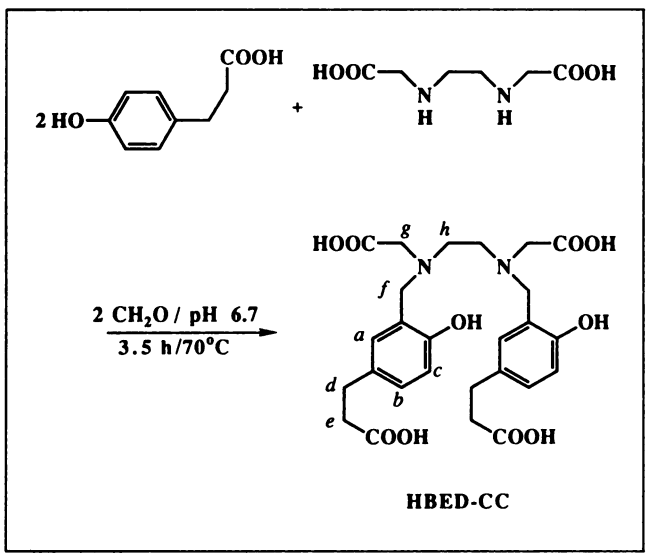

FIGURE 1. Chemical synthesis of HBED-CC.

Complexing of Ga^{3+} by HBED-CC

Twenty microliters of a 1.33 m*M* solution of Ga^{3+} [$Ga_2(SO_4)_3$ purriss. Fluka AG, Buchs, Switzerland] in 0.05 N HCl were labeled with 1.85 MBq of ^{67}Ga ($^{67}GaCl_3$ in 0.05 N HCl, 1.1 GBq per ml, Amersham Buchler, Braunschweig, FRG) and mixed with 3 μl of a 10 m*M* aqueous solution of HBED-CC. The addition of 400 μl 0.1 *M* morpholinoethane sulphonic acid buffer, pH 4.8, resulted in a pH of 3.0 and the mixture was allowed to react for 10 min. Subsequently, the solution was neutralized to pH 7.0 with 0.1 N NaOH. Complexing of Ga^{3+} was checked by ascending paper chromatography using Whatman No.1 as a support and methanol-water (55:45) as the solvent. Chromatograms were cut in 1-cm pieces and counted in a gamma counter. Ga^{3+} complexed by HBED-CC migrated with a Rf of 0.7, while uncomplexed Ga^{3+} formed an insoluble hydroxide which remained at the start. The complexing of indium and iron by HBED-CC was checked in the same manner as described for Ga^{3+} ($^{111}InCl_3$ in 0.04 N HCl, 1.8 GBq per ml; $^{59}FeCl_3$ in 0.5 N HCl, 1.9 GBq per mg of Fe, NEN-Du-Pont). After studying the competition of In-HBED-CC and Fe-HBED-CC with the Ga-chelate for Mab binding sites, the addition of ^{111}In and ^{59}Fe was omitted.

Monoclonal Antibodies

For immunization of mice, Ga-HBED-CC labeled with tracer amounts of ^{67}Ga was coupled to keyhole limpet hemocyanin (KLH) via 1-ethyl 3-(dimethylaminopropyl) carbodiimide (EDC). Separation from unreacted metal chelate was achieved by gel chromatography with Bio-Gel P-30 (Bio Rad, München, FRG). Coupling efficiency was determined by gamma counting and amounted to 14.5%, corresponding to 60 nmol of metal chelate per milligram of KLH. BALB/c mice were immunized four to six times with the Ga-HBED-CC-KLH antigen in complete and incomplete Freund's adjuvant (200 μg per mouse). Two days after a last i.v. injection, spleens from immunized animals were removed and lymphocytes were fused with Sp 2/0 myeloma cells according to the method of Köhler and Milstein (*13*). Supernatants of the clones obtained were screened for ^{67}Ga-HBED-CC binding by a standard RIA procedure (*14*) using microtiter plates coated with 10 μg of a goat anti-mouse antibody per well as the solid phase. From 60 positive clones resulting from three fusions, three high titer secretors 3-9, 8-16 (both IgG1) and 3A10 (IgG3) were recloned. Mabs were purified from ascites fluid by affinity chromatography on protein-A (Affi-Prep, Bio-Rad) (*15*), followed by anion exchange chromatography (Mono Q HR 5/5 Pharmacia, Freiburg, FRG) using a buffer 10-m*M* Tris-HCl (pH 8.0) with a gradient of 0.01-1.0 *M* NaCl for elution. Purity of Mabs was checked by SDS-polyacrylamide gel electrophoresis (*16*).

Preparation of 3-9 and 3A10 Mab-Sepharose

For conjugation of Mabs to CNBr-activated sepharose, the coupling procedure recommended by Pharmacia was followed (*17*). The final columns contained 3 ml of sepharose conjugated with 3 mg Mab (20 nmol) per ml gel. The columns were operated with 0.01 *M* PBS, pH 7.1. The elution of Ga-HBED-CC bound to Mab columns was achieved using a 3.0 *M* aqueous sodium thiocyanate solution 10 m*M* in phosphate buffer, pH 7.1.

Binding of Ga-HBED-CC to Anti-chelate Antibodies

The Farr assay, ammonium sulphate (AS) precipitation of the ^{67}Ga-HBED-CC-Mab complexes (*18*), was used for investigation of the various aspects of binding. Incubations of the chelate and Mabs were carried out in a final volume of 1.0 ml of 0.01 *M* PBS

containing 0.5 mg of BSA and 0.75 mg of a polyclonal pig IgG for coprecipitation if not stated otherwise. Incubations were kept for 2 hr at 4°C. Fifteen minutes after the addition of 1.0 ml of AS (saturated aqueous solution, 430 g/liter), the mixtures were centrifuged for 10 min at 7000 g. Precipitates were washed with 1.0 ml of a 50% saturated aqueous AS solution. Radioactivity in the supernatants and the precipitates was counted in a gamma counter. For evaluation of the maximum binding capacity, 100 pmol of Mab 3-9 were incubated with increasing amounts of Ga-HBED-CC (12.5-1000 pmol). Because the Ga-chelate binding to each of the Mabs was always incomplete, additional mixtures of two Mabs were used for precipitation (5 pmol of chelate, 50 pmol of each Mab). Furthermore, a column containing Mab 3-9 immobilized on sepharose was employed to separate the Ga-chelate in fractions with and without affinity to the Mab. The Ga-chelate fraction with no affinity to Mab 3-9 passing the column without retention was investigated for binding to Mabs 8-16 and 3A10 (5 pmol of chelate, 100 pmol of Mab).

The affinity constants of the chelate with Mabs 3-9 and 3A10 were determined using 1 pmol of Mab and increasing amounts of chelate (0.5-10.5 pmol). Data were plotted in a diagram B/F versus B and subjected to the Scatchard analysis (*19*). Competition of In-HBED-CC, Fe-HBED-CC and the free ligand HBED-CC with the Ga-chelate for antigen binding sites of Mabs 3-9 and 3A10 was measured with 1 pmol of Mab, 0.5 pmol of ^{67}Ga-HBED-CC and increasing amounts of the inactive competitors (0.5-500 pmol). Incubations with the metal chelates were carried out in a final volume of 1.0 ml; with use of the free ligand, the volume was reduced to 0.1 ml.

Circular Dichroism

The Ga-HBED-CC fractions eluted from a Mab 3-9 and Mab-3A10 column were investigated for the possibility of both fractions being enantiomers of a racemic mixture. Because circular dichroism is the most sensitive method for determination of molecular chirality (*20*), circular dichroism spectra were measured on a Jasco J-500 spectralpolarimeter coupled to a J-DPY data processor. Slit width was automatically maintained at 1 nm. Curves were recorded digitally and sent directly through the data processor for signal averaging and baseline subtraction.

Spectra were recorded at room temperature (ca. 22°C) in 0.01 *M* PBS at Ga-HBED-CC concentrations ranging from 5 to 15 μmol. A 1.0-cm quartz cuvette was used; samples were measured from 220 to 320 nm at a scan speed of 10 nm/min, a sensitivity of 1.0 mdeg/cm and a time constant of 1.0 sec. Spectra shown are the result of four repetitive scans with an equally signal averaged solvent base line subtracted. Spectra were adjusted to a 10-μmol Ga-HBED-CC concentration.

Animal Experiments

Biodistribution of ^{67}Ga-HBED-CC was measured in tumor-bearing mice. Eight-week-old athymic mice (CD-1 nu/nu) were inoculated subcutaneously with 5×10^6 MML-1 tumor cells derived from a human melanoma. Four weeks later, tumors had reached an average weight of 0.52 ± 0.23 g. Two groups of six animals were preinjected into the tail vein with 50 μg (333 pmol) of 3A10 Mab labeled with ^{131}I by the Iodogen method at a specific activity of 1.5 kBq per μg of Mab. Forty-eight hours later, one group of animals was injected intravenously with 45 ng (75 pmol) of the racemic Ga-chelate labeled with ^{67}Ga at a specific activity of 1 kBq per 1 pmol, while the other group received 75 pmol of the enantiomer eluted from a Mab 3-9 affinity column. As a control, a third group of mice, not preinjected with the Mab, received 75 pmol of the racemic Ga-chelate. Ninety minutes after the administration of the chelate animals were anesthetized with ether, bled from the retroorbital plexus and killed by cervical dislocation. Organs were removed, weighed and counted for radioactivity in a Ge well-type detector. For radioactivity excretion estimate, whole-body retention was calculated from the tissues measured, assuming blood, bone and muscle to be 6%, 10% and 40% of body weight, respectively.

RESULTS

Chemistry

The chemical structure of HBED-CC (Fig. 1) was fully confirmed by ^{1}H-NMR spectroscopy (Table 1). Chelation of Ga^{3+} by HBED-CC in an aqueous solution resulted in a stoichiometric 1:1 complex formation. Completeness of the Ga^{3+} chelation was checked by paper chromatography. Typically, more than 99.5% of the radioactivity present in the ^{67}Ga-HBED-CC preparations migrated with Rf 0.7. Quite similar results were found for the radiolabeled In- and Fe-HBED-CC chelates. No release of ionic Ga^{3+} from Ga-HBED-CC during the EDC-mediated coupling to KLH could be detected.

In-Vitro Binding

The binding of increasing amounts of Ga-HBED-CC to Mab 3-9 is presented in Table 2. With a 5-10-fold molar excess of chelate, the expected bivalency of binding (2 moles of chelate per 1 mole of Mab) was observed. Upon lowering the chelate concentration, however, binding was limited to approximately 50% of the chelate added. By using affinity chromatography with Mab 3-9 immobilized on sepharose, a quite similar result was obtained as with AS-precipitation: 51.4% of chelate appeared in the effluent, while 48.6% was retained on the column. Both the ^{67}Ga fraction, which passed the column, and the ^{67}Ga fraction, which was retained and subsequently desorbed with a 3 *M* NaSCN solution, proved to be tightly complexed as demonstrated by >98% radioactivity migration during paper chromatography.

TABLE 1
Five Hundred Megahertz ^{1}H-NMR Spectroscopy of HBED-CC in DMSO-d_6

Designation*	Shift (ppm)	Coupling constant J
e	2.42 tr 4H	7.7 Hz
d	2.67 tr 4H	7.7 Hz
h	2.75 s 4H	
g	3.27 s 4H	
f	3.69 s 4H	
c	6.65 d 2H	8.2 Hz
a	6.91 d 2H	2.3 Hz
b	6.94 d,d 2H	8.2 Hz;2.3 Hz

* For designation of protons, see Figure 1. s = singlet, d = doublet, and tr = triplet.

TABLE 2
Binding of Increasing Amounts of Ga-HBED-CC to Mab 3-9*

pmol Ga-HBED-CC		
Added	Bound to Mab	%Bound
12.5	6.2	49.5
25.0	12.7	50.7
50.0	25.0	50.0
100.0	48.0	48.0
200.0	94.4	47.2
500.0	188.0	37.6
1000.0	211.1	21.1

* As determined by AS precipitation. Mab = 100 pmol and incubation volume = 1.0 ml.

Extension of the Ga-HBED-CC binding studies Mabs to 8-16 and 3A10 demonstrated that Mab 8-16 precipitated the same fraction of the chelate as did Mab 3-9, while Mab 3A10 displayed a high affinity for the fraction not bound by Mabs 8-16 and 3-9. These results were supported by binding data obtained with the three Mabs and the Ga-HBED-CC fraction present in the effluent of a column containing Mab 3-9 immobilized on sepharose (Table 3). A repetition of the experiment 24 hr later indicated that no racemization of the eluted Ga-chelate fraction had occured.

Circular Dichroism

By measuring circular dichroism, it is possible to differentiate two enantiomers on the basis of their different interactions with left- and right-circularly polarized light. An intrinsically asymmetric chromophore will tend to absorb one form of circularly polarized light preferentially. The difference spectrum, $\Delta\epsilon = \epsilon_L - \epsilon_R$, will show a positive or negative ellipticity associated with the absorption maximum. The sign of such peaks will be reversed for the corresponding enantiomers. Figure 2 shows the spectra of the 50% fractions of Ga-HBED-CC present in the effluent of either a Mab 3-9 or a Mab 3A10 column. Ga-HBED-CC in the 3-9 effluent exhibits a positive ellipticity at 295 nm and a negative ellipticity at 241 nm. The signs of these bands are reversed for Ga-HBED-CC in the 3A10 effluent, but the two wave lengths at which these maxima occur and their molar ellipticity are unchanged, a behavior typical of optical isomers. The diagnosis of Ga-HBED-CC fractions in the 3A10 and 3-9 effluents as enatiomers is further supported by the total lack of ellipticity in the spectrum of untreated, racemic Ga-HBED-CC mixture (data not shown). The chemical structures of the Ga-HBED-CC enantiomers, as described by L'Eplattenier et al. (*21*), are shown in Figure 3.

TABLE 3
Binding of Racemic and Mab 3-9-Sepharose Eluted, Enantiomeric ^{67}Ga-HBED-CC to Different Anti-chelate Antibodies*

Exp. no	Mab	%Precipitated
1‡	3-9	49.0 (45.4–52.9)†
2	8-16	46.5 (44.2–48.2)
3	3A10	46.2 (43.8–48.3)
4	3-9 + 8-16	48.5 (47.0–50.1)
5	3-9 + 3A10	94.2 (92.1–98.3)
6	8-16 + 3A10	94.6 (91.8–96.1)
7¶	3-9	4.9 (3.8–5.6)
8	8-16	1.0 (0.7–1.3)
9	3A10	97.4 (96.2–99.2)
10	3-9	5.0 (4.1–6.2)
11	8-16	1.9 (1.4–2.5)
12	3A10	96.1 (94.1–98.4)

* Determined by AS precipitation. Molar ratio was always 100 pmol Mab to 5 pmol of chelate. Incubation volume is 1.0 ml.

† Mean + range, three determinations.

‡ Exp. 1–6 racemic ^{67}Ga-HBED-CC.

¶ Exp. 7–12 enantiomeric ^{67}Ga-HBED-CC: 7–9 = 2 hr after elution; 10–12 = 24 hr after elution.

Affinity and Competition

The association constant (K_A) for binding of Ga-HBED-CC to Mabs 3-9 and 3A10 were calculated from a Scatchard plot (Fig. 4). B/F data were corrected for the fact that only half of the chelate could virtually bind to the Mabs. Data thus represent the K_A for the enantiomer which could bind to the Mab.

Competition of inactive Ga-HBED-CC, In-HBED-CC, Fe-HBED-CC and the unchelated ligand with 0.5 pmol of ^{67}Ga-HBED-CC for binding to 1 pmol of Mab 3A10 is presented in Figure 5. To decrease the ^{67}Ga-HBED-CC binding to one-half of its initial value, 2.5, 3.0 and 200

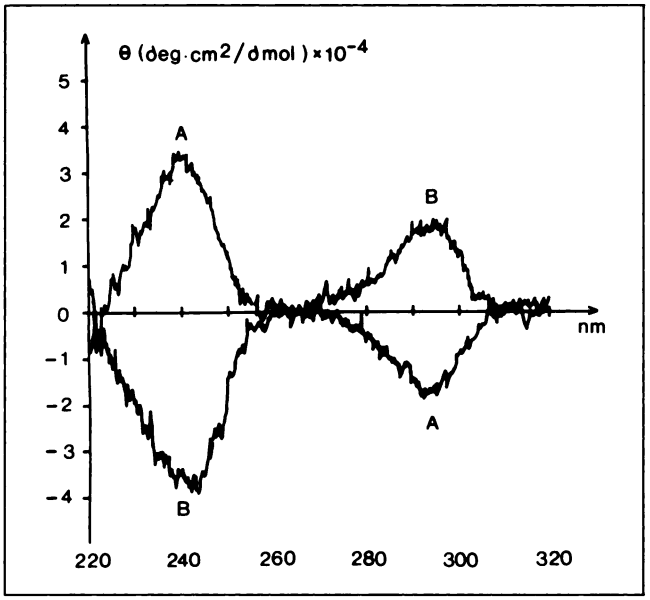

FIGURE 2. Circular dichroism spectra of Ga-HBED-CC present in the 0.01 *M* PBS effluent of Mab-columns. (A) Mab 3A10 immobilized on sepharose and (B) Mab 3-9 immobilized on sepharose. Spectra are adjusted to a 10 μmol solution of Ga-HBED-CC.

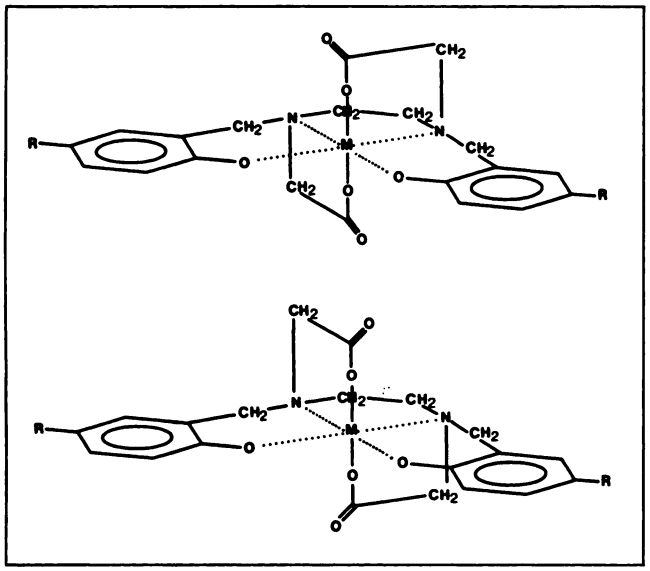

FIGURE 3. Enantiomers of the Ga-HBED-CC complex according to the chemical structure of HBED metal chelates described by L'Eplattenier (*21*). R=-CH_2-CH_2COOH.

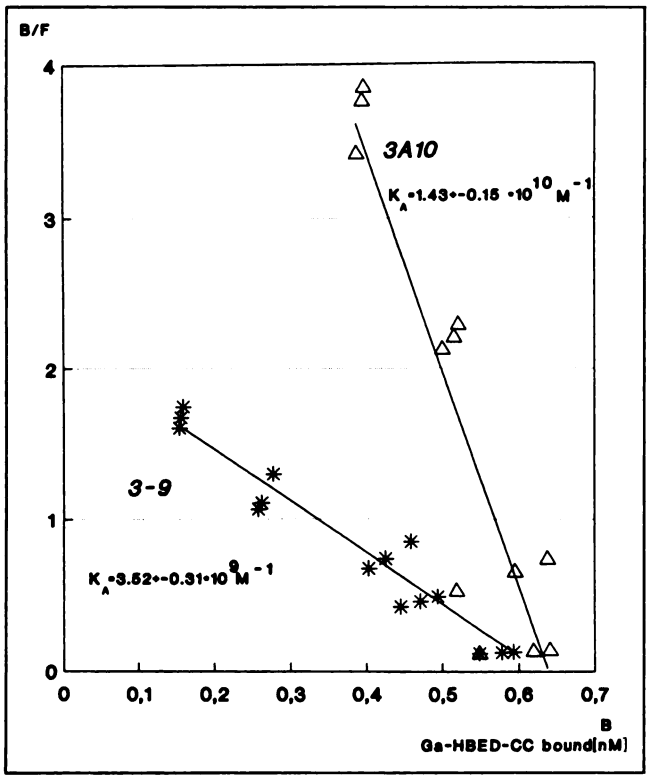

FIGURE 4. Scatchard plot of ^{67}Ga-HBED-CC binding to 3-9- and 3A10 anti-chelate antibodies as determined with AS precipitation. One picomole of Mab was incubated with 0.5-10.5 pmol of the chelate in a final volume of 1.0 ml. B/F data are corrected for the fact that only half of the chelate could actually bind to the Mabs. B = bound Ga-HBED-CC (precipitate) and F = free Ga-HBED-CC (supernatant). Incubations were done in triplicate. Non-specific binding to carrier IgG and BSA is 2.5%.

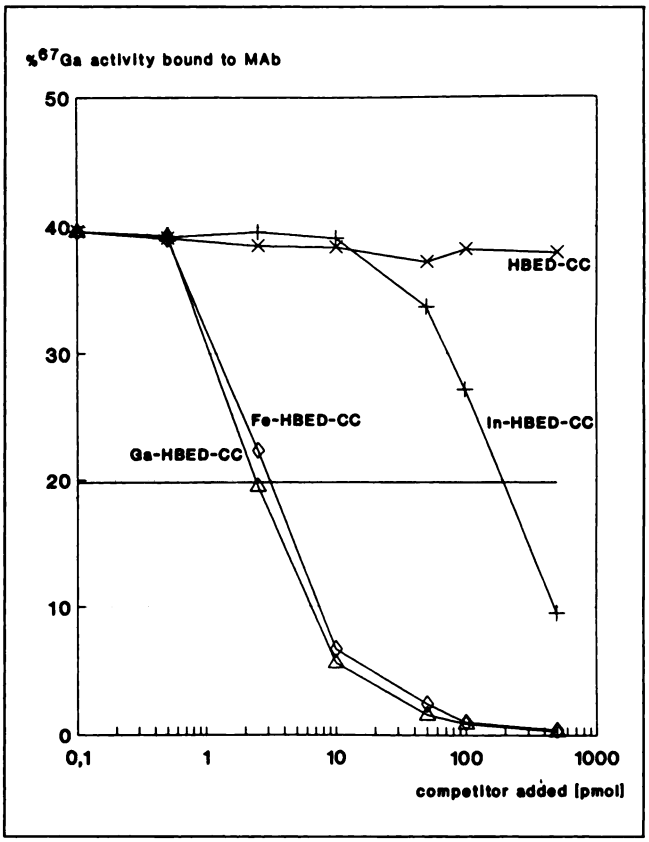

FIGURE 5. Competition of 0.5-500 pmol of Ga-, Fe-, In-HBED-CC and HBED-CC with ^{67}Ga-HBED-CC for Mab 3A10 antigen binding sites as determined with AS-precipitation. Incubations were done with 1 pmol of Mab and 0.5 pmol of ^{67}Ga-HBED-CC in a final volume of 1.0 ml using the metal chelates and 0.1 ml using the free ligand. Data are means from three determinations.

pmol of the Ga-, Fe- and In-chelate, respectively, were necessary, indicating a K_A for Fe-HBED-CC not significantly different from that of Ga-HBED-CC and for In-HBED-CC of $1.8 \times 10^8 M^{-1}$. No competition by the free ligand was noted. In a preceeding experiment, however, when using an incubation volume of 1.0 ml, the free ligand showed a competition between Ga- and In-HBED-CC. Chelation of very small traces of metal impurities, especially of Fe^{3+} which might still be present in the high purity PBS buffer used for dilution, was assumed to be the source of competition. A reduction in incubation volume to 0.1 ml or an increase in the Mab concentration (data not shown) confirmed this assumption. Competition of the In- and Fe-chelate with the Ga-chelate for Mab 3-9 binding sites was quite similar to that observed for Mab 3A10 (data not shown).

Animal Experiments

Preinjection of Mab 3A10 drastically altered the biodistribution pattern of the racemic ^{67}Ga-HBED-CC and increased whole-body retention of ^{67}Ga by a factor of 15 in comparison to animals not preinjected with the Mab (Table 4). Administration of the corresponding enan-

TABLE 4
Biodistribution of ^{131}I-Labeled 3A10 Anti-chelate Mab in Athymic Mice and of Racemic or Enaniomeric ^{67}Ga-HBED-CC in Mice Preinjected or Not Preinjected with 3A10

	^{67}Ga-HBED-CC			^{131}I-3A10
	Racemic	Racemic	Enantiomeric	
	Nonpreinjected	Preinjected		
Blood	0.11 ± 0.02*	10.17 ± 1.63*	24.45 ± 0.80¶	10.11 ± 1.04†
Liver	0.20 ± 0.11	1.90 ± 0.37	4.65 ± 0.64¶	2.03 ± 0.56
Spleen	0.04 ± 0.01	1.60 ± 0.37	3.58 ± 0.38¶	1.88 ± 0.50
Kidney	0.42 ± 0.13	2.53 ± 0.79	4.81 ± 0.56¶	2.03 ± 0.16
Muscle	0.04 ± 0.02	0.75 ± 0.21	1.00 ± 0.25	1.05 ± 0.22
Bone	0.06 ± 0.01	1.07 ± 0.46	2.12 ± 0.39¶	0.93 ± 0.14
Lung	0.15 ± 0.04	4.23 ± 1.20	9.23 ± 1.60¶	4.24 ± 0.76
Tumor	0.13 ± 0.06	1.82 ± 0.27	3.91 ± 0.71¶	3.01 ± 0.47
Whole-body retention‡	2.1 ± 0.8	31.3 ± 2.8	70.2 ± 3.1¶	43.5 ± 6.2

* % ID/g, mean ± 1 s.d. (n = 6), 90 min p.i.
† % ID/g, mean ± 1 s.d. (n = 12), 48 hr p.i.
‡ % ID mean ± 1 s.d., estimated from the organs measured assuming blood, bone and muscle to be 6%, 10% and 40% of body weight, respectively. Body weight of animals 28.1 ± 3.3 g, tumor weight 0.53 ± 0.23 g (n = 18).
¶ Significantly different from racemic Ga-HBED-CC, $p < 0.01$ (t-test).

tiomer of the ^{67}Ga-chelate increased ^{67}Ga retention by an additional factor of two, as expected from the 50% and 100% in-vitro binding to Mabs of the racemic and enantiomeric Ga-chelate, respectively. By comparing the biodistributions of the enantiomeric ^{67}Ga-chelate and the preinjected, nontumor-specific ^{131}I-labeled Mab 3A10, it is evident that the fraction of Ga-chelate taken up in most tissues is increased by a factor ranging from 1.9 to 2.4 relative to the fraction of Mab present. This high ^{67}Ga uptake demonstrates a similar quality of Mab to chelate binding in vitro and in vivo. The molar ratio of Mab binding sites present per animal at the time of chelate injection to chelate administered was close to 4:1 when calculated from the whole-body retention of ^{131}I 48 hr p.i., assuming all of the ^{131}I to be present in the form of intact labeled Mab. An increase in the Mab-to-chelate molar ratio, e.g., by reducing the chelate dose, should additionally increase the chelate uptake. At the same molar ratio but with markedly decreased amounts of Mab and chelate, a reduced ^{67}Ga uptake should be expected. No such marked increase in ^{67}Ga uptake was found in muscle and tumor. This appears to be due to a preferred trapping of the chelate by Mabs present in the blood and a lower perfusion of muscle and the melanoma xenograft. An increased tumor uptake of the chelate should, however, be obtained by a blockage of the chelate binding sites of the Mab still present in the circulation, as discussed earlier and as already demonstrated by Goodwin et al. (*8*).

DISCUSSION

Since the first report on an anti-In-benzyl-EDTA Mab for use in pretargeted immunoscintigraphy by Reardan et al. (*7*), an increasing number of anti-metal chelate Mabs have been established. Mabs described so far were directed against In-benzyl-EDTA (*7*), Co^{3+}-benzyl-EDTA (*8*), free DTPA (*22*) and In-DTPA (*9*). Binding of Mabs to the corresponding chelate ranged from 6×10^8 to 4×10^9 M^{-1}. The fine specificity of Mabs recognized the central metal ion of the chelate used for immunization and/or the chemical linker used for coupling of the chelate to the immunizing carrier protein. By changing to another metal ion in the chelate and/or a lack of the linker in the chelate-linker-hapten, there were markedly decreased K_A's.

None of the Mabs, however, was described as discriminating between the enantiomers of the metal chelates used for immunization. This is not readily understood since chirality at the metal ions is an inherent property of metal chelates containing multidentate ligands (*23*) and the recognition of chirality of amino acids or other organic compounds is a common feature of antibody-antigen binding. An example with interesting implications for organic synthesis, recently published by Lerner et al. (*24*), is the production of Mabs with lipase activity, showing a high enantioselectivity for the R- or S-form of the alcohol moiety in an ester. There might be two reasons for not realizing that anti-chelate Mabs are able to recognize enantiomers:

1. The stereochemical rigidity of the In-chelates used is too low, resulting in a rapid rearrangement of enantiomers into each other and allowing only antigen binding sites to be raised against the racemic form of the chelate. However, this is not valid for the Co^{3+}-EDTA, which is highly rigid. Separation of its enantiomers by fractional crystallization and gel chromatography has been reported (*25*).
2. The metal chelates of the aliphatic polyaminocarbox-

ylic acids EDTA and DTPA are not of high immunogenicity and binding sites of the Mabs are not mainly focused on the chelate itself, but rather to the linkers used for coupling of the chelate to the immunizing carrier protein. Such an influence of the linker group on binding affinity was found for the anti-In- and Co-benzyl-EDTA Mabs (*8*) and the anti-DTPA Mabs (*22*), but not for the anti-In-DTPA-Mabs (*9*).

In contrast, all Mabs raised against the Ga-HBED-CC chelate showed a specificity exclusively for one of the enantiomers, and K_A's up to $1.4 \times 10^{10} M^{-1}$ were obtained with the chelate that lacks the linker used for immunization. The application of pure enantiomers, easily prepared by affinity chromatography as described above, at the nanomole level led to a nearly 100% in-vitro binding of the chelate to Mabs and to a 70% in-vivo binding in animals preinjected with the Mab. The high affinity of the chelate-to-Mab binding and the rapid urinary excretion of the unbound chelate (biological half-life ~20 min), which enables the use of the short-lived positron-emitter ^{68}Ga (physical half-life 68.3 min), meets the requirements for introducing PET in pretargeted immunoscintigraphy.

ACKNOWLEDGMENT

Supported by a grant from the Tumor-Center Heidelberg-Mannheim.

REFERENCES

1. Halpern SE, Dillman RD, Witztum KF, et al. Radioimmunodetection of melanoma utilizing ^{111}In-96.5 monoclonal antibody: a preliminary report. *Radiology* 1985;155:493–499.
2. Siccardi AG, Buraggi GL, Callegro L, et al. Multicenter study of immunoscintigraphy with radiolabeled monoclonal antibodies in patients with melanoma. *Cancer Res* 1986;46:4817–4822.
3. Lamki LM, Patt YZ, Murray JL. In-111-monoclonal antibody immunoscintigraphy of colorectal cancer. In: Goldenberg DM, ed. *Cancer imaging with radiolabeled antibodies.* Boston/Dordrecht/London: Kluwer Academic; 1990;293–312.
4. Bound JP, Woodbury RG, Hart CE, Hellström I, Hellström KE. Quantitative analysis of melanoma associated antigen P97 in normal and neoplastic tissues. *Proc Natl Acad Sci USA* 1981;78:39–53.
5. Beatty JD, Beatty BG, O'Conner-Tressel M, Do T, Paxton RJ. Mechanisms of tissue uptake and metabolism of radiolabeled antibody. Role of antigen-antibody complex formation. *Cancer Res* 1990; 50 (suppl): 840–845.
6. Jain RK. Physiological barriers to delivery of monoclonal antibodies and other macromolecules in tumors. *Cancer Res* 1990;50(suppl):814–819.
7. Reardan RT, Meares CF, Goodwin DA, et al. Antibodies against metal chelates. *Nature* 1985;316:265–268.
8. Goodwin DA, Meares CF, McCall MJ, McTigue M, Chaovapong W. Pretargeted immunoscintigraphy of murine tumors with ^{111}In-labeled bifunctional haptens. *J Nucl Med* 1988;29:226–234.
9. Le Doussal JM, Gruaz-Guyon A, Martin M, Gautherot E, Delaage M, Barbet J. Targeting of ^{111}In labeled bivalent haptens to human melanoma mediated by bispecific monoclonal antibody conjugates: imaging of tumors hosted in nude mice. *Cancer Res* 1990;50:3445–3452.
10. Stickney DR, Slater JB, Kirk GA, Merchant B, Schweighardt SA, Frincke JM. A bifunctional antibody delivery systems: clinical studies demonstrate imaging and therapeutical potential [Abstract]. *J Nucl Med* 1990;31:734.
11. Taliaferro CH, Motekaitis RJ, Martell EA. New multidentate ligands. 2,2N,N′ dipyridoxylethylenediamine N,N′ diacetic acid. A new chelating ligand for trivalent metal ions. *Inorg Chem* 1984;23:1188–1192.
12. Kroll H, Simonian J, Knell H. Aromatic 1,2 alkylene or 1,2 cycloalkylene diamine N,N′ diacetic acids. German Patent 1072252; 1959.
13. Köhler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specifity. *Nature* 1975;256:495–497.
14. Matzku S, Brüggen J, Bröcker EB, Sorg C. Criteria for selecting monoclonal antibodies with respect to accumulation in melanoma tissue. *Cancer Immunol Immunother* 1987;24:151–157.
15. Ey PL, Prowse SJ, Jenkin CR. Isolation of pure IgG1, IgG2a and IgG2b immunoglobulines from mouse serum using protein-A sepharose. *Immuno Chem* 1978;15:429–436.
16. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:660–685.
17. Pharmacia, Uppsala Sweden. Affinity chromatography. Principles and methods. 1979.
18. Hudson L, Hay FC. *Practical immunology*, 3rd edition. Oxford, Blackwell Scientific Publications; 1989.
19. Scatchard G. The attraction of proteins for small molecules and ions. *Ann NY Acad Sci* 1949;51:660–672.
20. Reed J. Circular dichroism as a tool for investigating macromolecular structure. In: Tschesche H, ed. *Modern methods in protein and nucleic acid research.* Berlin, New York: Walter de Gruyter & Co; 1990:367–394.
21. L'Eplattenier F, Murase I, Martell EA. New multidentate ligands. VI. Chelating tendencies of N,N′ di(2-hydroxybenzyl) ethylenediamine N,N′diacetic acid. *J Am Chem Soc* 1967;89:837–893.
22. Gillette RW, Singleton J, Janowicz A, Gilman SC. Development and characterization of monoclonal antibodies with specificity for metallic radioisotope chelators linked to antibodies and other proteins. *J Immunol Meth* 1989;124:277–282.
23. Harrowfield JM, Wild SB. Isomerism in coordination chemistry. In: Wilkinson G, Gillard RD, McCleverty JA, eds. *Comprehensive coordination chemistry, volume 1.* Oxford, New York: Pergamon Press; 1987:179–212.
24. Janda KD, Benkovic SJ, Lerner RA. Catalytic antibodies with lipase activity and R or S substrate selectivity. *Science* 1989;244:437–440.
25. Strasak M, Bystricky S. Rapid optical resolution of anionic metal complexes by gel permeation chromatography. *J Chrom* 1988;450:284–290.