Pre-Targeted Immunoscintigraphy of Murine Tumors with Indium-111-Labeled Bifunctional Haptens

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A method of tumor imaging utilizing the nonspecific accumulation of antibody through leaky capillaries is described, in which the antibody and the radiolabel are administered separately. Nonradioactive antibody is given first (pre-targeted), and allowed adequate time to reach maximum tumor concentration. Depending on the antibody, this may take several days. At the time of maximum tumor concentration of nonradioactive antibody, the blood is quickly cleared of excess circulating nonradioactive antibody using a special i.v. "chase". The radiolabel then is given and imaging done in 1 to 3 hr. The use of short lived tracers (hours) to image antibodies that localize slowly (days) in-vivo is made possible by this method.

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That tumors can accumulate globulins and other macromolecules nonspecifically was first reported by Duran-Reynolds in 1939 (*1*). This phenomenon has already been used effectively in brain tumor imaging with iodine-131- (^{131}I) labeled human serum albumin (*2*). The success of this method of brain tumor visualization depended on the intact blood-brain barrier excluding the protein from normal surrounding brain. The absence of such a barrier in other tissues has made it difficult to image tumors with nonspecific proteins elsewhere in the body since tumor concentrations (although high) did not exceed the background.

Because of this large nonspecific component in tumor uptake of antibodies, Pressman et al. (*3*) developed a paired label technique in which specific anti-tumor antibody was labeled with one isotope and normal control globulin labeled with another. The two were mixed and injected into tumor bearing animals. In this way it was possible to determine how much of the uptake was due to the specificity of the antibody and how much was due to nonspecific factors.

In spite of the advent of monoclonal antibodies and improvements in labeling techniques, high background, especially in the blood, has remained a difficult problem in tumor imaging. Although maximum target concentrations may be achieved in one day, most investigators suggest waiting for an extended period to achieve acceptable target to background ratios (*4*).

When indium-111 chelate conjugates are used, high liver background is an additional problem (*5,6*). This prolonged retention of radioactivity may lower the target to background ratios for a lesion situated near the liver and may also increase the radiation dose to the patient.

Noncovalent labeling of antibodies with radioactive haptens provides a new approach to background reduction (*7,8*). Because of the reversibility of these complexes, it is possible to eliminate blood background shortly before imaging. This is done by administering a nonradioactive hapten chase intravenously 1–3 hr prior to the optimum imaging time. The chase immediately displaces the noncovalently bound radioactive hapten which then is cleared rapidly from the blood by the kidneys. In this approach (as with covalently labeled antibodies) the antibody and label are administered together and therefore the method is unsuited to short-lived radionuclides such as ^{99m}Tc or gallium-68 (^{68}Ga).

In the present study we report a novel method in which antibody and label are administered separately. The nonradioactive antibody is given first and allowed to localize in the tumor (pre-targeted), followed by

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administration of the radiolabel a few hours prior to imaging (*9*). With this technique short-lived radionuclides (hours) could be used for imaging antibodies that may require several days to localize.

MATERIALS AND METHODS

Chemistry

General: thin layer chromatography. Thin layer chromatography (TLC) was done using plastic backed silica gel plates (EM Science #5735 EM Science, Cherry Hill, NJ) and 10% (w/v) aqueous ammonium acetate-methanol (1:1) as the solvent phase.

HPLC. High performance liquid chromatography (HPLC) was performed using two Waters (Waters Chromatog. Div., Millipore Corp., Milliford, MA) 45 pumps controlled by a Waters 660 Solvent Programmer with an ISCO UA-5 detector ISCO, Lincoln, NE) at wavelengths of 280 nm (bleomycin compounds and proteins) and 254 nm (chelates). Column and gradient conditions were as follows:

A. C_{18} 10 × 250 mm column (Alltech Assoc., Inc., Deerfield, IL), 20 min linear gradient, aqueous 1% sodium acetate (pH 7), 1 m*M* EDTA, to 100% methanol.

B. Alltech C_{18} 10 × 250 mm column, 30 min linear gradient, 0.1 M ammonium acetate, pH 7: acetonitrile from 15%:85% to 50%:50%.

C. TSK-DEAE-5-PW 7.5 × 75 mm column (Biogel, Bio-Rad Labs, Richmond, CA), 20 min concave (#8) gradient, water to 2.0 *M* ammonium acetate, pH 5.5.

Spot tests. In the synthesis procedures which follow, various spot tests were used to determine the presence of certain functional groups. These tests are summarized below:

Fluorescamine tests: (*10*) Used to detect the presence of primary amine groups.

4-(p-Nitrobenzyl)pyridine (NBP) test: (*11*) Used to detect the presence of alkylating groups; in the cases below: $BrCH_2C(O)NH$-R.

5,5′-Dithiobis(2-nitrobenzoic acid) (Ellman's Reagent, DTNB): (*12*) Used to detect the presence of thiol groups.

KLH-2IT-BABE-Co(III): BABE-Co(III). To 0.6 mg (1.14 μmol) of p-bromoacetamidobenzyl-EDTA (BABE) (*13*) was added 65 μl of a 17.9 m*M* $CoCl_2$ solution (1.16 μmol Co, 2% excess). 30% Hydrogen peroxide (132 μl) was added and the pH was adjusted to 7 with sodium bicarbonate. The solution's color changed from deep blue to light reddish-purple on standing for 2 hr. The solution was lyophilized and the residue was used without further purification.

KLH-2IT-BABE-Co(III). To a solution of 33 mg of Keyhole Limpet Hemocyanin (KLH) in 650 μl of 0.15*M* sodium phosphate, pH 8, was added 150 μl of freshly prepared 27 m*M* 2-iminothiolaine solution (2IT, Traut's Reagent (*14*)) in 50 m*M* triethanolamine-HCl, pH 8.5, followed by 10 μl of 2-mercaptoethanol. The resulting solution was incubated at 4° for ~1 hr. Spin column chromatography (*15*) on 4 × 150 μl aliquots of the reaction solution removed the unreacted 2IT as well as other small molecules, and also changed the buffer containing the KLH-2IT conjugate to 0.1*M* sodium phosphate, pH 8. Each spin column receiver vial contained 25 μl of 11.4 m*M* aqueous BABE-Co(III) solution. The spin column effluents containing the BABE-Co(III) solutions were pooled, flushed with argon, sealed, and incubated at 4° for ~24 hr. Iodoacetamide (1 mg) was added to the KLH-conjugate solution to alkylate any remaining thiol groups and the reaction solution again was subjected to spin column chromatography (column buffer: 0.1*M* sodium phosphate, pH 8) to separate the KLH-2IT-BABE-Co(III) conjugate from unreacted small molecules.

Human Transferrin-2IT-BABE-Co(II). Transferrin-2IT-BABE-Co(II) was prepared by essentially the same method as for KLH-2IT-BABE-Co(III) above except that a metal free BABE solution was used in lieu of a BABE-Co(III) solution for the alkylation of Transferrin-2IT. The product was isolated by spin column chromatography (column buffer: 0.1*M* ammonium citrate, pH 5.5). Metal binding uv and analyses were conducted to determine the concentrations of protein and bound chelates in the product solution. A chelate to protein ratio of slightly more than 4:1 was achieved. A slight excess (1–2%) of cobalt (as a cobalt acetate solution) was added. The reaction solution was allowed to stand at room temperature for 15 min, 10 μl of a 10 m*M* EDTA · Na_2 solution was added to sequester free cobalt, and the resulting solution was passed through a spin column (column buffer: 0.1M sodium phosphate, pH 8) to yield the final product, Transferrin2-IT-BABE-Co(II).

1,4-DT-BABE. To a solution of 1.0 ml 1,4-butanedithiol and 1.0 ml triethylamine in 25 ml of methanol was added 19 mg of BABE (solid). The solution was stirred under nitrogen for 4.5 hr at which time the solution was negative to NBP. The methanol was removed by evaporation under a stream of nitrogen and the residue was dissolved in 25 ml of water. The aqueous solution was extracted with chloroform (3 × 5 ml) and ether (3 × 5 ml) and the aqueous layer was lyophilized. The lyophilization residue was dissolved in 1 ml of water and applied to a SEP-PAK C_{18} cartridge (Waters Chromatog. Div., Millipore Corp., Millford, MA) which had been activated with methanol and then equilibrated in water. The SEP-PAK was washed successively with water (3 × 1 ml), 25% aqueous methanol (3 × 1 ml) and 50% aqueous methanol (3 × 1 ml) while 1 ml fractions of each wash were collected. All fractions were tested with DTNB and those testing positive (which eluted with 25% aqueous methanol) were further examined by reverse phase HPLC (Condition A). Those fractions composed primarily of a product eluting at ~21 min retention time were pooled and lyphilized. Thin layer chromatography of the residue gave a single spot at Rf 0.9, which was DTNB positive and also bound metal as determined by ^{57}Co assay. The product was ~90% pure by HPLC. Yield: 16 mg (78%).

BLEDTA IV: Cobalt(III) blenoxane-1,4-butanedithiol (Co(III)BLM-1,4-DT). To a nitrogen flushed, stirred solution of 853 mg Co(III)BLM (*16*) in 20 ml of methanol was added 8 ml of 1,4-butanedithiol and 1.2 ml of triethylamine, and the reaction solution was stirred for 20 min at room temperature. The precipitate which formed was removed by centrifugation and the supernate was lyophilized to a brown oily residue. Water (~25 ml) was added to the residue and the resulting mixture was extracted with ether (3 × 15 ml). Thin layer chromatography of the aqueous layer gave three spots at Rf 0.35, 0.65, and 0.7, corresponding to the A_2, B_2 and A_2DM forms of Co(III)BLM. All three spots also gave a positive DTNB test indicating that a thiol containing "spacer" had been attached to the BLM. Reverse phase HPLC (Condition B) showed three major groups of peaks at retention times of

13, 18, and 23 min. The first group exhibited the same TLC and HPLC characteristics of the starting material, and lacked any thiol groups as evidenced by a negative DTNB test. On this basis, the first group was identified as the starting material, unreacted Co(III)BLM. The second group, eluting at 18 min, gave the same TLC characteristics as Co(III)BLM-A_2/-B_2; however, the TLC spots also gave a positive DTNB test. These factors indicated that the second group was a mixture of the Co(III)-BLM-A_2/-B_2 forms, each possessing a thiol containing spacer (Co(III)-A_2/-B_2-1,4-DT). The same criteria (TLC and DTNB) were used to suggest that the third product was the Co(III)BLM-A_2DM-1,4-DT. The second group was separated by HPLC and lyophilized prior to coupling with BABE. Yield from Co(III)BLM) to Co(III)BLM-A_2/-B_2-1,4-DT was 26% as determined by uv absorbance at 290 nm.

Co(III)BLM-1,4-DT-BABE (BLEDTA IV). To 8 ml of a 3.4 ml solution of Co(III)BLM-A_2/-B_2-1,4-DT (27.2 μmol) in 0.15*M* sodium phosphate, pH 8, was added 17 mg of BABE (33 μmol). The solution was flushed with argon, capped and stirred at room temperature in the dark for 3.5 hr, until a DTNB test indicated complete reaction of the thiol groups. The desired product, BLEDTA IV, was isolated from the reaction mixture by anion exchange HPLC (Condition C) followed by reverse phase HPLC (Condition B). The final material was analyzed by uv spectroscopy, thin layer chromatography, and ^{57}Co metal binding assay. Yield: 0.7 μmol (3%). Cobalt-57 metal binding analysis on the final product indicated that it was contaminated by DT-BABE (possibly from decomposition of the BLEDTA IV) to the extent of 13% of the total chelating groups. The product was used without further purification.

Lysine-2IT-BABE. To a solution of 508 mg (2.78 mmol) of lysine-HCl in 20 ml of 50 m*M* triethanolamine·HCl, pH 7.5, was added 283 mg (2.07 mmol) of 2-iminothiolane. The reaction flask was flushed with argon, capped and incubated at 4° for 3.5 hr. The solution was extracted with ether (3 × 10 ml), and the aqueous layer was lyophilized after removal of the last traces of ether under reduced pressure. An aqueous solution of the residue (110 mg/ml) was prepared and added in 100-μl aliquots to a solution of 38 mg BABE in 800 μl of 0.1*M* sodium phosphate, pH 6. The course of the reaction was followed using DTNB and NBP tests on the reaction mixture. A new aliquot of lysine-2IT was added each time a DTNB test was negative (indicating consumption of the thiol groups) until the NBP test was also negative (indicating consumption of all BABE). The reaction solution was applied to a SEP-PAK C_{18} cartridge (Waters Chromatog. Div., Millipore Corp., Millford, MA) which had been equilibrated in water. The SEP-PAK was washed with water (8 × 0.5 ml) while 0.5-ml fractions were collected. A fluorescamine positive product eluted in the third and fourth fractions. HPLC (Condition A) of these pooled fractions showed a major product (~90%) eluting at ~16 min retention time and a minor product (~10%) eluting at ~7 min. The pooled material was lyophilized and used without further purification. The crude material gave a single spot at Rf 0.85 identified by uv quench. The spot was positive to fluorescamine and DTNB, and also bound ^{57}Co.

Monoclonal Antibodies

Using standard hybridoma technology with the antigen KLH-2IT-BABE-Co(III) (Fig. 1B) we prepared antibodies which bound to a 1,4 dithiol spacer group coupled covalently to BABE-Co(III) (Fig. 1C,1D) (*17*). BALB/c mice were immunized intraperitoneally with 10 μg of the above KLH conjugate in complete Freunds adjuvant. Two weeks later this was repeated in incomplete Freunds adjuvant followed in a further 2 wk with an i.v. booster dose of the conjugate alone. The mice were then screened in 1 wk by measuring the 24-hr retention by whole body counting using a dual probe scintillation counter (Picker Medical, San Francisco, CA) of i.v. Indium-111 BLEDTA IV, bleomycin conjugate containing a 1,4 DT space (Fig. 1D). The mice with >50% retention (compared with <1% in control nonimmunized mice) were hyperimmunized with 30 μg i.v. conjugate and 3 days later their spleen cells were fused with PX63 AG8 myeloma cells. Hybridomas were selected in HAT medium. Clones obtained were screened by ELISA using human transferrin-2IT-BABE-In(III) (Fig. 1B) as the solid phase antigen coated on microtiter plates. Positive clones were recloned by limiting dilution and three high titer secretors, WC3A11 (IgG3), WC4B7 (IgG2a), and WC3F5 (IgG2a) were selected. These hybridomas were grown in ascites fluid and purified by ammonium sulfate precipitation twice. Protein concentration was determined both by Lowry's method (*18*), and by 280 nm absorbance. The antibody concentration was ~80% of the total protein as judged by gel electrophoresis. The relative binding affinity (Kb = Ka = 1/Kd) of the three monoclonals for four bifunctional haptens was measured in duplicate in vitro with an indirect ELISA assay (*19*).

Biologic half-life

The complex of WC3A11 and ^{111}In-1 labeled bifunctional hapten made by mixing a molar excess of antibody with hapten was given i.v. to normal BALB/c mice and the half-life disappearance measured by whole-body counting in a dual probe scintillation system. In five experiments, 50 μg (0.33 nmol), 100, 200, 600, and 800 μg of WC3A11 was used with 30 μCi (0.1 nmol) of ^{111}In bifunctional hapten. Four radiolabeled chelates not containing the spacer; [^{111}In]EDTA, [^{111}In]LBEDTA(L-benzyl EDTA), [^{111}In]BLEDTA II (*13*), and ^{57}Co-labeled bleomycin, as well as two bifunctional chelates containing the spacer; 1,4-DT-BABE and BLEDTA IV labeled with either ^{111}In or ^{57}Co were studied.

Competitive inhibition of binding (chase) in vivo was studied in animals with circulating radiolabeled complex by injecting a large excess (~1.4 μmol) of nonradioactive chase IV and observing the change in half-life (*20*). Chase compounds not containing spacer; Ca Na_2EDTA, EDTA-Co(II), LBEDTA(Ca), LBEDTA-Co(II) as well as those containing spacer; 1,4-DT-BABE-Co(III) (Fig. 1C), and BABE covalently coupled through spacer to human transferrin (Fig. 1B) were studied. Covalent chase compounds were synthesized to contain three to four molecules of hapten per molecule of protein in order to allow crosslinking and lattice formation to occur in vivo.

Organ Distribution

The dose response with increasing amounts of chase was studied in normal BALB/c mice (n = 3 in each group) with ^{125}I-labeled WC3A11. Organ distribution was determined 1 hr after various amounts of chase in animals injected 21 hr previously with ^{125}I antibody.

Tumor and organ assay was done 24 hr after injection of WC3A11 in BALB/c mice (n = 3) with KHJJ tumors (*21*).

The i.v. antibody injection was followed in 20 hr by covalent chase i.v., and in ^{111}In-labeled hapten i.v. 1 hr later (at 21 hr postantibody). The animals were killed 3 hr after administration of tracer (at 24 hr post-antibody), and concentrations determined in blood, heart, lungs, liver, spleen, kidneys, tumor, muscle, bone + marrow (femur), skin, and gut. In control experiments either the chase or the pre-targeted antibody was omitted.

Animal Imaging

Whole-body pinhole gamma camera images of BALB/c mice with KHJJ tumors in the flank were made immediately and 3 hr after i.v. injection of 100–500 μCi (~1.0 nmol ^{111}In) bifunctional hapten. The mice were sedated with chloral hydrate and 200k count gamma camera images obtained with the pinhole 3 in. above the dorsum of the animal. The percent remaining was calculated from the decay corrected counts expressed as a fraction of the counts obtained immediately after injection.

Pre-targeted animals were prepared by injecting 50 μg of WC3A11 IV. This was followed in 20 hr by i.v. transferrin-2IT-BABE-Co(II) chase. One hour was allowed for the chase to clear the blood of antibody before injecting the ^{111}In bifunctional hapten. In control experiments either the chase or the pre-targeted antibody was omitted. Images were then made in the same way 3 hr after ^{111}In bifunctional hapten injection.

RESULTS

In Vitro

The relative affinities of the three monoclonal antibodies are shown in Table 1. Four different haptens were tested to observe the effect of small changes in structure. The highest affinity was shown for the hapten with a structure similar to that of the antigen; 1,4-DT-BABE-Co(III); much lower affinity was observed when the metal ion was changed to indium. The 1,4-DT spacer (Fig. 1C, 1D) differs slightly from the 2IT spacer (Fig. 1B). Using the original antigen 2IT spacer attached to lysine, to mimic the attachment of the hapten to the protein antigen (KLH) increased the affinity significantly. Indium BLEDTA IV (Fig. 1D), which contains the 1,4-DT spacer had only slightly lower affinity for WC3A11 than the original hapten. The larger size of the bleomycin molecule may have had favorable steric effects. WC3A11 was therefore selected for further in vivo studies.

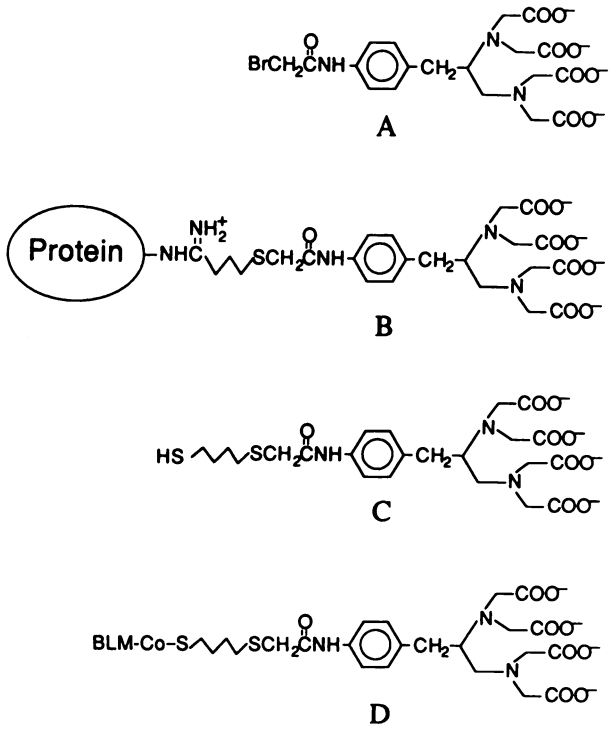

FIGURE 1
Structural formulas for (A) bromoacetamidobenzyl-EDTA (BABE); (B) the conjugate of bromoacetamidobenzyl-EDTA with a protein (e.g., keyhole limpet hemocyanin or human transferrin) that has first been treated with 2-iminothiolane; (C) the product of the reaction between bromoacetamidobenzyl-EDTA and butane-1,4-dithiol; (D) BLEDTA IV.

In Vivo Binding

The amount of [^{111}In] BLEDTA IV retained at 24 hr (an index of the biologic half-life) was dependent on the amount of WC3A11 injected, increasing from 56% with 200 μg to 70% with 800 μg. Less was retained with the same amounts of WC4B7 in keeping with its lower affinity for [^{111}In]BLEDTA IV. Adding lysine to the end of the spacer increased the 24-hr retention with WC3A11 from ~30% with 1,4-DT-BABE to ~45% with lys-2IT-BABE.

The in vivo biologic half-life of chelates not containing the epitopic function; [^{111}In]EDTA, [^{111}In] LBEDTA, [^{111}In]BLEDTA II, and [^{57}Co]bleomycin, was unchanged by WC3A11 with <5% remaining at 24 hr. In contrast, the complex of WC3A11 and bifunctional chelates containing the spacer; [^{57}Co]1,4-DT-

TABLE 1
Association (Binding) Constant: (Ka = Kb) of Hapten by Antibody (Duplicates; Mean)

Clone	IgG SubClass	1,4 DT BABE Cobalt (III)	1,4 DT BABE Indium (III)	Lysine-2IT BABE Indium (III)	BLEDTA IV Indium (III)
WC3A11	IgG3	6.0×10^8	2.3×10^6	2.6×10^7	1.6×10^8
WC4B7	IgG2a	5.6×10^8	4.2×10^5	1.2×10^5	3.6×10^7
WC3F5	IgG2a	3.3×10^8	3.6×10^5	1.9×10^6	5.9×10^7

BABE, [^{111}In]1,4-DT-BABE, [^{111}In]lysine-2IT-BABE, and [^{111}In]BLEDTA IV all had >30% remaining at 24 hr.

In competitive inhibition experiments in vivo even a large excess (~1.4 μmol) of chelate without spacer failed to inhibit binding and did not change the biologic half-life. However, with 200 μg WC3A11 only 4 nmol of transferrin-2IT-BABE-Co(II) (Fig. 1B) reduced 24 hr whole-body retention of [^{57}Co]1,4-DT-BABE from 50% to 13%.

In Vivo Distribution

A total of 48 mice with antibody circulating for ~20 hr have been given i.v. transferrin-2IT-BABE-Co(II) chase. It was noted that 29 of these had a moderate but transient reaction beginning 5 min after injection and lasting 1–2 hr. It appeared allergic as the mice scratched, hyperventilated, and remained less active for this time. All mice had apparently complete recovery. The other 19 showed no apparent ill effects. One mouse that had received 600 μg antibody died immediately following injection.

The effect of increasing amounts of chase on the organ distribution of ^{125}I-labeled WC3A11 is shown in Figure 2. The maximum effect on lowering the blood and increasing the liver concentration was seen at an antigen to antibody ratio of slightly less than 1:1, with a drop in liver concentration at higher ratios. There was a small increase in spleen concentration but not significant lung or kidney uptake with increasing amounts of chase. No reaction was noted in any of these mice.

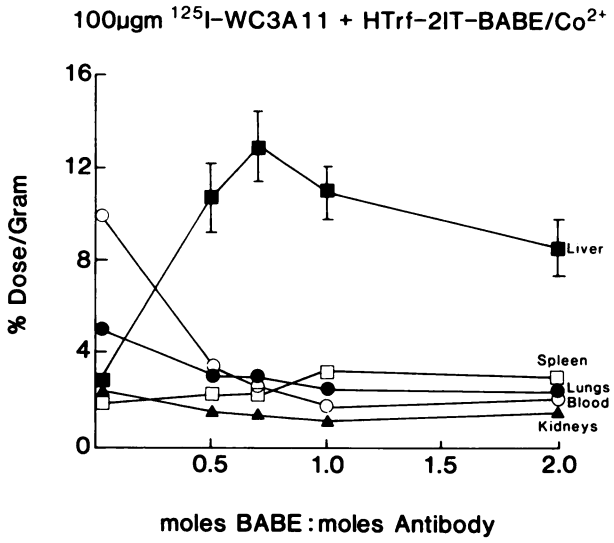

FIGURE 2
Dose-response curve in mice pre-targeted with 100 μg WC3A11, 20 hr prior to injection of increasing amounts of covalent antigen (chase). The liver, spleen, lungs blood, and kidney concentrations are plotted against the molar ration of antigen to antibody.

The 3-hr organ and tumor distribution of [^{111}In] BLEDTA IV alone and [^{111}In]BLEDTA IV in animals pre-targeted with 50 μg WC3A11, with and without chase, is shown in Table 2. Without pre-targeting the tumor concentration was 0.72%/g, and this increased to ~4%/g when antibody was present. However, with-

TABLE 2
Three-Hour Organ and Tumor Distribution in BALB/c Mice with KHJJ tumor
(n = 3, % injected dose/g, T/O tumor/organ ratios, ± s.d.)

	A*			B†			C			D
	[^{111}In]BLEDTA IV§			Ab + [^{111}In]BLEDTA IV¶			Ab + CH + [^{111}In]BLEDTA IV**			
O	%/g	±	T/O	%/g	±	T/O	%/g	±	T/O	C/B
BL	0.40	0.03	1.80	6.05	0.57	0.68	0.16	0.05	8.72	0.03
HT	0.21	0.01	3.41	1.72	0.11	2.36	0.11	0.01	13.04	0.06
LU	0.50	0.04	1.44	3.01	0.29	1.35	0.36	0.06	3.81	0.12
LI	1.03	0.16	0.71	22.54	3.59	0.18	0.47	0.09	2.93	0.02
SP	0.39	0.02	1.84	1.39	0.09	2.92	0.24	0.05	5.95	0.17
KI	12.75	0.44	0.06	9.64	1.42	0.43	6.15	0.90	0.22	0.64
TU	0.72	0.05		4.07	0.33		1.40	0.61		0.34
MU	0.10	0.003	7.27	0.63	0.12	6.75	0.08	0.03	16.54	0.13
BO	0.57	0.09	1.27	1.06	0.07	3.85	0.33	0.06	4.24	0.31
SK	0.72	0.30	1.12	0.82	0.03	4.93	0.38	0.09	3.66	0.46
GU	0.76	0.05	0.95	1.52	0.65	2.99	0.48	0.22	3.16	0.32

* A: [^{111}In]BLEDTA IV.
† B: [^{111}In]BLEDTA IV 21 hr after WC3A11.
‡ C: [^{111}In]BLEDTA IV 21 hr after WC3A11, but chase given 1 hr prior to tracer.
C/B ratio of organ background in pretargeted animal with chase (C)/pretargeted no chase (B). A measure of the efficiency of the chase in lowering background.
§ Conjugate of bleomycin and BABE through four-carbon spacer
¶ Animal injected 24 hr previously with WC3A11.
** Chase given 1 hr prior to [^{111}In]BLEDTA.

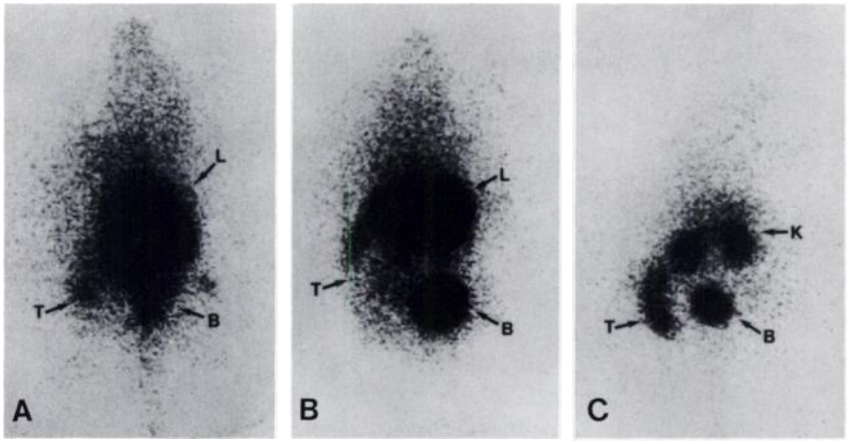

FIGURE 3
Posterior gamma camera pinhole images of BALB/c mice with KHJJ tumor in left flank. 200k counts were collected in each image 3 hr after 100–500 μCi ^{111}In bifunctional hapten. Arrows point to: T = Tumor, L = Liver, K = Kidney, B = Bladder. A: Control; [^{111}In]BLEDTA IV alone. B: Control; [^{111}In]BLEDTA IV 21 hr after pre-targeting with 50 μg WC3A11 (image at 24 hr). C: Chase experiment; [^{111}In]BLEDTA IV 21 hr after pre-targeting with 50 μg WC3A11, but covalent chase given 1 hr prior to tracer injection (at 20 hr after pre-targeting; image at 24 hr after pre-targeting).

out chase just prior to [^{111}In]BLEDTA IV, the blood levels were higher than tumor and T/O ratios were low (blood, liver, and kidney <1). When the chase was given the background blood levels as well as liver, spleen, and bone + marrow concentrations were much lower. The tumor concentration also fell from 4.07%/g to 1.40%/g. However, because the background in all other organs fell proportionately more than the tumor, the tumor to organ (T/O) ratios were >1 for all organs except the kidney, which was the major route of excretion.

Animal Images

The 3-hr images made with [^{111}In]BLEDTA IV alone, and with [^{111}In]BLEDTA IV in pre-targeted mice with and without chase are shown in Figure 3. With BLEDTA IV alone (Fig. 3A) 5.2% of the injected dose remained at 3 hr. The tumor is not well seen and contained only ~1% of the counts (0.08% dose) by digital ROI analysis. With pre-targeting (Fig. 3B), the absolute amount in the tumor increased but the tumor was not better vizualized because of the large background (50% of the dose remaining). With chase (Fig. 3C), the tumor stands out much better against the lower background. Only ~7% of the dose remained in the whole body at the 3-hr imaging time with greatly improved T/O ratios and contrast. The tumor contained ~7% of the counts (~0.5% dose) by ROI analysis. Note the low liver background in the chase image (Fig. 3C).

DISCUSSION

The problem of background reduction in radioimmunoimaging has been approached in several different ways. Reducing the molecular size of the antibody and eliminating the Fc fragment increased the excretion rate and reduced nonspecific binding. This resulted in lower blood levels and liver background but also somewhat lower target concentrations (*22,23*).

The injection of second antibody specific for the first, while decreasing blood background, caused a reciprocal increase in liver background. Whole body radiation was not decreased, since none of the activity was excreted (*24,25*).

Local injection of antibody subcutaneously or into body cavities limited the distribution of antibody to regional nodes, peritoneum, pericardium, or pleural space (*26,27*). This improved local uptake but tumor imaging was limited to the region near the injection site.

Recently the development of metabolizable chelates has provided a more rapidly excreted label for antibodies, that lowered the blood and whole body background (*28*). Like antibody fragments, the target concentrations were also somewhat lower due to the shorter plasma half times and lower effective plasma concentrations.

We have recently proposed the use of reversibly labeled antibody-hapten complexes, since blood background can be eliminated just prior to imaging (*7,8*). However, as with covalently labeled antibodies, 24 hr was required for the reversibly labeled antibody to localize so the method was not suited to short lived tracers like technetium-99m (^{99m}Tc) or gallium-68 (^{68}Ga).

The pre-targeting method we describe here avoids some of the above problems, such as prolonged radiation exposure, the need for long lived tracers (^{131}I, ^{111}In), and high blood and liver background (*9*). The three steps we used are shown schematically in Figure 4, the broken column representing the capillary wall.

The first step was i.v. injection of antibody with slow diffusion from blood through the extracellular fluid to the tumor target, as depicted in the top of Figure 4. It may take several days to reach maximum target concentrations due to the slow pharmacokinetics of antibodies. Tumor uptake in this animal model occurs primarily because of the presence of relatively leaky capillaries. Here the use of bifunctional antibodies, either hybrids or chimeric molecules, that could bind both a chelate and a tumor antigen, would further improve tumor uptake (*29,30*).

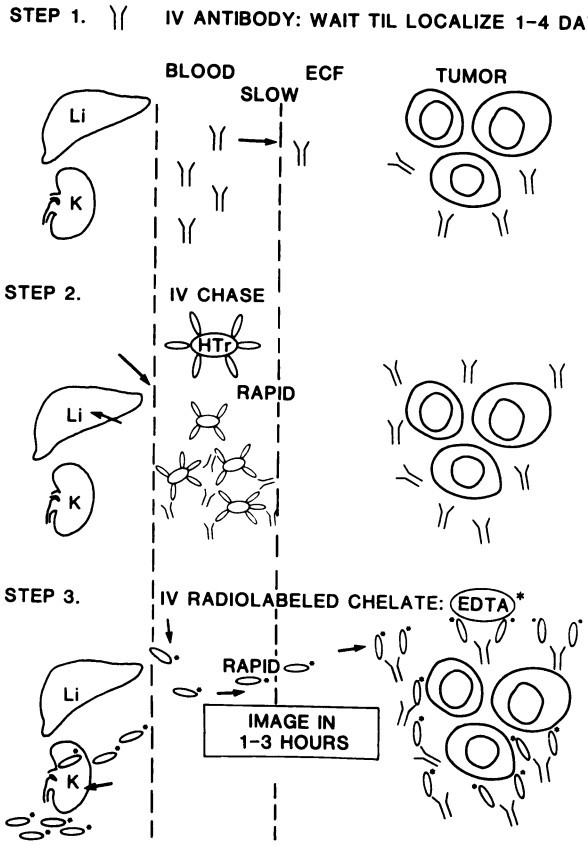

FIGURE 4
Diagramatic representation of three-step pre-targeted radioimmunoscintigraphy. The dotted lines represent the capillary walls with the major organs involved (liver and kidneys) on the left, and the extra cellular fluid space (ECF) with target tumor cells on the right. The first step; slow diffusion of antibody thru capillarys and ECF to target takes days. The second and third steps are rapid and require only hours.

The second step required rapid blood clearance of excess circulating antibody (~15–25% injected dose) after localization in the target had occurred. It was necessary to do this without greatly reducing the amount of antibody in the target. This was accomplished by using antigen covalently bound to a slowly diffusable serum protein (human transferrin) as a chase. A mean of approximately four molecules of antigen per molecule of protein was used to allow cross-linking and lattice formation in the plasma. The aggregates thus formed were rapidly removed by the RE cells of the liver. The unreacted chase presumably remained predominantly within the vascular space due to its large size and did not have time to diffuse into the target or other tissues before the tracer was administered. This process is shown in the middle of Figure 4.

The amount of transferrin-2IT-BABE-Co(II) chase necessary to obtain maximum liver sequestration of antibody in mice with 100 μc circulating for 20 hr is shown in Figure 2. In agreement with classic antibody-antigen reaction theory, the largest aggregates (and, hence, liver concentrations) formed in vivo at antigen-antibody equivalence, with less at either antigen or antibody excess. No reaction was seen in any of these mice.

The transient toxicity seen in some mice with transferrin-2IT-BABE-Co(II) chase did not occur with chase injection alone, and seemed related to the amount of circulating antibody. Further investigation of toxicity with various doses and in other species is planned.

It was noted using ^{125}I-labeled antibody that no significant kidney or lung uptake of aggregates occurred following the chase. This may have been due to the size being less than the minimum 7 μ necessary for lung capillary entrapment to occur. Immune complex disease involving the kidneys has been reported in patients with autoimmune diseases so it will be important to avoid renal deposition (*31*). This condition usually results from long exposure to large amounts of small complexes within a narrow size range such that glomerular sequestration can occur which is not the case in single injection imaging procedures. No renal accumulation of ^{125}I-labeled WC3A11 was seen in these chase experiments. This suggests that the high renal activity seen in the three step method after the radiolabel is given is probably a result of the rapid excretion of chelate by glomerular filtration rather than complex sequestration. Immunogenicity of the chase in humans can be kept to a minimum by the use of human serum proteins as carriers.

In the final and third step, the desired tracer was given i.v. in the form of an epitopically derivatized bifunctional chelate which was small, rapidly diffusable, and quickly cleared predominantly by the kidneys. This is shown in the bottom of Figure 4. Many metal chelates and drugs have the property of rapid distribution in the extracellular fluid and prompt renal clearance, since they are small hydrophilic molecules. It follows that a large number of currently available pharmacologic agents are potential diagnostic or therapeutic candidates for use in this system. The advantage would be increased target concentrations of drug relative to other tissues. This would be especially important in decreasing toxic side effects from radioimmunotherapeutic agents (bone marrow), anticancer drugs and certain antibiotics, but it could also reduce drug efficacy by sequestering active drug molecules.

Future improvements in hapten binding antibodies include development of a monoclonal to an epitope completely separate from the chelate. Any chelate (or drug) derivatized with the epitope would then bind to the antibody. Such a bifunctional chelate would bind to the antibody with the same Kb regardless of the metal ion it contained and could be used with ^{111}In, ^{99m}Tc, ^{68}Ga, or Yttrium-90 (*32,33*).

A potential drawback to the method is the relatively large amount of antibody needed to bind the tracer in

the target. This is inversely related to the binding constant Kb of the antibody (1.6×10^8). In these experiments 50 to 100 μg of WC3A11 were needed which on a body weight basis is equivalent to ~100 to 200 mg for a human. Only small improvements can be expected in Kb for antibodies; possibly to 10^{10}. However, other noncovalent systems, for example avidin and biotin, do have affinities much higher than antibodies (10^{15}) and may be employed here to reduce the amount of binding protein needed (*34*). We are currently investigating an anti-tumor antibody coupled to streptavidin for use with a chelate biotin derivative in a mouse lymphoma tumor model (*29,35*). Until such bifunctional antibodies are developed the method will depend on nonspecific factors such as leaky tumor capillaries for imaging tumors.

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