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RECENT DEVELOPMENTS IN MONOCLONAL ANTIBODY RADIOLABELING TECHNIQUES

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INTRODUCTION

Monoclonal antibodies (MAbs) have shown the potential to serve as selective carriers of radionuclides to specific in vivo antigens. Accordingly, there has been an intense surge of research activity in an effort to develop and evaluate MAb-based radiopharmaceuticals for tumor imaging (radioimmunoscintigraphy) and therapy (radioimmunotherapy), as well as for diagnosing nonmalignant diseases (1-3). A number of problems have recently been identified, related to the MAbs themselves (3-6) and to radiolabeling techniques (4, 7-8), that compromise both the selectivity and the specificity of the in vivo distribution of radiolabeled MAbs. This paper will address some of these issues and primarily discuss recent developments in the techniques for radiolabeling monoclonal antibodies that may help resolve problems related to the poor in vivo stability of the radiolabel and may thus produce improved biodistribution. Even though many issues are identical with therapeutic radionuclides, the discussion will focus mainly on radioimmunoscintigraphic labels.

GENERAL CONSIDERATIONS

Efficient labeling of monoclonal antibodies depends on a number of factors including the characteristics of the radionuclide itself and the method and manner of its incorporation into the protein. The chemical changes inherent in the labeling procedures often cause significant effects on the functional integrity of antibodies and their fragments. Table I lists the various radionuclides that based on considerations of half-life, emission characteristics, and availability factors are well suited as MAb labels for radioimmunoscintigraphy (2,9,10). Out of these, iodine nuclides, ^{111}In, and more recently ^{99m}Tc have almost exclusively been utilized in most investigations. The usual criteria for an imaging isotope for a radiopharmaceutical also apply to monoclonal antibodies; however, because of the variable retention and excretion characteristics of the radiolabel, certain nuclides may be preferable over others. These factors require individual and separate evaluation. It should be pointed out that what one is imaging with radiolabeled antibodies is the radioactivity distribution which may not necessarily correspond with the distribution of the antibody itself. Unless it can be proven that the distribution of the radionuclide is totally

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and exclusively guided by the antibody at all times after injection, the *in vivo* behavior of the dissociated radionuclide has to be carefully accounted for and considered. These aspects are exemplified from the data in Table II.

TABLE I
NUCLIDES FOR RADIOIMMUNOSCINTIGRAPHY[a]

Radionuclide	Z	Half-Life	Decay Properties Energy keV (Abundance %)
Fluorine-18	9	1.83 h	β^+, 635 max (97%) $\gamma^{\pm}$, 511 (194%)
Cobalt-55	26	17.9 h	β^+, 1500 max (81%) $\gamma^{\pm}$, 511 (160%)
Copper-64	29	12.8 h	β^+, 656 max. (19%) $\gamma^{\pm}$, 511 (38%)
Copper-67	29	61.9 h	β^-, Avg. 141 γ, 92 (23%), 184 (40%)
Gallium-67	31	78.3 h	γ, 93 (40%), 184 (24%) 296 (22%), 388 (7%)
Gallium-68	31	1.13 h	β^+, 1900 max (88%) $\gamma^{\pm}$, 511 (176%)
Bromine-75	35	1.65 h	β^+, 1700 max (90%) $\gamma^{\pm}$, 511 (180%)
Bromine-76	35	16.7 h	β^+, 3600 (62%) $\gamma^{\pm}$ 511 (133%)
Zirconium-89	40	79.0 h	β^+, 900 max (22%) $\gamma^{\pm}$, 511 (44%), others
Technetium-99m	43	6.02 h	γ, 140 (89%)
Ruthenium-97	44	69.2 h	γ, 216 (86%), 325 (10%)
Indium-111	49	67.9 h	γ, 171 (91%), 245 (94%)
Iodine-123	53	13.2 h	γ, 159 (83%)
Iodine-131	53	8.04 d	β^-, Avg. 182 γ, 284 (6%), 364 (81%), 637 (7.3%)
Lead-203	82	52.0 h	γ, 279 (81%), 401 (3.4%)

[a]Positron emitters for PET imaging are also included.
Data taken from Reference 9.

TABLE II

TUMOR UPTAKE AND TUMOR-TO-BLOOD RATIOS IN NUDE MICE OF LABELED ANTIMELANOMA MAb 225.28s, 96 HR FOLLOWING INJECTION (n = 6)

Radionuclide	Tumor/blood	% ID/g in Tumor
^{111}In	4.9	16.1 ± 4.1
$^{109}Pd^{a}$	63	18.9 ± 4.4
^{65m}Zn	15	3.0 ± 0.9
^{125}I	5.6	2.3 ± 0.1

[a]48 hr after injection

Shown in this table are the results from a previous study (4) where the biodistribution in nude mice of an antimelanoma antibody was investigated. The tumor uptake and the tumor-to-blood ratios of radioactivity for this antibody were different for the four radionuclides studied. Variations in the stability of attachment of the radionuclide to the antibody (strength of iodine-carbon bond or stability of chelated metal), as well as the transformed in vivo properties of the labeled antibody (which vary with the radiolabel used) would appear to account for the observed differences. Had the antibody suffered minimal alterations following radiolabeling and if the radioactivity was guided totally by the antibody to specific binding sites, the distribution would have been identical. Certain antibodies may indeed be more susceptible than others to labeling conditions and thus produce different results with different radiometals. In addition, the in vivo kinetic stability of different radiometal chelates themselves contributes to differences in the localization and excretion of radioactivity with the same antibody system.

The chemical form of the radionuclide used for labeling antibodies can vary according to the element used. In the case of iodine, one can use the simple I^- ion for direct labeling, or a "prosthetic" group which is iodinated prior to conjugation to the antibody. With radiometals, either direct labeling using the metal cation in its most commmon (or reactive) oxidation state, or indirect labeling using a MAb-chelator conjugate are employed. The effectiveness of a particular labeling method depends upon a number of factors. Important considerations include the rate of formation and the radiochemical yield of the labeled product, and its thermodynamic and kinetic stability especially in the presence of serum proteins and other in vivo competing ligands. These factors also relate to the inertness of the metal chelate towards dissociation and its propensity to undergo ligand exchange,

transchelation, or redox reactions. Even though *in vitro* methods such as serum incubation and ligand challenge are good indicators of *in vivo* stability, the latter must be determined by actual experiments *in vivo* since a positive correlation between *in vitro* and *in vivo* behavior has not always been obtained (4,11,12).

RADIOIODINATION METHODS

The radionuclides of iodine (Table I) have enjoyed wide popularity as protein labels. This is due to the fact that many iodine nuclides, particularly ^{125}I and ^{131}I and, to a lesser extent ^{123}I, have become available in sufficient quantities to carry out extensive investigations. In addition, the chemistry of protein radioiodination is well established (1,7, 13-16).

For radioimmunoscintigraphy, ^{123}I is most suitable because of its nuclear characteristics. Indeed, its use has been increasing at a steady pace (17) even though the problems of cost and availability still remain. However, for certain antibody systems, the 13 hr half-life of ^{123}I is too short and thus ^{131}I with an eight day half-life, in fact, has been the iodine nuclide of choice (15). Its use has drawbacks, which include the presence of β-emission that adds to the radiation dose, and the high-energy gamma emissions that compromise image quality; these shortcomings have nevertheless been outweighed by its inexpensive cost and ready availability. Iodine-125 has mainly been useful for biodistribution studies since its long half-life and low-energy gamma emission are not well suited for patient imaging.

Antibody iodination methods (1,7,13,14,16) include the common electrophilic substitution methods that utilize oxidants such as chloramine T, iodine monochloride, iodogen, lactoperoxidase and electrolytic methods, as well as the classic Bolton-Hunter conjugation method in which the "prosthetic" group N-succinimidyl 3-(4-hydroxyphenyl) propanoate is first iodinated using one of the above oxidants and then conjugated to the antibody. Recently introduced conjugation methods have utilized the new prosthetic groups para- (18) and meta-iodobenzoyl (19,20) conjugates shown in Fig. 1, or an adduct of tyramine cellobiose (21). These latter conjugation methods produce labeled antibodies that, in animal experiments, have exhibited much less *in vivo* deiodination compared to the conventional direct electrophilic methods (18-21). A kit method for antibody iodination through attachment of a para-iodophenyl-maleimide derivative to an antibody sulfhydryl group has also been described (22). The superiority, if any, of these newer methods for tumor imaging in man remains to be demonstrated.

The direct electrophilic substitution methods involve oxidation of radioiodine to a reactive cationic species that then replaces a hydrogen atom in the ortho position of an activated phenyl ring of antibody tyrosine

Fig 1. Halogen Labeling of Antibodies Through an Amine Group

residues and, in some cases, histidine. At neutral pH, iodination occurs mainly at the tyrosine residues (23). Direct attachment can also take place through the formation of a sulfur-iodine bond such as with the cysteine residues. However, the sulfur-iodine bonds are less stable in vivo compared to covalent carbon-iodine bonds. The conjugation methods employ a somewhat different mechanism. The prosthetic groups are iodinated first, and then attached to antibodies through lysine amino acids (Fig. 1) or through sulfhydryl groups by means of activated esters or maleimide groups. In these systems, electrophilic iodination occurs at the activated ortho carbon of a phenyl ring as in the Bolton-Hunter reagent, or by displacement of an organometallic species such as trialkyltin (Fig. 1) or mercuric acetate (22) from a phenyl ring. The site-specific nature of these reactions insures the formation of aromatic carbon-iodine bonds which are more stable in vivo than aliphatic carbon-iodine bonds. In addition, the labeling of a prosthetic group eliminates the need for contact between the antibody and oxidant which eliminates oxidative damage to the antibody. A combination of these factors accounts for the improved stability and biodistribution of MAbs iodinated using these methods. Prosthetic groups should also allow the use of other radiohalogens. For example, PET imaging with MAbs may be possible using ^{75}Br and ^{76}Br. Also, MAbs recently have been labeled with the α-emitter ^{211}At using the ATE compound (Fig. 1) (20).

All directly iodinated antibodies have encountered the problem of in vivo dehalogenation (1,3,7,8). Dehalogenation produces free iodide which results in a slow washout of the radioactivity from the tumor and also causes its accumulation in the thyroid and the stomach. This creates background problems in imaging. As much as 50% of radioiodine is excreted into the urine (24).

Low-molecular-weight iodinated antibody fragments have also been found in the urine (25). The dehalogenation is presumed to be caused by *in vivo* deiodinases which cannot distinguish between thyroxine and the iodinated tyrosine in antibodies. The recently described conjugation methods (18-22) produce iodinated antibodies that, as mentioned above, result in substantially decreased *in vivo* deiodination in animal experiments. If these results are duplicated in human studies as well, radioiodinated antibodies may continue to play a useful role in radioimmunoscintigraphy.

RADIOMETAL LABELING

Radiometal labeling of monoclonal antibodies is gentler than most oxidative methods of iodination. Radiometals also help circumvent the problem of *in vivo* deiodination of iodinated MAbs in both tumor and normal tissues, discussed above. This is particularly true in the case of antibodies that are internalized into the cells following binding to the surface antigens. Use of radiometals also allows a wide variety of choices of half-life and emission characteristics for radioimmunoscintigraphy (Table I) as well as for radioimmunotherapy. Nuclear and physical properties can be better matched with the *in vivo* pharmacokinetics of particular antibody systems thus allowing for better imaging contrast at early time periods following injection. Positron emitters (Table I) can also be used to allow better quantification of biodistribution by PET imaging (26-28). This can be very useful for obtaining better dose estimates prior to high dose radioimmunotherapy. As described below, antibodies can be labeled with radiometals either directly or through the use of a "bifunctional" chelating agent.

Direct Labeling

In the direct method, where the endogenous complexing groups on the antibody are used, the label stability is often poor and the sites of attachment of the metal are generally nonspecific and not clearly defined. It is difficult to manipulate the chemistry in order to modify *in vivo* pharmacokinetics and clearance of the labeled antibody. Despite these shortcomings, direct methods are simple to perform, require minimal antibody manipulation, and can often be conducive to a kit formulation. Thus far, direct labeling of MAbs with radiometals has been limited to technetium.

Direct Labeling with Technetium-99m

Even though the 6 hr half life of ^{99m}Tc may be a limitation for use with antibodies that display slow tumor uptake and background clearance, it is an ideal nuclide for imaging (29) provided that sufficient contrast can be achieved rapidly following administration. Indeed, its use for antibody

labeling has picked up considerably during the last three years, and examples of ^{99m}Tc-labeled antibodies producing radioscintigraphic images of adequate quality within 2-24 hours following injection have steadily increased (8, 30-34).

Several direct methods have recently been developed and studied for labeling antibodies with ^{99m}Tc. One of these involves reacting the antibody or the fragments with a stannous tin reagent. This presumably reduces some disulfide bonds to free sulfhydryl groups that then bind technetium (35). Clinical use of this method has met with some success (30-31). However, this method has not been generally applicable to all MAbs. Two more methods that involve the same principle of attachment of ^{99m}Tc to reduced sulfhydryl groups have been reported. These employ much milder reducing conditions that presumably better maintain the integrity of the antibody. In these methods, a weak complex of ^{99m}Tc is presented to the reduced antibody for transchelation. Fab' fragments of an antifibrin antibody and an antimyosin antibody have been labeled in this fashion with a kit method using ^{99m}Tc-glucarate (36). Dithiothreiotol was used to reduce the $F(ab')_2$ disulfide bonds and produce Fab' fragments. A similar method first reported in 1987 involves reduction of the antibody disulfides with 2-mercaptoethanol followed by exchange labeling using ^{99m}Tc-phosphonate or pyrophosphate (37). Clinical results using this method have been claimed to be quite satisfactory (34,38) although more work, in particular pharmacokinetic studies, will be required in order to establish its general usefulness.

Indirect Labeling Methods

Indirect radiometal labeling of antibodies is generally accomplished through the use of "bifunctional" chelating agents. A number of such chelating agents have been developed and evaluated for antibody labeling with various radiometals. These are listed in Tables III and IV. Indirect labeling can be carried out following either one of the two following approaches. In the first approach, the radiometal chelate is formed first and then conjugated to the antibody. The advantages of this approach are that the metal chelate structure and stability are better defined and, since the metal chelate is formed first, the antibody is not exposed to the chelation conditions which, in the case of some metal-ligand systems, can indeed be quite harsh. The major disadvantage is that often multiple steps are required as well as post-labeling purification, and these make a kit formulation very difficult. In the second and more widely applicable indirect labeling approach, the bifunctional chelating agent (ligand) is first covalently attached to the antibody to form an antibody-ligand conjugate (immunoconjugate) which then binds the radiometal.

TABLE III

BIFUNCTIONAL CHELATES FOR RADIOMETAL LABELING OF MONOCLONAL ANTIBODIES.

I. POLYAMINOCARBOXYLATES

Chelate	Radiometal(s)	Bond	Reference
1-(p-benzenediazonium) EDTA	In	Azo	Sundberg et al. (39)
DTPA-mixed anhydride	In	Amide	Krecjarek et al. (40)
1-(p-carboxymethylbenzyl) EDTA	In	Amide	Meares et al. (41)
1-(p-SCN-benzyl) EDTA	In	Thiourea	Meares et al. (42)
DTPA-bicyclic anhydride	In,Y,Tc	Amide	Hnatowich et al. (43)
1-(p-SCN-benzyl) DTPA	In, Y	Thiourea	Brechbiel et al. (44)
Propionic-EDDHA	Ga	Amide	Matzku et al. (45)
Cyclohexyl EDTA monoanhydride	In,Co,Pb,Ru	Amide	Mease et al. (46)

TABLE IV

BIFUNCTIONAL CHELATES FOR RADIOMETAL LABELING OF MONOCLONAL ANTIBODIES

II. MACROCYCLIC AND OTHER MISCELLANEOUS COMPOUNDS

Chelate	Radiometal(s)	Bond	Reference
Desferrioxamine (DFO)	Ga,In	Imine	Yokoyama (47) Ward (48)
p-SCN-benzyl TETA	Cu	Thiourea	Moi et al. (49)
Dithiosemicarbazone (DTS)	Tc	Amide	Arano et al. (50)
Diamide dimercaptide system (N_2S_2)	Tc	Amide	Fritzberg et al. (51)
Triamide mercaptide system (N_3S)	Tc	Maleimide	Dean et al. (36)
p-Nitrobenzyl DOTA	Cu,Y	Thiourea	Deshpande et al. (52)
Br-phenyl HBED	In,Ga	Amide	Mathias et al. (53)
N-Benzyl carboxyphenyl tris-sulfophenyl porphine	Cu	Amide	Roberts et al. (54)
Macrocyclic tetraamines	Cu, Tc	Maleimide	Franz et al. (55,56)
Triaza, tetraaza compounds	Pd,Rh	Amide	Troutner et al. (57,58)

Indirect Labeling with Technetium-99m

Methods using prechelation of ^{99m}Tc have been developed and studied by Fritzberg and coworkers (51), and Franz and coworkers (56). The method of Fritzberg uses a diamide dimercaptide chelator which is first labeled with

^{99m}Tc and then conjugated to antibodies (Fig. 2). Adapted as a kit procedure (51), the method has recently been evaluated with a number of antibodies in patients and given encouraging results (32). The second approach for indirect labeling of MAbs with ^{99m}Tc has been developed and used by Dean and coworkers (36). In this method, the free sulfhydryl groups of an Fab' fragment are used to attach a triamide monomercaptide chelating ligand. The use of sulfhydryls to conjugate the MAb with the ligand removes the site of ^{99m}Tc binding away from the antigen binding region. While this method is more convenient, it is generally restricted to the use of Fab' fragments.

Fig. 2 Preparation of ^{99m}Tc Labeled Antibodies Using the Prechelation Method (Fritzberg et al., Ref. 51)

Indirect Labeling with Other Radiometals

The most commonly used ligand for indirect labeling of MAbs with ^{111}In and other radiometals is DTPA. It is generally conjugated to antibodies via bicyclic DTPA anhydride, which forms a covalent amide bond between an antibody amine (most likely on a lysine) and one of the DTPA carboxylate groups (43). Although the method is convenient, its use with ^{111}In yields complexes which show high liver retention and slow body clearance (1,7,8, 59-61). Use of liposome-encapsulated ^{111}In-^{14}C-DTPA has shown that ^{14}C-DTPA is cleared through the kidney into urine while ^{111}In remains in the liver (61). This suggests that bioretention of indium is due to its transchelation in the liver following its detachment from DTPA; these results also suggest that multidentate ligands which form more stable chelates will be necessary for ^{111}In-complexes to survive liver catabolism and be excreted intact through the kidney.

One strategy that has been used in an effort to resolve the problem of

retention of indium in the liver is the covalent attachment of DTPA moieties via one of the carboxylates to the Fc-oligosaccharide regions of antibodies (62). Since this coupling is restricted to the carbohydrate chain, and since the carbohydrates are found predominantly on the constant Fc region, this "site-specific" procedure is believed to reduce the effect on immunoreactivity, of the metal binding in too close a vicinity to the antigen-binding hypervariable regions on the MAb, and thus produce more stable preparations. Animal studies showed greatly reduced liver retention and higher tumor uptakes when this method of conjugation was used (63). Recent clinical studies, however, were not particularly encouraging; radioactivity in the liver was found to be comparable to that obtained using the conventional DTPA dianhydride method (8).

The incorporation of a p-isothiocyanato- or p-bromoacetamido- substituted benzyl group into the backbone of either EDTA or DTPA has been shown to increase the serum stability of the corresponding ^{111}In labeled complex or immunoconjugate (41,44,64,65). These para-substituted benzyl ligands are shown in Fig. 3. In mice, liver retention of the ^{111}In was reduced with a para-substituted benzyl EDTA immunoconjugate, and ^{111}In detected in the urine was shown to be the intact benzyl EDTA ^{111}In chelate (66). This work suggests that benzyl EDTA is a better ligand for ^{111}In than ordinary DTPA. Also, high tumor uptake with low liver retention was reported recently with ^{111}In (p-isothiocyanatobenzyl) DTPA immunoconjugates (64,67). Improvements observed with benzyl EDTA and benzyl DTPA may result from a combination of factors. First, with these ligands the antibody is bound to the radiochelate via covalent linkage with the benzyl ring; unlike the case with DTPA dianhydride, all carboxylate groups remain free to complex the metal. In addition, steric bulk of the benzyl group may improve <u>in vivo</u> radioimmunoconjugate stability by blocking the approach of competing ligands and reducing the volume of space the carboxylates can occupy, thereby providing some preorientation for metal binding. In spite of these improvements, benzyl EDTA and benzyl DTPA are not optimal ligands for all metals. For example, ^{67}Cu-benzyl EDTA immunoconjugates are very unstable in serum and thus, macrocyclic ligands or porphyrins are required to form ^{67}Cu-complexes with appreciable <u>in vivo</u> stability. The macrocycles studied include para-isothiocyanatobenzyl TETA (49) and para-bromoacetamidobenzyl DOTA (Fig. 3) (52). The increased stability of the macrocyclic complexes results in part from structural rigidity, which prevents coordination sites on the ligand from rotating away from the metal. These and other macrocyclic ligands, included in Table IV have been developed and evaluated for use with copper, yttrium, palladium, and rhodium (54-58). PET imaging with ^{64}Cu-MAbs can be performed as well using these ligands (55).

Since a major effort is necessary in order to develop and evaluate "tailor-made" ligands for individual radiometals (more than a dozen are potentially useful and have been proposed for imaging and/or therapy), our approach at

Fig. 3 Para-substituted Benzyl Polyaminocarboxylate Chelating Agents for Radiometal Labeling of Monoclonal Antibodies. (1) p-SCN-Bz-EDTA, Ref.42; (2) p-SCN-Bz-DTPA, Ref. 44; (3) p-SCN-Bz-TTHA, Ref. 77; (4) and (5) p-SCN-Bz-EDTA and DTPA modified at the acetyl carbon, Ref. 78; (6) p-BABE-HBED, Ref. 53; (7) p-SCN-Bz-TETA, Ref. 49: (8) p-BABE-DOTA, Ref. 52.

Brookhaven has been to combine the rigidity of macrocycles and porphyrins with the general utility of polyaminocarboxylates (10, 27, 46, 68, 69). The starting point in our study has been to investigate the semi-rigid ligand trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid (Fig. 4). CDTA has the ethylenediamine portion of EDTA incorporated into a cyclohexane ring. This ring locks the nitrogens into the trans-diequatorial configuration and preorients a portion of the ligand in a position favorable for metal chelation. The ring also shields the chelated metal from competing ligands. In general, CDTA metal complexes have formation constants 1-3 orders of magnitude higher than those of EDTA and exhibit slower rates of metal transchelation (70-72).

The monoanhydride of CDTA (CDTAMA) and the mono-NHS ester of CDTA (CDTA-NHS) (Fig. 4) were prepared and used to conjugate CDTA to anticolon carcinoma antibody 17-1A and to anti-CEA antibody. We have also synthesized cyclohexyl derivatives of DTPA (CDTPA) and triethylenetetraaminehexaacetic acid (CTTHA, Fig. 4) and converted these polyaminocarboxylates to their respective N-hydroxysuccinimide (NHS) esters. These bifunctional ligands were also conjugated to 17-1A, and labeled with ^{111}In and ^{57}Co. The positive effect of

Fig. 4 Cyclohexyl Polyaminocarboxylates (Ref. 46,68,69)

chelate rigidity was apparent in the biodistribution of these preparations in human tumor xenografted nude mice: with indium (Table V) the order of tumor uptake was CDTA ≈ DTPA ≈ CDTPA ≈ CTTHA > TTHA ≈ EDTA, while with cobalt (Table VI) the order was CDTA > CDTPA > CTTHA > DTPA > EDTA (27,69). In general, the rigidity of the cyclohexane ring increased complex stability (serum stability was higher; data not shown), allowing greater tumor uptake compared with non-rigid chelates. The observed effect was greatest with CDTA, where the highest percentage of binding sites (2 of 5, 40%) are locked into place by the cyclohexane ring.

When investigating new chelates, especially those that contain more than one reactive site which can conjugate the antibody, characterization using a sensitive analytical technique such as HPLC is mandatory. Whereas conjugation with DTPA dianhydride at a protein concentration of 15-20 mg/ml gives an average of 5-15% high molecular weight material (most likely cross-linked antibody), conjugation with the dianhydride of CDTA and EDTA at the same protein concentration yielded 40-50% high molecular weight material (68). The use of these preparations in biodistribution studies without removal of the

high molecular weight material by HPLC results in higher liver retention and lower tumor uptake (68,69). The use of CDTAMA or CDTA-NHS, which have only one site capable of conjugating the antibody, eliminated the formation of high molecular weight material and thus did not require post labeling purification.

Immunoconjugates containing an internal cleavable linking group between the MAb and chelated indium have demonstrated faster background clearance and modified liver retention (73,74). In one study (73), a linking group containing the diester, ethyleneglycol bis (succinimidyl) succinate, was added sequentially to DTPA-p-(aminoethyl)anilide and the MAb. Faster whole body clearance of ^{111}In and higher tumor to tissue ratios were obtained in mice. In another study (74), linking groups containing a disulfide, ester, thioether thiourea, or peptide group were attached to p-aminobenzyl EDTA and its

TABLE V

BIODISTRIBUTION (% DOSE/G) OF ^{111}In LABELED 17-1A CONJUGATES IN NUDE TUMOR MICE[1]

Ligand[2]	Time	n	Blood	Liver	Kidney	Bone	Tumor	Whole Body[3]
EDTAMA &	24	2	3.2	7.3	24.4	7.2	6.2	90
EDTADA	96	6	0.5	7.4	12.7	4.8	2.8	60
CDTAMA	24	4	4.2	9.0	4.2	6.0	14.0	96
	96	4	1.0	7.1	4.3	2.5	6.0	63
CDTA-1NHS	24	4	6.8	7.5	4.7	2.6	12.3	85
	96	5	0.3	7.7	3.7	2.2	3.0	50
DTPADA	24	6	5.6	8.8	8.8	5.2	11.2	96
	96	6	2.6	6.5	11.6	3.6	9.8	71
DTPA-2NHS	24	3	11.6	6.8	12.0	4.6	14.1	94
	96	3	0.4	6.6	11.5	6.3	6.0	64
CDTPA-2NHS	24	3	9.8	7.2	7.3	5.5	16.8	100
	96	3	0.4	8.7	5.3	7.1	5.9	68
TTHA-1NHS	24	3	2.0	5.4	26.3	7.8	5.4	80
	96	3	0.3	6.0	14.8	6.7	2.9	61
CTTHA-2NHS	24	3	6.7	5.9	13.8	6.4	11.8	78
	96	3	2.9	5.6	9.4	5.1	9.5	71

[1]All data were obtained using HPLC-purified monomeric fractions and are normalized to 25 g body weight. Standard deviations omitted for clarity.

[2]DA-dianhydride; MA, monoanhydride; NHS, N-hydroxysuccinimide.

[3]Percent dose retained.

Table VI.

BIODISTRIBUTION (% DOSE/G) OF ^{57}Co LABELED 17-1A CONJUGATES IN NUDE TUMOR MICF[1]

Ligand[2]	Time	n	Blood	Liver	Kidney	Bone	Tumor	Whole body[3]
EDTAMA &	24	3	2.2	4.1	3.0	1.0	3.1	35
EDTADA	96	3	0.2	2.0	1.2	0.5	0.5	10
CDTAMA	24	5	12.9	5.7	3.5	2.5	12.8	79
	96	5	1.5	2.7	1.9	1.4	4.1	31
CDTA-1NHS	24	5	6.0	7.2	3.3	4.4	14.2	78
	96	4	5.5	3.2	2.5	1.3	11.2	41
DTPADA	24	6	2.2	5.8	3.6	1.9	5.6	41
	96	7	0.4	2.2	1.6	0.6	0.9	10
DTPA-1NHS	24	3	5.2	6.1	3.8	0.8	6.2	41
	96	3	0.3	1.8	1.7	0.5	0.8	11
CDTPA-2NHS	24	3	3.7	4.8	3.0	2.0	6.2	44
	96	3	1.0	1.8	1.5	0.7	2.4	16
CTTHA-2NHS	24	2	1.9	5.9	3.9	1.7	5.9	35
	96	2	0.6	2.3	2.0	0.4	1.4	13

[1]HPLC-purified monomeric labeled preparations were injected. The data are normalized to 25 g body weight. Standard deviations omitted for clarity.

[2]DA, dianhydride; MA, monoanhydride; NHS, N-hydroxysuccinimide.

[3]Percent injected dose retained.

derivatives, and isolated prior to immunoconjugation. In nontumor mice at 72 hr, the liver uptake of these conjugates was 2-3%, 5-10%, 10-15%, 5-10% and 20-25% of the injected dose per g, respectively. While the disulfide clearance is promising it is thought that this occurred in the circulation and may be too rapid for use with antibodies. Recently, we have added symmetrical linking groups to one of the COOH groups of CDTA by two routes that utilize CDTAMA as the starting material (75). The ease of synthesis of CDTAMA allows subsequent syntheses to be on a scale sufficient for the isolation and characterization of all products. In the first route, symmetrical amines (ethylenediamine, 4,4'-methylenedianiline, and 4-aminophenylether) were either reacted in excess with CDTAMA or converted to mono t-BOC derivatives before reacting with CDTAMA. Hydrolysis of the t-BOC group, followed by treatment

with thiophosgene, produced the isothiocyanates for immunoconjugation. In the second route, a symmetrical diacid, ethyleneglycol-bissuccinate, was converted to the corresponding NHS ester and reacted in excess with CDTA-ethylenediamine from the first route. These linkers were conjugated to MAbs and are currently under study in mice. Due to the ease of synthesis of CDTAMA, this approach is attractive for the continued investigation of the ability of linking groups to modify the biodistribution of indium immunoconjugates (75).

So far, the immunoconjugates with the semi-rigid polyaminocarboxylates have all utilized one of the carboxylates for conjugation to the antibody. This type of conjugation is not optimal since it uses up one of the carboxylates, and places the metal complexation site in close proximity to the antibody conjugation site. A better situation would be to place the antibody conjugation site at a position removed from the metal complexation site such as in p-SCN-benzyl EDTA or DTPA derivatives. Such a compound would be 4-amino-cyclohexyl EDTA (4-NH_2CDTA). The synthesis of this compound and conversion of NH_2 to the isothiocyanate, have been described briefly in the patent literature (76). It is not clear, however, if the stereochemistry of the two amino groups was carefully controlled. The stereochemistry is important since trans-diequatorial nitrogens are in a better position to chelate metals than cis axial-equatorial nitrogens. We have recently synthesized 4-cyclohexenyl EDTA where the nitrogens are in the trans-diequatorial position. Derivatization of the olefin in this compound is currently underway to allow conjugation to MAbs. Another sterically hindered chelating agent which does not use a carboxylate for attachment to the MAbs and has shown promise with trivalent metals including ^{111}In is the bromoacetamidobenzyl derivative of HBED (53).

Demonstration of the effectiveness of these newer generation chelating agents and new conjugation technology for immunoscintigraphy, although promising in animal experiments, must await detailed clinical evaluation.

CONCLUSION

Ideal nuclides for radioimmunoscintigraphy are essentially the same as those for other conventional radiopharmaceuticals. However, in the case of labeled antibodies, a better matching of the nuclear and physical characteristics of the radionuclide with the in vivo radioantibody pharmacokinetics is necessary in order to achieve maximum contrast and sufficient photon yield as early as possible after injection. Iodine-131 and ^{111}In have been the most commonly used nuclides but recently, shorter lived ^{123}I and ^{99m}Tc have also been found effective with certain antibody systems particularly when fragments are utilized. Other radionuclides suitable for immunoscintigraphy include ^{67}Ga, ^{97}Ru, and ^{203}Pb, but their usefulness remains

to be determined. Positron emitters ^{18}F, ^{55}Co, ^{64}Cu, ^{68}Ga, ^{75}Br and ^{76}Br are also potentially useful for PET imaging of antibody biodistribution, in particular for obtaining better dose estimates before initiating radioimmunotherapy with particle emitting radionuclides of chemically similar elements. Factors related to the production and availability of many of the above radionuclides will have to be addressed and the development of better radiometal-antibody labeling techniques will be required.

Even though much research effort has recently focused on the development of new radiometal chelating agents and novel methods of conjugation to antibodies, required improvements in in vivo radioactivity distribution have not yet been realized. New more rigid chelating agents that are presently being synthesized are, however, expected to yield metal chelates with substantially higher in vivo stability. These developments may lead to a significant reduction of the nonspecific uptake of radioactivity and to improved blood and body clearance of non-tumor radioactivity, and thus allow imaging with higher contrast early after injection. Indeed, many recent and promising results covered in this article offer renewed hope that, in the years to come, radioimmunoscintigraphy will continue to be a fact, not fiction.

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