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Review

Approaches to Radiolabeling of Antibodies for Diagnosis and Therapy of Cancer

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The development of monoclonal antibodies of high affinity and selectivity for tumor antigens has supported the development of radiolabeled antibodies for diagnostic localization and targeted delivery of therapeutic radionuclides. Several radionuclide chelating agent systems have been developed for indium-111 and technetium-99m that have shown good sensitivity and specificity for tumor detection in patients. Feasibility for therapy has been shown in animal models and a few patient studies with iodine-131 and yttrium-90. This review covers selection of radionuclides and chemistry of antibody radiolabeling.

KEY WORDS: antibodies, monoclonal; radionuclides, tumor-targeted delivery; tumor antigens; cancer, radiotherapy; cancer, diagnostics.

INTRODUCTION

The ability of antibodies to recognize epitopes on tumor-cell antigens provides a basis for targeted delivery of radioactivity, drugs, and toxins to malignant tumors. The development of monoclonal antibodies (MoAbs) in 1975 (1) provided antibodies that are uniform reagents which can be continuously produced by immortalized clones. Much effort in the last decade has gone into the development of MoAbs of high affinity for antigens selectively expressed on tumor cells. Moreover, the antigens should be in high numbers per cell and be expressed on most, if not all, cells of the target tumor type. More recently efforts have focused on optimizing conjugation chemistry with desired drug forms. In the case of radioactive drug forms, the focus of this review, matching of physical half-lives with *in vivo* pharmacokinetics and reduction of Fc-directed uptake has led in some cases to the use of antibody fragments, including $F(ab')_2$, Fab', and Fab.

A number of reviews have appeared on the use of radiolabeled antibodies for tumor imaging and radioimmunotherapy. Several emphasize radiochemistry and labeling methodology (2–4) and others more generally discuss *in vitro* immunology of labeled MoAbs, animal studies, and results of studies in patients (5–9). As the field has been extremely active over the last 10 years, it is not practical to include all studies in this review. Instead, the focus is selected studies on radionuclides and chemistry of antibody labeling, with some perspectives on applications of radiolabeled antibodies for diagnosis and therapy.

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RADIOLABELED ANTIBODIES FOR *IN VIVO* DIAGNOSIS

Requirements for radiolabeled antibodies for *in vivo* diagnostic application include the ability to localize in tumor tissue on the basis of tumor-associated antigen recognition and retention for the time necessary for clearance of radioactivity from normal tissue. Whether lesions are imageable or not depends most importantly on lesion-to-surrounding tissue ratios and to a lesser extent on absolute amounts of radioactivity delivered (9,10). Superficial lesions may require only 2:1 target-to-background ratios, while increasingly greater ratios are required for the visualization of deeper and smaller lesions. The need for high ratios may be decreased by tomographic imaging techniques which remove overlying and underlying radioactivity about the lesions. Unfortunately, these techniques are time-consuming and require high photon fluxes.

Physical Properties of Radionuclides and Immunoscintigraphy

The ideal radionuclide for diagnostic applications has (i) a high abundance of photons between 100 and 200 keV, (ii) a physical half-life similar to the biological process being studied, and (iii) minimal particulate radiation (Table 1). Technetium-99m, with 140-keV photons, no particulate radiation, and a 6-hr half-life as well as a very low cost and convenience of supply is the single-photon radionuclide of choice in nuclear medicine. However, the 6-hr half-life and complex redox and chelate chemistry of technetium have resulted in limited successful application in immunoscintigraphy.

Most immunoscintigraphy studies have used iodine-131 or indium-111. Their advantages and shortcomings are discussed later. As can be seen from Table I numerous alternatives exist with physical properties useful for imaging. How-

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Table I. Radionuclides for Immunoscintigraphy

Radionuclide	Half-life	Photon energy (keV)	Decay mode
F-18	1.83 hr	511 (97%)	Positron
Ga-67	3.26 days	93 (10%), 185 (24%) 296 (22%)	EC
Br-76	16.1 hr	511 (38%)	Positron
Br-77	2.38 days	240 (30%), 520 (24%)	EC
Zr-89	3.26 days	511 (22%)	Positron
Ru-97	2.88 days	215 (91%), 324 (8%)	EC
Tc-99m	6.0 hr	140 (90%)	IT
In-111	2.83 days	172 (89%), 247 (94%)	EC
Sn-117m	14.0 days	159 (87%)	IT
I-123	13 hr	159 (83%)	EC
I-131	8.05 days	364 (82%), 637 (7%)	Beta
Pb-203	2.2 days	279 (81%)	EC

ever, practical considerations such as radionuclide production and specific activity attainable may restrict choices.

The wide range in physical half-lives requires that some may be too short or others may be longer than necessary, resulting in needless radiation. The use of antibody fragments provides a means to match physical half-life with biological requirements for time to target radioactivity to tumor cells and clear surrounding nontarget tissues. Thus, physical half-lives of a few days or longer are needed with whole antibodies, while fragments allow the use of the desirable I-123 and Tc-99m radionuclides. Typically, a usable window for imaging is four half-lives. Thus, Tc-99m can provide imaging to about 24 hr. If sufficient uptake and nontarget clearance has taken place in 8 hr, which has been seen with Tc-99m-labeled antimelanoma 225-285 $F(ab')_2$ (11) and NR-M1-05 Fab (12), even the 2-hr-half-life F-18 may be used. This would allow quantitative *in vivo* assessment of localization by positron emission tomography (PET) techniques.

Radionuclides and Antibody Labeling

Radiohalogenation (Iodine-123, -125, and -131, Bromine-76 and -77, Fluorine-18)

There are several halogen radioisotopes suitable for diagnostic imaging with labeled monoclonal antibodies. In particular, radioisotopes of iodine have been extensively used as radiolabels for antibodies because of their availability and the wealth of experience with radiolabeling of proteins with these isotopes. Iodine-131, with its 364-keV gamma emission and 8.05-day half-life, is the most frequently used iodine isotope for imaging. However, I-131 also decays by beta emission, which adds to the radiation dose to the patient, and several higher-energy minor gamma emissions add to the degradation of image quality. Iodine-123, with its 159-keV gamma and 13-hr half-life via electron capture decay, is more suitable than I-131 for imaging with current gamma cameras. However, iodine-123 is cyclotron produced and thus is relatively expensive. Several bromine radioisotopes may find use in imaging applications. Bromine-77, electron capture decay, has a gamma emission at 239 keV with a half-life of 57 hr; however, its application to imaging is difficult due to its equally abundant 511-keV gamma and other less abundant high-energy photons. Bromine-76, with a half-life of 16.1 hr, can potentially be used for positron imaging. Finally, fluorine-18 is an easily produced positron emitter with a short half-life of 1.83 hr.

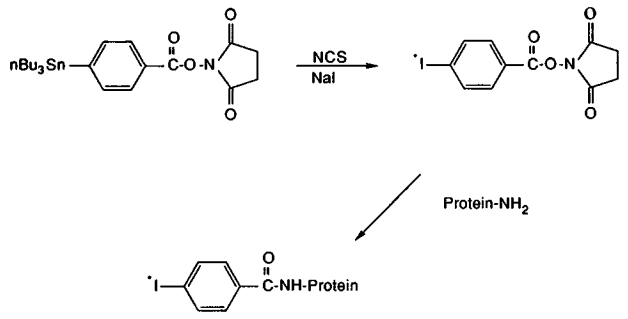
The chemistry of directly attaching radioisotopes of iodine to proteins in general has long been established (13,14). Thus, radioiodine has played a premier role in the development of radiolabeled monoclonal antibodies. Standard radioiodination methods employ oxidants such as chloramine-T and iodogen that maintain radioiodine specific activity for the generation of electrophilic iodide species, which in turn attack principally the activated aromatic ring of tyrosyl residues on the protein. The iodine becomes covalently attached to the 3-position ortho to the hydroxyl group of the tyrosine residue. Another less common and less stable site for incorporation of the reactive iodine is the histidine residue.

A limitation of labeling antibodies with typical iodination conditions is exposure of the antibody to varying levels of oxidizing reagents during the labeling process. Some antibodies may not tolerate these conditions and their immunoreactivity may be decreased. In addition, derivatization of a critical tyrosine residue in the antigen binding site may also result in the loss of immunoreactivity. To circumvent these problems indirect methods for radioiodination of antibodies have been considered. Indirect methods involve covalently attaching the radioiodine onto a small molecule which is subsequently conjugated to the protein. Most commonly the radioiodine is attached to the activated aromatic ring of the conjugating agent in a similar fashion to the direct labeling of tyrosine residues of proteins. For example, the Bolton–Hunter reagent, *N*-succinimidyl 3-(4′-hydroxyphenyl)propionate, can be radioiodinated in the 3′ position of the aromatic ring and then conjugated at basic pH to a lysine residue of the antibody. A similar conjugating agent for radioiodinating proteins is methyl 4-hydroxybenzimidate.

A major limitation to the use of either directly or indirectly radioiodinated antibodies for tumor imaging and therapy is that they frequently suffer extensive *in vivo* deiodination. Significant localization of radioiodine in both the thyroid and the stomach is often observed. Iodinated tyrosines and activated conjugating agents such as the Bolton–Hunter reagent, where the iodine is attached adjacent to the hydroxy group on an aromatic ring, are structurally similar

to the thyroid hormones. These hormones are known to be rapidly dehalogenated by enzymes found in the liver, kidney, and thyroid (15).

A new radioiodinating reagent, *N*-succinimidyl 4-iodobenzoate, referred to as PIP for *p*-iodophenyl, has been developed to stably attach radioiodine to antibodies (16) (Scheme I). This reagent is prepared by iododestannylation



Scheme I

of the corresponding 4-tributyltin derivative. The resulting PIP reagent is conjugated at basic pH to lysine residues on the antibody. Extensive *in vitro* and *in vivo* studies have demonstrated that deiodination of PIP antibody conjugates does not occur to any significant extent. It is believed that the absence of a hydroxyl group adjacent to the iodine on the aromatic ring of PIP is responsible for the absence of deiodinase activity. Recent reports suggest that similar results are observed with the 3-iodo isomer of PIP (17).

There are limited reports in the literature on radiobrominated antibodies (18–20). Radiobromination is more difficult than radioiodination because of the lower oxidation potential of bromide compared to iodide. Direct radiobromination of proteins has been accomplished with several enzymes. Chloroperoxidase has been used to radiobrominate a variety of proteins; however, this enzyme has an optimum pH of 2.8 and is completely inactive above 4.5. The low-pH conditions for labeling may not be compatible with many antibodies. Myeloperoxidase and bromoperoxidase operate efficiently at pH values around neutrality. The resulting radiobrominated proteins are more stable *in vivo* than the corresponding radioiodinated proteins.

A single report on the radiofluorination of antibodies has recently appeared (21). Two F-18 reagents, 3-fluoro-5-nitrobenzimidate and 4-fluorophenacyl bromide, were successfully conjugated to several proteins and antibodies. Conjugation yields in a model IgA antibody were only 15% for the benzimidate and 46% for the bromoketone at 22 mg/ml. Limited *in vivo* studies of these conjugates were described.

Indium. The widespread uise of In-111 in nuclear medicine is due to its favorable nuclear properties ($t_{1/2}$ = 67 hr, 173,247-keV gamma) and its availability. Bifunctional chelating agent technology has been developed which results in effective targeting of In-111 to tumors despite drawbacks of deposition of In-111 in the reticuloendothelial system after metabolism or transchelation to transferrin (2,9). Imaging studies utilizing In-111-labeled monoclonal antibodies are now quite common.

Polyaminocarboxylate ligands dominate the chemistry of In-111-labeled antibodies and proteins. Ethylenediaminetetraacetate (EDTA) and diethylenetriaminepentaacetate (DTPA) have been used most frequently. Variations in the method by which the ligand is attached to proteins are most interesting and are discussed below.

Direct attachment of DTPA to proteins was first achieved using a mixed anhydride (22) with isobutylcarbonate to label human serum albumin (HSA). Tumor imaging using monoclonal antibodies (23) labeled by this method was among the earliest studies using metal chelates as labels. The method now is used extensively. In this method, the anhydride reacts with a lysine amino group to form a covalent amide linkage. Reaction with histidine, tyrosine, or cysteine leads to hydrolytically unstable products. A monoanhydride in which only one of the carboxylates from DTPA is used appears most effective since the probability of cross-linking proteins is negligible.

Another method for direct attachment of DTPA to proteins utilizes the cyclic (or bicyclic) anhydride, in which the anhydride is formed intramolecularly. This method was first applied to fibrinogen (24) and albumin (25). Application to antibodies (26,27) followed shortly. This method takes advantage of the greater stability of the cyclic versus mixed anhydride toward hydrolysis. However, due to the presence of two anhydrides in a single molecule, the possibility of forming cross-linked aggregates is increased (28).

A third method for direct attachment of DTPA to antibodies makes use of a carbodiimide coupling reaction (29). This method has not been applied as widely as the methods using anhydrides.

Another method for direct attachment of DTPA utilizes the reactivity of the *N*-hydroxysuccinimide ester (30,31). The amino group of lysine reacts with the ester to produce an amide linkage. While the monoester is expected to give negligible amounts of cross-linked aggregates, the pentaester showed significant amounts of higher molecular weight forms (32).

Attachment of DTPA by direct bonding of one of the carboxylates reduces the number of groups available for coordination to indium. Stability studies (33,34) have shown that DTPA attachment through a linker bonded to the ethylene backbone produces a chelate which holds indium more firmly, thereby giving higher target-to-nontarget ratios. This added stability is presumed to be due to the availability of all five carboxylates of the DTPA. Several methods for backbone attachment of DTPA and EDTA are discussed below.

Diazoniumphenyl carbon backbone derivatives of EDTA were first coupled with HSA and fibrinogen (35–37). These studies proved that the concept of using bifunctional chelating agents for bonding metal ions to proteins was feasible. However, the diazonium coupling is difficult to control and has not been applied to antibodies.

Two methods of linking EDTA to antibodies (38) utilize 1-(*p*-aminobenzyl)EDTA activated either as the *p*-bromoacetamidobenzyl (BABE) or the *p*-isothiocyanatobenzyl derivative (CITC). The lysine amino groups, histidine imidazole ring, and terminal amino groups are suggested to be the dominant sites of reaction with the BABE chelate. For the isothiocyanate, amino groups are usually considered to

be the site of attachment. This method was expanded to include the DTPA chelate (33,34). In these studies, the 1-(*p*-isothiocyanatobenzyl)DTPA derivative was coupled to antibodies.

Carbohydrate oligosaccharides, when oxidized, have been utilized as a site for attachment of DTPA-containing molecules (39). The production of aldehydes through oxidation produces sites that are reactive toward amines, hydrazines, and hydrazides. The attachment of the chelate at oligosaccharides results in better immunoreactivity compared to attachment at lysines since the oligosaccharides are distal to the antibody-combining site whereas the lysines may be distributed throughout the protein, perhaps even at the combining site.

Finally, a novel approach to the problem of nonspecific localization of radioisotope has been the incorporation of metabolizable linkers in the chelate (40). The linker is composed of a central ethylene glycol which is bound through succinate esters to both the antibody and the chelate (*p*-aminobenzylhydroxyethylethylenediaminetriacetic acid). The clearance of the activity from the blood and nontarget organs is more rapid than for antibody with a nonmetabolizable linker, suggesting that the envisioned mechanism may be operable. Similar results have been reported by others using an ester approach (41,42). Preliminary results were presented on thioether, disulfide, and hydrocarbon chains (43).

Gallium. Monoclonal antibodies have been labeled with Ga-67 ($t_{1/2}$ = 78 hr, photon energies of 93, 185, and 296 keV) through the use of DTPA conjugates. The DTPA conjugates were prepared through either the mixed anhydride (23) or the cyclic anhydride (44) method. Chelation of Ga-67 to DTPA-derivatized antibodies has been shown to be unstable, with the Ga-67 bound adventitiously to the antibody.

Proteins (HSA) have been labeled with Ga-67 using desferrioxamine (DF) conjugates (45,46). The DF is coupled to the protein using glutaraldehyde, which bonds to the amine groups of the DF and the HSA (47).

Technetium-99m. Proteins have been an object of Tc-99m labeling nearly as long as it has been available. It is easy to achieve an apparent Tc-99m-labeled protein by reduction of Tc-99m pertechnetate and nonspecific binding of the reduced technetium to donor atoms present in the protein, namely, thiol, amide, amino, and carboxylate. While these donor atoms form highly stable complexes when optimal five-membered chelate rings of sufficient number result in small molecule ligands, the likelihood of finding four or more donor atoms in a preferred arrangement in the protein is low. The result typically has been loss of much or most of the radioactivity from the protein once subjected to *in vivo* conditions. In addition, complications of colloid-bound Tc-99m have been observed from stannous ion reduction procedures (48).

Because of the simplicity of direct labeling of Tc-99m to antibodies, a significant effort has been made to optimize conditions for binding of a high fraction of the radioactivity to high-affinity sites (49). A pretinning approaching has been described (50) in which stannous ion is incubated with a $F(ab')_2$ fragment for up to 24 hr and then lyophilized. Labeling takes place during incubation with Tc-99m pertechnetate. Rapid disappearance of radioactivity from the blood (11) and renal uptake (51) indicate a significant fraction of weakly bound Tc-99m. However, clinical studies in melanoma using this labeling approach have shown successful tumor imaging (11). Others have attempted to eliminate stannous ion problems by acid reduction of pertechnetate and then concentration to dryness (52) or reaction of pertechnetate with azide in HCl to give a reduced chloronitrido-reactive intermediate after evaporation (53). The addition of buffered antibody results in labeling. These are laborious processes with no apparent gain in binding to high-affinity sites on the antibody.

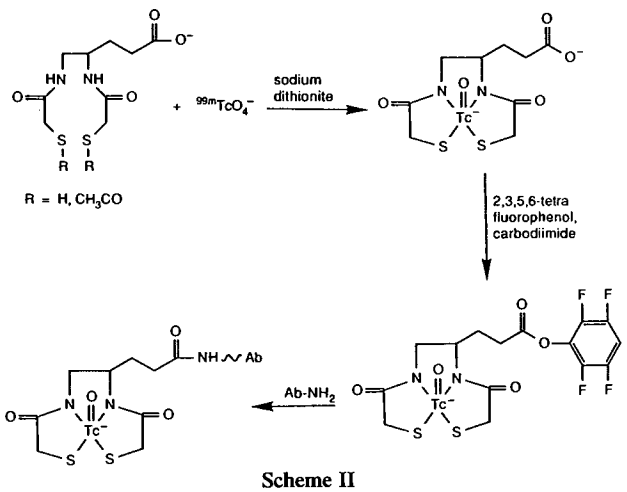
Efforts have been made to label antibodies with Tc-99m using the DTPA-antibody conjugate technology developed for In-111. In one approach (54) dithionite was used as both reducing agent for Tc-99m and transfer ligand. It was determined that the optimal yield of Tc-99m protein resulted from the use of 4×10^4 molar excess dithionite over Tc-99m plus long-lived Tc-99. While analysis indicated appropriate molecular weights for the Fab (50,000) and fibrinogen (340,000) and binding assay values, nonspecific binding of Tc colloids was problematic and elimination of colloids required extra column or $(NH_4)_2SO_4$ precipitation steps. Final yields were 32%.

Based on studies that showed the hexadecylamine monoamide of DTPA to result in a Tc complex several times more stable than uncoupled Tc-99m DTPA, Tc-99m labeling of DTPA-antibody was done in the presence of free DTPA (55). Optimization of ratios of stannous ion, DTPA, DTPA–antibody, and pH resulted in useful yields of Tc-99m-labeled antibody. However, a minimum of 15% nonspecific Tc-99m binding to antibody was seen. Several DTPA moieties per antibody were used, which has been shown to affect immunoreactivity (5). As Fab or $F(ab')_2$ fragments almost certainly are required for Tc-99m applications due to the short 6-hr half-life, the relative effects of the needed DTPA groups per antibody may be significantly detrimental.

The use of the dithiosemicarbazone (DTS) group as a bifunctional chelating agent for Tc-99m has been studied (56). The latest in a series of DTS ligands, *p*-carboxyethylphenylglyoxaldi(*N*-methylthiosemicarbazone) (CE-DTS), appears to enhance *in vivo* stability of Tc-99m antibody. Ratios of over 1:1 CE-DTB:antibody degrade immunoreactivity. Exchange of the Tc-99m to the 1:1 CE-DTB:antibody increased over a 3-hr period. While *in vivo* evidence was presented to show increased Tc-99m antibody stability, no results were presented with antibody fragments.

Metallothionein, a 6000 molecular weight metal binding protein with a high proportion of cysteines, has been used as a protein-derived chelating agent (57). While metallothionein efficiently binds Tc-99m, the larger size of metallothionein may affect antibody biodistribution properties, especially on antibody fragments. An *in vivo* study indicated relatively rapid disappearance of Tc-99m from the blood (58). Whether this was due to cleavage of the Tc-99m metallothionein from the antibody, catabolism of the conjugate, or direct release of Tc-99m was not determined.

Another highly stable chelating agent system that has been developed for antibody fragment labeling with Tc-99m is the N_2S_2 diamide dimercaptide (59,60) (Scheme II). In



Scheme II

order to label with this system and maintain well-defined chemistry, a preformed chelate (PFC) approach was used. Thus, Tc-99m 4,5-dithioacetamidopentanoate ($C_5N_2S_2$) was prepared, esterified with 2,3,5,6-tetrafluorophenol, and finally conjugated with antibody at basic pH. These steps allow characterization of the Tc-99m complex, active ester and labeling via well-known acylation chemistry. Stability was high, with 2% or less lost in 24 hr by challenge at 37°C with serum, chelating agents DTPA, $C_3N_2S_2$, and urea denaturation. *In vivo* targeting was shown with D_3 F(ab')$_2$ in guinea pigs with Line 10 tumors (Fig. 1). Clinical studies have shown expected stability in patients with tumor targeting demonstrated in melanoma with Tc-99m $C_5N_2S_2$ 9.2.27 F(ab')$_2$ and Fab' (12). Results with both fragments were 81% detection of known metastases, visualization of tumors as small as 250 mg, and uptake of 0.03% dose per gram tumor determined for Tc-99m F(ab')$_2$. Average serum half-lives were 11 hr for Tc-99m F(ab')$_2$ and 2 hr for Tc-99m Fab. Importantly, liver retention was not observed allowing visualization of liver metastases. Apparently metabolism of Tc-99m N_2S_2 antibodies results in soluble catabolites that are excreted from the liver and kidneys.

Radiolabeled Antibodies for Radioimmunotherapy

Successful targeting of radiolabeled antibodies using diagnostic radionuclides suggests that substitution with radionuclides that emit particulate radiation should result in targeted radiation therapy. A body of knowledge exists from external beam therapy with regard to radiation dose and tumor response as well as experience in the treatment of thyroid carcinoma with I-131 (5,6). On this basis studies began as early as 1957 by Bale and Spar (61).

Choice of Radionuclides

A number of particle-emitting radionuclides have been recommended as being potentially useful for radioimmunotherapy (RIT) (62–64). The properties of those that have been studied are shown in Table II. Some properties may be clearly desirable, while others lack sufficient understanding to be overriding. Minimal penetrating radiation is desired as a nonspecific radiation dose results. However, a low abundance of gamma photons allows the determination of targeting of the therapeutic conjugate by imaging. A high spe-

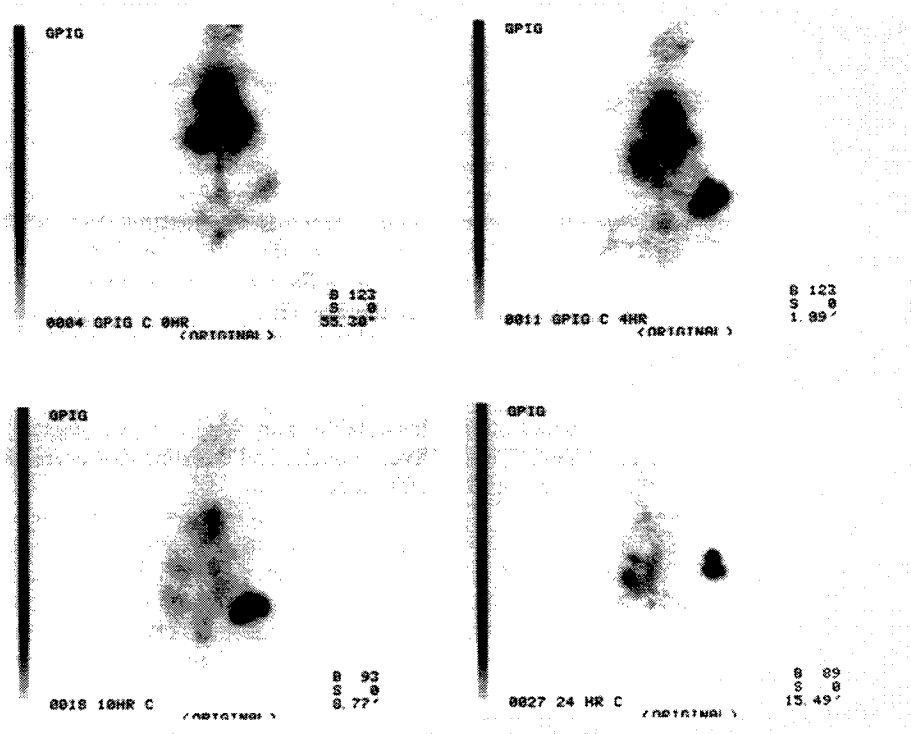


Fig. 1. Gamma camera scintillation images of Tc-99m N_2S_2-D_3 F(ab')$_2$ antibody fragment in a guinea pig with a line 10 tumor at 0, 4, 10, and 24 hr post-injection. At 24 hr the tumor was excised and imaged adjacent to the animal. (Reproduced with permission from Ref. 59).

Table II. Potentially useful Radionuclides for Radioimmunotherapy

Radionuclide	Half-life	Particle	E_{max} (MeV)	Photon (keV)	Source	Specific activity
^{32}P	14.3 days	Beta	1.7	—	n, p	High
^{47}Sc	3.43 days	Beta	0.600	163 (73%)	—	—
^{67}Cu	2.58 days	Beta	0.575	184 (40%)	p, 2p	High
^{90}Y	2.66 days	Beta	2.27	—	Gen.	High
^{105}Rh	1.44 days	Beta	0.568	319 (20%)	n, gamma, p	High
^{109}Pd	0.56 days	Beta	1.028	88 (5%)	n, gamma	Low
^{131}I	8.05 days	Beta	0.606	364 (82%)	n, gamma, beta	High
^{153}Sm	1.95 days	Beta	0.80	103 (28%)	n, gamma	Low
^{186}Re	3.7 days	Beta	1.02	137 (7%)	n, gamma	Low
^{188}Re	0.71 days	Beta	2.12	155 (15%)	Gen.	High
^{211}At	7.2 hr	Alpha	5.87	—	Alpha	High
^{212}Bi	1.01 hr	Alpha	6.09	—	Gen.	High
$^{212}Pb/^{212}Bi$	10.6 hr	Beta/alpha	0.58/6.09	—	Gen.	High

cific activity is desired since the load capacity of MoAbs and their fragments is limited and doses of several hundred millicuries of beta emitters may be necessary. The capability of preparation of useful amounts is clearly necessary. The energy of beta particles and beta versus alpha particles is currently not sufficiently well understood in terms of microdosimetry and efficacy to allow clear choices to be made.

Some generalizations and characterizations can be made, however. High-energy beta emitters such as Y-90 and Re-188 have maximum penetration ranges of about 10 mm or 1000 cell diameters. The average energy is about one-third or about 3 mm penetration. These radionuclides have the potential of creating an effective field effect that may kill cells heterogeneously dispersed in tumor. However, the high penetration increases the nonspecific dose to other organs. Lower-energy beta emitters such as Cu-67, I-131, and Re-186 modify the extent of field to average penetrations of 1 to 2 mm. The far end of the range is represented by Auger and Coster–Kronig electrons of a few electron volts in which the penetration range is 5 to 10 nm (65). These radiations, when near the nucleus inside the cell, are very potent and only a few hits are required. Despite the high potency and lack of effect outside the cells, radioimmunotherapy with these radionuclides which include I-125, Br-77, and Tl-201 would require targeting to tumor cells, internalization of the radionuclide, and transfer to the vicinity of the nucleus. The last major category of radionuclide is alpha emitters. Emission of alpha particles results in high-energy transfer in which several mega-electron volts of energy is given up in several cell diameters. Alpha emitters kill cells with three to six hits (66), but with their low penetration nearly uniform cell targeting is required. Astatine-211 and Bi-212 are examples of alpha emitters that are under consideration for radioimmunotherapy.

Yttrium. The labeling of antibodies with Y-90 ($t_{1/2}$ = 64.1 hr, maximum beta energy = 2.3 MeV) has used DTPA conjugates (67–69) which were formed by the cyclic anhydride method (22). Affinity chromatography shows that Y-90- and In-111-labeled antibodies behave similarly (67). Biodistribution indicates substantial uptake into the liver (68), which is a concern for therapy. The release of Y-90 from the DTPA may perhaps be mediated by iron mobilization proteins such as transferrin. Bone uptake was observed from the administration of Y-90–antibody conjugates (70). This raises concern for bone marrow toxicity, typically the dose-limiting organ in radioimmunotherapy.

Iodine-131. Most radioimmunotherapy studies have been done with I-131. Concerns for therapy applications are scale-up and radiation exposure. *In vivo* deiodination is of greater concern, as released iodide will target the thyroid and may decrease thyroid function despite blocking of the thyroid with cold iodide. The application of stabilized radioiodinated antibodies (16,17) may have important advantages for therapy.

Rhenium-186 and -188. Rhenium-186 has been suggested as an excellent RIT radionuclide candidate by Wessels and Rogus (63). Its 3.7-day half-life and 1.07-MeV maximum particle energy are similar to those of I-131, while its low abundance of 137-keV photons allows imaging without significant contribution to the nonspecific radiation dose. Rhenium-188 has particle energies similar to those of Y-90, but with a shorter half-life and imageable 155-keV photon. Rhenium has a structural chemistry similar to that of technetium and the diamide dimercaptide preformed chelate approach has been applied to Re-186 and Re-188 essentially as described for Tc-99m (71). Good *in vivo* stability and tumor targeting identical to that of Tc-99m-labeled antibody fragments support their use as a diagnostic (Tc-99m)/therapy (Re-186, -188) matched pair.

Scandium-47. Scandium-47 has favorable properties for therapy (Table II). A feasibility study with more readily available Sc-46 was carried out (45). Labeling with DTPA–antibody conjugates occurred in lower yields than In-111 and Sc-46 DTPA–antibody was observed to be significantly less stable than the In-111 conjugate. Uptake was seen in liver, muscle, and intestine that corresponded with Sc-46 acetate localization.

Copper-67. Copper-67 has been of considerable interest for therapy despite concerns of reliable supply. A number of chelating agents for copper have been evaluated, with variable results. Isothiocyanatobenzyl-EDTA and the cyclic anhydride of DTPA were conjugated to antibodies (3:1–4:1 and 4:1 chelates/antibody, respectively), followed by the addition of Cu-67 at pH 5.5–7.5 (72). However, both antibody conjugates lost copper to serum albumin *in vitro*. A new bifunctional chelate, 6-(*p*-bromoacetamidobenzyl)-1,4,8,11-tetrazocyclotetradecane *N,N′,N″,N‴*-tetraacetic acid (*p*-bromoacetamidobenzoyl-TETA), formed a highly

stable complex with copper-67. However, it was necessary to incorporate the copper into the chelate at pH 7 prior to conjugation with the antibody at pH 9.0–9.5. A 16:1 ratio of Cu-TETA:Ab conjugation level could be obtained. Alternatively, 2-iminothiolane (Traut's reagent) could be used as a spacer between the antibody and the *p*-bromoacetamidobenzoyl-TETA chelate, followed by labeling with copper-67 (73). In this case 5 ± 1 TETA/Ab were incorporated, compared to 0 TETA/Ab when no spacer was employed. There has been speculation that the spacer is needed because the metal ion is sterically hindered in its approach to the macrocycle (74).

The TETA–antibody conjugates exhibited excellent stability in human serum *in vitro* (72,74). It was shown that the small loss of radionuclide (less than 1% per day) was from the chelate–antibody conjugate itself (75). Neither the chelate nor the antibody was degraded *in vitro*. It is interesting to note that the *in vitro* stability results are in direct contrast to the stability predicted by equilibrium constants; i.e., the TETA derivative would be the least stable chelate at physiologic pH compared to the EDTA or DTPA derivatives (74). This observation suggests that *in vitro* equilibrium constants may not predict the *in vivo* fate of metal chelates conjugated to antibodies. Furthermore, *in vitro* predictions concerning metabolism of the chelate and/or antibody may not hold *in vivo*.

A functionalized derivative of cyclam, 1-(3-aminopropyl)-4-methyl-1,4,8,11-tetrazacyclotetradecane, which should also bind copper-67, has been studied using copper-64 for convenience (75). Several chemical reactions were necessary in order to bind this functionalized cyclam to antibody using lysines. The antibody was reacted with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide (MBS) at pH 7.5 to yield Ab–MBS. The functionalized cyclam was treated with 2-imiothiolane-HCl at pH 9.0. This product was reacted with the Ab–MBS derivative at pH 7.5–8.0. The copper-64 was added at pH 7.5–8.4. A labeling efficiency of 0.82 copper atom/Ab or lower, depending on the conditions, was found. The stability in serum *in vitro* was excellent, with a good recovery of immunoreactivity. Since affinity columns were used during the purification with elution at acid pH, the recovery of immunoreactivity may vary with the antibody tested.

Since porphyrins chelate with copper to form stable copper complexes, functionalized porphyrins labeled with copper-67 have been conjugated to antibodies by three different methods (76). *N*-Benzyl-5,10,15,20-tetrakis(4-carboxyphenyl)porphine [*N*-benzyl(HTCPP)] was directly conjugated to antibody using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl (EDAC). An activated carboxylate of *N*-benzyl(HTCPP) was formed by reaction with 1,1′-carbonyldiimidazole before conjugation with antibody. An activated carboxylate of *N*-benzyl(HTCPP) can also be generated by reaction with *N*-hydroxysuccinimide in the presence of EDAC before conjugation with antibody. Metalation with copper-67 results in a loss of the *N*-benzyl group to form ^{67}Cu–TCPP under mild conditions. No other experimental details were given and *in vivo* studies have not been reported.

Palladium-109. Palladium-109 is a possible therapy radionuclide with a half-life of 13.4 hr and a principal maximum beta emission at 1.03 MeV. Two different approaches to add a chelate to antibodies using lysine have been reported. A monoclonal antibody to the high molecular weight antigen associated with human melanoma was conjugated to the cyclic anhydride of DTPA at pH 7.0 and subsequently labeled with Pd-109 at pH 5–6 (77). The radiolabeled antibody maintained immunoreactivity and was injected into nude mice with human melanoma. The antibody targeted the tumor (18.5% injected dose/g); however, kidney (10.0% injected dose/g) and liver (4.3% injected dose/g) uptake was noted.

Using a different approach, the carboxylate group of the bifunctional ligand 6-(5-carboxypentyl)-5,7-dioxo-1,4,8,11-tetraazoundecane was coupled to the lysines in antibody (78) using EDAC. Since palladium binds to proteins in general, the Pd-109 complex is prepared first at pH 9, followed by coupling to the antibody using EDAC at pH 5. Approximately 2.0–2.7 atoms of palladium were linked to the antibody. The radiolabeled antibody was stable to 100-fold molar excess challenges of EDTA at pH 7. To become an effective radioimmunotherapy agent, the efficiency of the coupling reaction must be increased since only 20–30% of the radioactivity is incorporated into the antibody. Conjugation of the chelate to the antibody followed with palladium-109 does not work since the radionuclide binds to the antibody nonspecifically. However, if this approach increased the coupling efficiency, the nonspecifically bound palladium could be removed by challenges with a better chelating agent.

Bismuth-212. Bismuth-212 is of interest as an alpha emitter, with an alpha emission of 6.05 MeV and a 60.4-min half-life. Initial studies indicated labeling of DTPA–antibody with bismuth-2112 with retention of immunoreactivity (79). Attempts to form the metal chelate first followed by conjugation to the protein failed. *In vivo* studies indicated that free bismuth accumulates in the liver and other organs, whereas the chelated complex cleared rapidly through the kidneys in normal and leukemic mice.

An anti-Tac monoclonal antibody directed against the human interleukin 2 (IL-2) receptor was conjugated with the isobutylcarboxycarbonic anhydride of DTPA at pH 8.0 and labeled with bismuth-212 at pH 6.0 (80). Approximately three chelates per antibody were incorporated and specific activity was 2–3 μCi/μg. The immunoreactivity was not adversely affected. *In vitro* studies demonstrated specific killing of the IL-2 receptor-positive adult T-cell leukemia line. The killing was specific and not mediated through Fc binding since a nonspecific monoclonal antibody labeled in the same fashion exhibited little effect. The anti-Tac antibody labeled with bismuth-212 may be a very effective immunocytotoxic agent in order to remove IL-2 receptor-positive cells (leukemic T cells) *in vivo*.

In another preliminary report, a monoclonal antibody against a Class I BN alloantigen conjugated with DTPA through the carbohydrate groups and labeled with bismuth-212 (sp act, 2–4 μCi/μg) effected specific killing of malignant cells *in vivo* (81). Presumably, the oligosaccharide moieties were oxidized to aldehydes by $NaIO_4$ at pH 6.0, followed by incubation with either glycyltyrosyllysyl-DTPA or *p*-aminoaniline, followed by the addition of sodium cyanoborohydride (82). In these studies, the radiolabeled anti-

body was injected 2 hr after tumor inoculation. The results are encouraging but need further investigation.

Astatine-211. Astatine-211, with a half-life of 7.2 hr, has excellent radiation characteristics, decaying with a 42% alpha branch at 5.87 MeV and a 58% electron capture branch. The election capture decay daughter, ^{211}Po, is a very short-lived, 0.5-sec, alpha emitter at 7.45 MeV. Thus, alpha emission is associated with 100% of the decays of At-211.

Direct labeling of proteins with astatine has been accomplished using chloramine-T and H_2O_2 as oxidants. Unlike radioiodine, the attachment of astatine to proteins appears to be not through tyrosine residues but through a weaker thiol–astatine bond (83). Thus, the astatine is not bound in a stable fashion and is easily released from the protein, especially *in vivo*.

In order to stabilize At-211, a method has been developed to astatinate the phenyl ring of a benzoic acid, which is subsequently conjugated. In this approach, 4-diazobenzoic acid was reacted with astatine to generate 4-astatatobenzoic acid. Conjugation of 4-astatatobenzoic acid to the protein was accomplished using a mixed anhydride method (84). There are several limitations to the use of this method to label antibodies. Competitive hydrolysis of the diazonium salt during the astatination procedure can lead to 4-hydroxybenzoic acid, which if not removed will become conjugated to the protein. In addition, the labeling procedure can take up to 3 hr to complete, resulting in significant loss of At-211 from decay.

In Vivo Therapy of Radiolabeled Antibodies

Since the report by Bale and Spar (61) a number of studies have shown efficacy in animal models (Ref. 85 and references cited therein). As an example, Cheung *et al.* (86) studied an IgG_3 MoAb against disialoganglioside GD_2, 3F8. Radioiodinated 3F8 was given to mice bearing neuroblastoma xenografts. Only specific radioiodinated 3F8 caused tumor response (Fig. 2). Doses of less than 3900 rads resulted in regression but recurrence, while doses greater than 4200 rads resulted in cures. The experiments involved doses of 0.125 to 1.0 mCi to tumors of 0.5 to 2.0 cm^3.

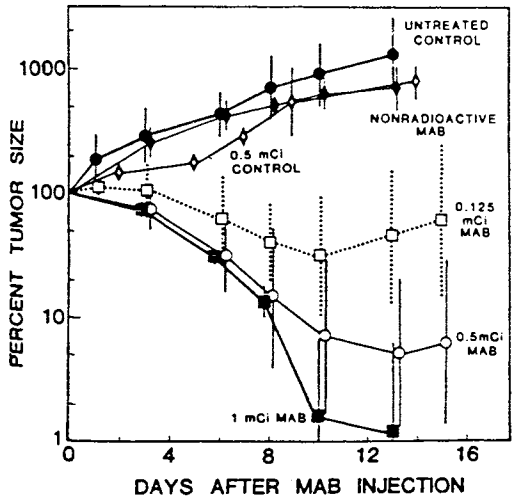


Fig. 2. *In vivo* treatment of human neuroblastoma xenografts. Established tumors were treated with varying doses: (■), 0.5 (0), and 0.125 mCi (□) I-131 labeled 3F8 (100 μg 3F8). Tumor response over time (days) was expressed as percent of original tumor volume at the beginning of experiment. Groups of 3–7 mice were used. The geometric means ± SD of the percent tumor sizes are shown. The control group (●) mice did not receive any treatment. The nonradioactive MoAb (MAB) group (◆) received 100 μg unlabeled 3F8 iv. The 0.5 mCi group (◇) received 0.5 mCi of I-131 radiolabeled anti-sheep red blood cell MoAb iv. (Reproduced with permission from Ref. 86.)

Clinical studies are still somewhat anecdotal, but increasing numbers of tumor responses are being reported. Order and co-workers have administered I-131 and Y-90 anti-ferritin polyclonal antibodies and achieved regressions of bulky hepatomas (87,88). A major limitation is the low typical tumor uptake of 0.01 to 0.05% dose/g in most favorable antibody targeting situations in humans. In the near-future cures and significant responses are likely to come from compartmental administration such as intraperitoneal, pericardial, and intrapleural in which much higher doses can be achieved (89,90).

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