Abstracts in this section pertain to papers to be presented at the meeting of SNM's Radiopharmaceutical Science Council, "Radiochemical Labeling and Characterization of Proteins," June 1, 1985, Houston. Program Chairman: Harold A. O'Brien, PhD.

Immunological Assessment of Immunoconjugates. R.W. Schroff, J.W. Pearson, C.S. Woodhouse, G. Pavanasasivam, and A.C. Morgan, Jr. *NeoRx Corporation, Seattle, WA; and the Biological Response Modifiers Program, BTB, DCT, NCI-Frederick Cancer Research Facility, Frederick, MD.*

Monoclonal antibodies in the form of drug, toxin, or radioisotope immunoconjugates offer the potential for specific tumor delivery. The preclinical assessment of immunoconjugates presents a formidable challenge, requiring a wide variety of methodologies and expertise. This process can be categorized into five phases of evaluation. Phase I involves determining the specificity of the monoclonal antibody by microfluorimetry and indirect immunoperoxidase staining. Phase II testing involves characterization of the antigen, including its biochemical nature, its degree of cell surface expression (sites/cell), and the epitope recognized by antibody. The third phase of evaluation includes assessment of conjugate integrity, the presence of free antibody or ligand by HPLC/FPLC chromatography (gel filtration, ion exchange, hydrophobicity) and SDS-PAGE, and immunoreactivity. Phase IV includes evaluation of conjugate potency by in vitro cytotoxicity or soft agar cloning assays as well as biodistribution by organ distribution or radioimaging. We are currently studying a combination of in vitro assays designed to evaluate nonspecific uptake of conjugate, by way of Fc or complement receptors. Conjugates found to be acceptable by these criteria are then evaluated in human tumor xenograft-nude mouse models. This evaluation may consist of inhibition of palpable or prepalpable tumors, mortality studies, inhibition of experimental metastasis, or combination therapy with chemotherapeutic, radiation, or surgical cytoreductive techniques. Conjugates successfully completing the initial four phases of evaluation proceed to the final phase, consisting of small animal toxicology studies and IND testing. This systematic approach is designed to identify and evaluate those antibodies and conjugates with the greatest potential for clinical efficacy.

Modification and Fragmentation of Monoclonal Antibodies. B.A. Brown, F.A. Liberatore, C.B. Dearborn, P.S. Shaw, and B.M. Gallagher. *Immunopharmaceutical Research, E. I. duPont de Nemours, Co., Inc., North Billerica, MA.*

Radiolabeled monoclonal antibodies (MAbs) have been shown to be useful in the diagnosis and treatment of some cancers. Useful techniques for the coupling of chelators to these proteins have been examined. Specific clinical applications may require using either intact MAb or fragments. Although adequate techniques for fragmentation exist for polyclonal antibodies, many MAbs behave quite differently. The classical procedures utilizing papain and pepsin for the generation of $F(ab')_2$, Fab', and Fab from several subclasses of murine IgG's have been studied along with alternate methods for fragment production which have proven useful for recalcitrant MAbs such as B72.3 as well as strategies for the purification of these fragments. DTPA, covalently coupled to a MAb, is a valuable chelator for subsequent labeling with radioisotopes such as indium-111 (^{111}In). The carbodiimide activation method and the dianhydride method of conjugation have been compared and alternate methods of attaching DTPA have been addressed. As a necessary adjunct to MAb modification and fragmentation, the determination of the intactness of structure and function with reference to the reported techniques has also been studied. The final criterion for judging the success or failure of the modifications and fragmentations is the MAb's in vivo behavior. It is obvious to those working with numerous MAbs, even ones of the same subclass, that a variety of approaches must frequently be tried before conjugations or fragmentations yielding viable MAbs can be accomplished.

Elimination of Immunogenicity and Fc Binding of Antibodies. W.L. Anderson and T.B. Tomasi. *University of New Mexico Medical School, Albuquerque, NM.*

Affinity purified rabbit, goat, and rat antibodies as well as a mouse monoclonal antibody were modified with the active ester of monomethoxypolyethylene glycol succinate (mPEG) (average molecular weight = 5,000). These chemically modified antibodies show no change in affinity and also retain their ability to specifically bind to target cells. PEG-modified antibodies and immune complexes prepared from these antibodies exhibit little or no binding to cell surface Fc receptors on either mouse splenocytes or P388 Dl cells. Rabbit antibody with between 13 and 18% of the lysine residues modified with mPEG fail to elicit an immune response in Swiss mice following primary, secondary, or tertiary exposure to antigen. mPEG also provides an ideal "spacer arm" for the attachment of chemotherapeutic, radioimaging, or nuclear magnetic resonance imaging agents to antibody molecules. This possibility has been evaluated by preparing monofluorescein-PEG, monorhodamine-PEG, monoprotophorphyrin-IX-PEG, and monoperfluorohexane-PEG and then attaching these derivatized PEG molecules to rabbit and mouse monoclonal antibodies. The ability of the modified antibody to specifically deliver the attached moiety to a target cell has been shown. PEG-modified antibodies exhibit excellent stability, low Fc binding obviating the necessity of preparing F(ab')2 fragments as well as providing a means of attaching a variety of therapeutic or imaging agents to antibody molecules. The results suggest that PEG-modification of antibodies may provide advantages for use in in vitro diagnostic procedures and warrant further studies for possible use in vivo in both diagnostic imaging and therapy with antibodies.

Techniques of Radiolabeling Antibodies with Copper-67 Utilizing Porphyrin Chelators.* J.A. Mercer-Smith, S.D. Figard, D.K. Lavallee, and Z.V. Svitra. *Medical Radioisotope Research Group, Los Alamos National Laboratory, Los Alamos, NM; and Hunter College, CUNY, New York, NY.*

We have developed methods to label antibodies with copper-67 (^{67}Cu), a potentially useful medical radioisotope. Porphyrins were selected as chelating agents since they are known to form stable copper complexes, and they can be synthesized with a variety of functional groups for conjugation to proteins. We have examined conjugation and metalation reactions utilizing rabbit IgG and *N*-benzyl-5,10,15,20-tetrakis(4-carboxyphenyl)porphine[*N*-benzyl(HTCPP)].

There are three useful approaches to the conjugation reactions: (a) direct conjugation of *N*-benzyl(HTCPP) using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC); (b) formation of an activated carboxylate of *N*-benzyl(HTCPP) by way of reaction with 1,1′-carbonyldiimidazole prior to conjugation with IgG; and (c) formation of an activated carboxylate of *N*-benzyl(HTCPP) by reaction with *N*-hydroxysuccinimide in the presence of EDAC, followed by conjugation with IgG. *N*-benzyl(HTCPP) can be metalated faster and under milder conditions than can H_2TCPP. Metalation accompanied by loss of the *N*-benzyl group to form [^{67}Cu]TCPP can be accomplished with $^{67}CuCl_2$ in 30 min at 40°C in aqueous buffered solution. Thus, attachment of *N*-benzyl(HTCPP) to IgG followed by rapid metalation with radiocopper appears to be a very promising method to radiolabel antibodies with copper.

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The Use of Metalloproteins as Bifunctional Chelators. G.L. Tolman, R.A. Hadjian, M.M. Morelock, C.A. Drozynski, and T.A. Cormier. *Immunopharmaceutical Research, E. I. duPont de Nemours, Co., Inc., No. Billercia, MA.*

The low-molecular-weight metalloprotein metallothionein (MT) has been successfully used as a bifunctional chelator for the labeling of monoclonal antibodies directed against tumor associated antigens [Tolman GL, *et al: J Nucl Med* 25: P24, 1984 (abstr)]. MT is a cysteine-rich metalloprotein and is composed of two distinct metal-binding cluster domains capable of binding seven to 20 metal atoms per molecule. Its high affinity for heavy metals has been exploited for the labeling of monoclonal antibodies (MAbs) with several radionuclides of diagnostic and therapeutic interest including technetium-99m (^{99m}Tc) and gold-199 (^{199}Au).

The labeling of MT is accomplished by exchange of labile Zn^{2+} from ZnMT with the desired radionuclide at physiological pH. The chemistry of exchange labeling with ^{99m}Tc and ^{199}Au will be discussed. The long-lived radionuclide ^{99m}Tc was used to characterize the nature of metal binding of ^{99m}Tc in MT. The generation of exchangeable Au(I) complexes and their ability to label MT will also be discussed.

Metallothionein can be conjugated to MAbs as well as other biologically active molecules through the amine groups of the lysine residues of MT. The availability of surface groups on MT for conjugation to MAbs has been examined. The changes in the metal-binding characteristics of MT which arise upon modification of the lysines or other available functional groups will be discussed. MT has been crosslinked to MAbs using glutaraldehyde, dimethylsuberimidate, and other widely-used cross-linking agents. The conjugation of MT to MAbs has been accomplished so that the targeting capability of the MAb and the excellent metal-binding properties of the MT are preserved.

Labeling Antibodies with Metals Using Bifunctional Chelates. W.C. Eckelman and C.H. Paik. *National Institutes of Health Bethesda, MD; and George Washington University Medical Center, Washington, DC.*

Metallic radionuclides, especially technetium-99m (^{99m}Tc) are attractive radiolabels because of their superior nuclear properties for imaging with the Anger camera. Much of the antibody labeling to date has been with iodine. As with other radiopharmaceuticals, the trend is to radiolabel antibodies with metallic radionuclides, especially indium-111 (^{111}In) and ^{99m}Tc, in order to improve the imaging characteristics. Although there have been attempts to radiolabel antibodies directly with metallic radionuclides, especially ^{99m}Tc, in general, these are weak chemical bonds. Therefore, the general approach of using a bifunctional chelate has been used. A number of chelates have been synthesized especially for the purpose of linking a chelating agent to a specific functional group of the antibody. In addition, available chelating agents such as diethylenetriamepentaacetic acid have been linked to an antibody by forming an active ester. Although this technique could be expected to produce a stable metal protein bond, the concentration of the chelating agent is so low that some nonspecific binding results. In addition, the introduction of the chelating agent decreases the immunologic activity of the antibody. We have shown that the nonspecific binding can be minimized by radiolabeling in the presence of excess chelating agent. We have also shown the effect of an increasing number of chelating agents on immunologic activity but this trend appears to be different for each antibody. Radiolabeling has been carried out by exchange labeling with specific activities in the range of 1 to 10 μCi/μg. Both ^{111}In- and ^{99m}Tc-labeled antibodies have the potential to be sensitive diagnostic agents.

DTPA-Coupled Proteins: Procedures and Precautions. D.J. Hnatowich. *University of Massachusetts Medical Center, Worcester, MA.*

While demonstrating that the cyclic anhydride of diethylenetriaminepentaacetic acid (DTPA) may be used to covalently attach this chelator to proteins, it was necessary to develop several new procedures and techniques. We were able to show that coupled proteins may be labeled with indium-111 (^{111}In) simply by transcomplexation from a weaker complex such as the acetate to avoid subjecting the protein, even momentarily, to the acidity of the chloride. However, we have also observed that too high a concentration of acetate and other complexing agents such as citrate will interfere with protein labeling by competing with DTPA for the radioactivity. In agreement with other investigators using bifunctional chelate methodology, we have observed that trace metals may interfere with the labeling. Accordingly, we developed a simple paper chromatographic assay which may be used to establish whether trace metals are present at interfering concentrations and to identify their sources. We have also employed a hydrolyzed control assay to determine whether and to what degree the radioactivity is bound to protein other than at the DTPA groups. We have developed a simple method of measuring the average number of DTPA groups per protein molecule which involves labeling the products of coupling with ^{111}In and de-

termining the fraction of radioactivity which is protein bound. The stability of ^{111}In and other radioisotopes on coupled protein has been determined in 37°C serum by injecting serum directly into an high performance liquid chromatgraphy to separate by size exclusion the product of transcomplexation and oxidation of the label. Using these and other techniques, we have shown that the coupling can be effective for a variety of proteins, even at low protein concentrations (in the μg/ml range) and that the coupled protein may be effectively labeled with a variety of radionuclides.

Consideration for the Preparation of Radiolabeled Monoclonal Antibodies with High Specific Activity. M.L. Thakur, P. Thiagrajan, S. McKenney, C.H. Park, and P. Maurer. *Thomas Jefferson University Hospital, PA.*

One of many potential applications of radiolabeled monoclonal antibodies (MAb) is their use as an agent to label specific type of blood cells in whole blood. We have developed MAb specific for human platelet membrane glycoprotein III.a and determined that each human platelet has 16,000–22,000 glycoprotein III.a receptors. One of the antibodies (B59.2) also reacts with canine platelets.

Thirty milliliters human blood drawn for platelet labeling normally contains $\sim 5 \times 10^9$ platelets totaling $\sim 10^{14}$ receptors. These will be saturated by 25 μg MAb (1 μg MAb = 4×10^{12} molecules). Since such saturation may adversely affect platelet function, not more than 10 μg MAb (4×10^{13} molecules) are cautiously considered safe for platelet labeling.

Indium-111 (^{111}In) has been chosen as a radioactive tracer for labeling MAb through diethylenetriaminepentaacetic acid (DTPA) bifunctional chelation. In order to avoid MAb polymerization and to preserve their biological activity only 0.3 DTPA molecules per MAb molecule are considered desirable [*J Nucl Med* 24: 1158–1163, 1983; *Int J Appl Radiat Isot* 35: 554–557, 1984]. This makes the total DTPA molecules available for chelating ^{111}In to be $\sim 1.2 \times 10^{13}$. Theoretically, these should be able to chelate $\sim$1 mCi ^{111}In (1.3×10^{13} ions, 1:1 ratio) and provide labeled MAb with specific activity 13 Ci/m*M*.

Faced in achieving such a high specific activity, however, are several practical problems. Examples are (a) handling 10 μg MAb without their loss due to nonspecific absorption during DTPA conjugation, purification, and storage; (b) the low MAb concentration for optimal labeling [300 μg/ml are considered lowest acceptable (*J Nucl Med* 24: 1158–1163, 1983)]; and (c) eliminating metal ion impurities from reagents, etc.

We have labeled MAb with ^{111}In (5 Ci/m*M*) and iodine-125 (^{125}I) (iodogen, 2.4 Ci/m*M*) and found them to have saturable binding to human platelets. Scatchard analysis of data revealed high MAb affinity (^{111}In MAb 83-133 n*M* compared to that of ^{125}I MAb 120–150 n*M*) to human platelets. In vivo evaluation of (B59.2) ^{111}In MAb is in progress.

A Practical Approach to the Preparation of High Specific Activity Indium-111-DTPA-Labeled Bioactive Proteins. S.S. Zoghbi, R.D. Neumann, and A. Gottschalk. *Yale University School of Medicine, New Haven, CT.*

The use of high specific radioactivity of labeled biologically active proteins makes the detection of a limited number of receptor sites possible and the radiolabeling procedure economical and technically easy to perform. The conjugation of diethylenetriaminepentaacetic acid (DTPA) to 100–500 μg protein in concentrations of 0.5–1.0 mg/ml is feasible by a modification [*Radiology* 149(P): 243, 1983] of the carboxy carbonic anhydride method of Krejcarek [*Biochem Biophys Res Comm* 77:581, 1971]. Concentrated anhydride of DTPA is added to the protein solution at a molar ratio of 800:1. The free DTPA is removed by minicolumn gel filtration and centrifugation (2,000 x *g* for 1 min) resulting in an average of 0.3 DTPA per protein molecule. Using the minicolumn gel filtration does not cause significant protein dilution. The radiolabeling with indium-111 (^{111}In) is done by diluting a small aliquot of the conjugated protein in citrate or acetate buffer system containing the $^{111}In^{3+}$ at any pH between 3.5 and 6.0. pH is selected so that it is away from the isoelectric point of the protein. These buffers act as auxillary chelating agents for ^{111}In and the nonspecific binding of ^{111}In to unconjugated protein at the abovementioned pH range is 1.8–7.3% in the acetate buffer and a constant of 3.7% in the citrate buffer. The highest possible specific activity can be achieved by titrating a desired amount of $^{111}In^{3+}$ with aliquots of the conjugated protein while monitoring the radiolabeling efficiency with instant thin layer chromatography and three developing solvents until no free $^{111}In^{3+}$ is detected. Such preparation results in $\sim$4–27 μCi/μg [*Hybridoma* 3: 79, 1984]. Furthermore, using the same conjugated protein with $^{111}In^{3+}$ that has been purified from interfering contaminants such as cationic impurities the specific activity is improved by a factor of three approaching the theoretical maximum of 100 μCi/μg [*Invest Radiol* 19: 532, 1984]. Such high specific radioactivity preparations showed no deleterious effect on the bioactivity and specificity of the labeled antibody as measured by in vitro cell binding.

Quality Control of Iodinated and Chelated Radiolabeled Monoclonal Antibodies and Their Fragments by High Performance Liguid Chromatography, Immunoreactivity, and Serum Stability Before and After Patient Injection.* S.J. DeNardo, G.L. DeNardo, C.F. Meares, J-S. Peng, and W.C. Cole. *University of California; and Davis Medical Center, Sacramento, CA.*

Monoclonal antibodies present a unique and powerful tool for the development of new radiopharmaceuticals. These same molecules also require meticulous quality control in each step of the pharmaceutical development and production in order to achieve the desired results in vivo, or understand and modify deviations from these results.

Molecular variations may occur at any time in the hybrid clone production of a chosen monoclonal antibody and these changes may affect immunoreactivity, fragment production, radiolabeling and in vivo biodistribution. We, therefore, evaluate each chosen monoclonal antibody and each new production batch for evidence of change by a stepwise pH gradient elution of protein A, as well as HPLC-DEAE (HPLC-DEAE) and HPLC TSK-3000 chromatography.

Following radioiodination or bifunctional chelate attachment (with and without the radiometal), we perform rechromatography on HPLC-DEAE and TSK-3000. The radiolabeled peaks are also evaluated for radioimmunoreactivity by serial dilutions in solid phase assay against cell membrane or

fix cell antigen. These methods of characterization demonstrate any major immunochemical alterations caused by the labeling process. We have found that excess chloramine-T/protein, excess chelate/protein and major changes from physiologic conditions may result in aggregates, macromolecules, and degraded fragments.

In vitro stability studies in serum have provided us with an effective challenge of these radiopharmaceuticals by the competitive protein environment they experience in vivo. Indium-111, cobalt-57, and copper-67 (^{67}Cu), attached by diethylenetriaminepentaacetic acid (DTPA) or BEDTA, and ^{67}Cu by TETA have demonstrated varied lability of these radiometal chelate labeled antibodies in the presence of protein ligands competing for the radiometal. Correlation of the radiopharmaceutical with plasma and urine samples from in vivo studies, studied by the same chromatographic methods, allows combined evaluation on in vivo and in vitro characteristics, and will lead to better understanding of the clinical relevance of any radiochemical or immunochemical changes in these complex agents.

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In Vitro Measurement of Avidity of Radioiodinated Antibodies. C.C. Badger, K.A. Krohn, I.D. Bernstein, and J.A. Ledbetter. *Fred Hutchinson Cancer Research Center, University of Washington and Genetic Systems Corp., Seattle, WA.*

An in vitro evaluation of the binding of radiolabeled antibodies to their target antigen prior to their in vivo use is essential both in the selection of antibodies for study and to evaluate the effects of the labeling procedure. The majority of investigations using radiolabeled antibody have emphasized assays of the proportion of antibody that will bind to the target antigen, usually under conditions of antigen excess, as a measure of immunoreactivity. However, the binding of radiolabeled antibody to cell surfaces is also dependent on antibody avidity. Avidity can be determined from a Scatchard analysis of binding at varying antibody concentration. In addition, since Scatchard plots vary in characteristic manner with contamination of the antibody preparation by radiolabeled molecules that do not bind antigen, these assays can provide a useful measure of antibody purity.

Using examples from our studies of antibody therapy of murine lymphoma we have reviewed the method of Scatchard analysis and the determination of antibody avidity. The usefulness, as well as the limitations, of this technique to examine the effects of iodination on antibody binding and to compare different monoclonal antibodies against the same target antigen has been examined.

Nondehalogenation Mechanisms for Excretion of Radioiodine After Administration of Labeled Antibodies.* G.L. DeNardo and S.J. DeNardo. *University of California; and Davis Medical Center, Sacramento, California.*

Rapid clearance from tissues and excretion into the urine of radioactivity after i.v. administration of radioiodinated antibodies has been reported by several investigators. These investigators have also reported that the concentration of radionuclide in cancerous tissue was less after administration of a radioiodinated antibody than after administration of the same antibody radiolabeled with a metal chelate. Unfortunately, there has been a propensity to assume that these observations are solely due to in vivo dehalogenation of the antibody without considering other mechanisms. While we have also observed discrepancies between the behavior of a radioiodinated and a metal chelate radiolabeled antibody, our observations suggest that the conditions for radioiodinating, storing, and administering the antibody can contribute to accelerated loss of radiolabel from the antibody in vitro and in vivo.

Three different monoclonal antibodies and their fragments have been radioiodinated at varying ratios of chloramine-T to protein and iodine to protein. Molecular sieving high performance liquid chromatography, cellulose acetate electrophoresis, live cell and solid phase immunoreactivity, and in vivo quantitative pharmacokinetics revealed significantly greater degradation of antibody and release of radiolabel when the antibody is radioiodinated at greater levels of chloramine-T. Hepatic accumulation and catabolism of antibody also is a mechanism for release of radiolabel and appears to be dependent upon amount of administered antibody.

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Correlating Labeling Chemistry and In Vitro Test Results with the Biological Behavior of Radiolabeled Proteins.* S.C. Srivastava. *Brookhaven National Laboratory, Upton, NY.*

A wide variety of iodination techniques as well as metal chelation methods are currently utilized for radiolabeling of proteins, including monoclonal antibodies and their fragments. These methods, particularly iodination, frequently give products with altered biological behavior. Sensitivity to labeling chemistry, however, is quite variable for different proteins and antibodies. Additionally, different aspects of in vivo behavior are affected, e.g., specificity of binding to in vivo antigens (for antibodies), kinetics of blood and tissue uptake and clearance rates and routes of excretion, hormonal activity, etc. The effect of labeling chemistry and purification schemes on these parameters must be clearly understood and minimized as much as possible. It is necessary to establish a correspondence between the in vitro test results and the observed in vivo behavior in order to obtain predictable and reproducible data. Various factors affecting the binding to platelets of an antiplatelet monoclonal antibody, 7E3, following iodination with iodine-123, iodine-125, iodine-131, and chelation labeling with indium-111 and technetium-99m (^{99m}Tc) have been investigated. A critical evaluation of the techniques for purification and in vitro characterization has been studied using the ^{99m}Tc-tin-human serum albumin model system as an example, as well as a comparison of both direct and indirect methods of labeling with respect to substitution level, specific activity, etc. These results and our experience with various antibodies indicate that in order to achieve maximum efficacy in imaging or therapy applications, individual antibodies will require a careful optimization of labeling and purification procedures with diffeent radionuclides.

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Comparison of Monoclonal Antibodies Labeled by Iodination with Those Labeled with Indium-111 Using Bifunctional Chelates. F.L. Otsuka and M.J. Welch. *Washington University School of Medicine, St. Louis, MO.*

In the past, much interest has been focused on the use of radioiodinated antibodies; either polyclonal or monoclonal, in

tumor imaging. Recently, bifunctional chelates have been used to label antibodies with radioisotopes of metals such as indium-111 (^{111}In). To compare iodinated antibodies with those labeled with ^{111}In we have developed a model system that does not involve the use of tumors but instead uses agarose beads to localize a synthetic antigen (dinitrophenyl) in rats' lungs. Using this system, the biodistribution of whole antibody (specific for dinitrophenyl) labeled with iodine-125 or ^{111}In and the corresponding $F(ab')_2$ or Fab fragments have been compared. We have evaluated deferoxamine, diethylenetriaminepentaacetic acid (DTPA) and 1-(para-bromoacetamidobenzyl)-ethylenediaminetetraacetic acid as bifunctional chelates for use with ^{111}In. All methods resulted in labeled antibodies or fragments that retained their capacity to bind to dinitrophenyl in vitro. When iodinated or ^{111}In-labeled antibodies and fragments were tested in animals, high uptake in the target tissue was observed with a maximum occurring between 4 and 24 hr following injection of the antibodies. Iodinated whole antibody cleared rapidly from the liver such that by 48 hr the %ID/g for this tissue was quite low. Similarly, iodinated fragments were cleared rapidly from the kidneys. In contrast to the results obtained for the iodinated whole antibody or fragments, the level of ^{111}In remained high in the liver or kidneys throughout the course of the experiment. From analyses such as these we hope to determine the preferred method to radiolabel antibodies for use in imaging studies.