Direct Labeling of Monoclonal Antibodies with Technetium-99m by Photoactivation

Thomas R. Sykes, Thomas K. Woo, Richard P. Baum, Pei Qi and Antoine A. Noujaim

Biomira Research Inc., Alberta, Canada; Dentistry-Pharmacy and Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Canada; and Johann Wolfgang Goethe University Medical Centre, Frankfurt/Main, Germany

Direct radiolabeling methods currently rely on the addition of exogenous chemical reagents to create the necessary binding sites for ^{99m}Tc binding to monoclonal antibodies (MAbs). This work describes the use of ultraviolet (UV) light to facilitate photoactivation of MAbs for ^{99m}Tc radiolabeling. **Methods:** The parameters of exposure wavelength and solution composition were investigated to provide a basis for further development. Based on these results, various murine MAbs and a chimeric MAb were photoactivated using a 300-nm (nominal) wavelength, eight-lamp (3.9 W each) photochemical reactor providing exposure for defined time periods. The MAb preparations were stored frozen and subsequently labeled by the addition of pertechnetate. For MAb-170, the photoactivated preparation was compared to a stannous ion reduced preparation by radiochemical (radiolabeling yield, serum stability cysteine challenge), biochemical (SDS-PAGE, IEF, SE-HPLC) and immunochemical (immunoreactivity) assays and biodistribution studies in mice. **Results:** Photoactivation produced high radiolabeling yields for all the MAbs studied and MAb-170 produced comparable in vitro quality control profiles and in vivo biodistribution data. **Conclusion:** The use of this relatively simple, short and easily controlled photoactivation process for MAbs facilitates facile radiolabeling with ^{99m}Tc and provides an alternative to the direct chemical radiolabeling procedures.

Key Words: technetium-99m; monoclonal antibodies; photoactivation

J Nucl Med 1995; 36:1913–1922

Radioimmunoscintigraphy has progressed considerably since its humble origins using ^{131}I-labeled polyclonal antibodies (*1*). The advent of more reproducible antibodies, most notably from monoclonal sources and more favorable radioisotope attachment methods for ^{99m}Tc has enabled further successes in the evaluation of these radioimmunopharmaceuticals. As diagnostic agents, current practice suggests an appropriate combination of ^{99m}Tc with a suitable whole antibody or its fragments can be successfully used to localize specific antigen sites in vivo, most notably from thrombotic, infectious, inflammatory or cancerous lesions (*2–4*).

The two primary approaches for accomplishing ^{99m}Tc binding to antibodies are based on the creation of binding sites for reduced ^{99m}Tc by modifying existing proteinaceous structures or by conjugating external agents capable of supplying this function. These have been termed direct and indirect, respectively, which identify whether endogenous structures or exogenous groups are used to facilitate ^{99m}Tc binding to the protein. Current methods of direct labeling make use of inorganic reagents such as stannous ion (*5*), sulphydryl reagents such as mercaptoethanol (*6,7*) and glutathione (*8*) or organic reagents such as phosphines (*9*), borohydrides (*10*) or ascorbic acid/dithionite (*11,12*) to generate sulphydryl groups. Direct labeling can also be accomplished by derivatization of pertechnetate into a more reactive species and its subsequent binding to native (*13,14*) or modified (*15*) immunoglobulins. Numerous ligands, such as DTPA (*16*), thiosemicarbazones (*17*), BATO derivatives (*18*), hydrazino nicotinimide (*19*), iminothiolane (*20*), macrocycles (*21*) and various other nitrogen and sulfur-based compounds (*22–24*) have been evaluated as bifunctional ligands in the indirect approach. The most commonly cited concerns regarding the indirect approach involve the lack of general availability of suitable bifunctional ligands, the potential for nonspecific protein binding of the reduced ^{99m}Tc, the need for postconjugation purification of unreacted ligand and potential alterations in monoclonal antibody (MAb) immunoreactivity (*25–27*). Previous work with direct methods has demonstrated some requirements for intermediate purification steps, their potential for generating fragments (especially from $F(ab')_2$), problems in reaction control and the relative instability of the ^{99m}Tc protein bond in comparison to specific direct and indirect methods (*25–27*).

This work describes the development of a nonchemical process for direct labeling of MAbs which makes use of ultraviolet (UV) light exposure of the protein to facilitate ^{99m}Tc attachment, suitable for use as radioimmunopharmaceuticals for diagnostic clinical applications.

Received May 31, 1994; revision accepted Feb. 14, 1995.

For correspondence or reprints contact: T.R. Sykes, PhD, Biomira Research Inc., 1134 Dentistry-Pharmacy, University of Alberta, Edmonton, Alberta, Canada T6G 2N8.

TABLE 1
Effects of Monoclonal Antibody Matrix and Stannous Ion Formulation on the Photoactivation of MAb-170*

MAb matrix solution (concentration; pH) A	Stannous ion complexing solution† (concentration; pH) B	Reaction solution volume ratio A/B	Stannous ion amount (μg/mg MAb)	% Radiolabeled protein‡
Tris 0.1 *M*; pH = 7.5)	Phosphate (0.5 *M*; pH = 7.4)	60/2	20	89
Acetate (0.05 *M*; pH = 5.5)	Phosphate (0.5 *M*; pH = 7.4)	60/2	20	85
Phosphate (0.05 *M*; pH = 7.4)	Phosphate (0.5 *M*; pH = 7.4)	60/1	10	96
		60/2.5	25	91
		60/3.2	32	81
Phosphate (0.05 *M*; pH = 7.4)	Pyrophosphate (0.1 *M*; pH = 7.4)	60/1	10	97
		60/2	20	96
		60/4	40	92
Phosphate (0.05 *M*; pH = 7.4)	EDTA (0.1 *M*; pH = 7.4)	60/1	10	38
		60/2	20	54
		60/5.2	52	55

*MAb-170 = 1.0 mg per vial at a concentration of 5 mg/ml. Photoactivation conditions = 30 min at 300 nm.
†Stannous ion = 3.0 mg/ml. ‡Mean of duplicate analysis.

MATERIALS AND METHODS

Monoclonal Antibodies

Several MAbs were used to evaluate the potential of the photoactivation process: MAb-B80 (reactive against human prostate-specific antigen), MAb-174 and MAb-ch174 (reactive against human squamous-cell carcinoma cell lines and tissue) and MAb-B43.13 and MAb-B27.1 (reactive against CA-125 antigen). Further optimization and a more complete analysis of the process was conducted with MAb-170 (reactive against human adenocarcinoma cell lines and tissue). These MAbs were obtained from Biomira Inc. (Edmonton, Alberta). MAb-425, obtained from Merck (Darmstadt, Germany), is reactive against the human epidermal growth factor (EGF) receptor. The MAbs were purified from ascitic fluid or cell culture supernatant prior to receipt at a final purity of greater than 92% monomeric IgG by size-exclusion high-performance liquid chromatography (SE-HPLC).

Kit Preparation

Monoclonal antibodies were prepared in 50 m*M* sodium dihydrogen phosphate and 150 m*M* sodium chloride at pH 7.4 (PBS) at a concentration of 1.8–5 mg/ml. A stannous phosphate solution was prepared by dissolving 150 mg stannous chloride dihydrate in 25 ml 500 m*M* sodium dihydrogen phosphate/150 m*M* sodium chloride, pH 7.4, to form a solution which is 3.16 mg/ml in stannous ion concentration. The final kit solution consisted of a 20–40:1 (V/V) mixture of the MAb solution and the stannous phosphate solution. This was dispensed as 0.21–0.46-ml aliquots (1.0 mg MAb; 32–35 μg stannous ion) into sterile, nitrogen-purged, presealed, 2-ml tubular injection vials which served as the reaction vessel for the photoactivation process. Stannous ion reduced (SnR) MAb-170 was produced by treating the MAb with stannous ion (in a tartrate/phthalate buffer) according to a modification of a method by Rhodes (*5*). The solution was incubated at room temperature for 48 hr to allow for complete reaction. Vials of SnR MAb-170 and photoactivated MAbs were stored at −20°C. The MAb-170 kits produced by these methods were used as a comparison for quality control measurements, stability assays and biodistribution studies.

Photoactivation Unit and Conditions

The photoactivation unit consists of a photochemical reactor equipped with three replaceable sets of eight UV lamps of nominal 300 (3.9 W each), 254 (8.0 W each) and 365 nm (4.5 W each) wavelength emission installed concentrically along the inner wall of the reactor chamber. Cooling is provided by a fan unit positioned at the bottom of the chamber. The samples for photoactivation (eight per run) were positioned on a merry-go-round unit which can be lowered into the chamber and rotates the samples to ensure uniform exposure. Sample volumes were normally standardized to 0.4 ml. After the experimental photoactivation time (up to 120 min), samples can be removed from the photochemical reactor and radiolabeled with ^{99m}Tc-sodium pertechnetate or stored frozen at −20°C until required.

Matrix and Stannous Ion Studies

The effects of solution composition (matrix) and stannous ion formulation were evaluated by preparing MAb-170 in three buffer solutions (0.1 *M* Tris, pH 7.5; 0.05 *M* acetate, pH 5.5; 0.05 *M* phosphate with 0.15 *M* sodium chloride, pH 7.4) at approximately 5 mg/ml and adding selected stannous ion complexes [stannous phosphate, stannous pyrophosphate, stannous ethylenediaminetetraacetic acid (EDTA) at various concentrations (Table 1)]. These solutions were photoactivated for a standardized period of 30 min, radiolabeled with ^{99m}Tc and analyzed by SE-HPLC (as described later).

Wavelength Studies

To compare the effect of incident wavelength on the radiolabeling yield, it was necessary to use band-pass filters to isolate selectively a narrow spectrum from the relatively broad range emission of the three available lamp sources. The filters permitted

the following wavelengths to be transmitted: 254 ± 5 nm, 280 ± 5 nm, 289 ± 5 nm, 317 ± 5 nm, 334 ± 5 nm and 365 ± 5 nm. For each wavelength study, the filter was inserted in front of the vials containing the MAb solution in identical geometry so that only exposure to the filtered UV light was permitted and extraneous lamp emissions were physically blocked. Given the differing relative intensities of the source lamp emissions as a function of wavelength, however, it was necessary to apply a correction factor to the filtered emissions to normalize the intensity of the incident light. This was accomplished by estimating the emission intensity at the appropriate wavelength (which matched that of the filter) for the appropriate lamp (which was closest to the filter wavelength) and varying the length of exposure so that the total incident flux for each wavelength study was equivalent (time of exposure multiplied by the intensity equals a constant). Quartz vials, which are totally transparent to all these wavelengths, were used for this study. The results are expressed as the percentage of radiolabeling yield relative to the highest radiolabeling yield obtained for this study.

Radiolabeling and Quality Control

The MAb kits were radiolabeled with ^{99m}Tc by the direct addition of ^{99m}Tc-sodium pertechnetate (up to 2.0 ml, approximately 740–1480 MBq) to the (thawed) vial containing the photoactivated (or SnR) MAb. The solution was allowed to incubate for 20 min before testing or use.

Routine radiochemical quality control on the radiolabeled MAbs utilized SE-HPLC to separate high molecular weight impurities (dimeric and polymeric forms), monomeric MAb, low molecular weight impurities (fragmented forms), reduced ^{99m}Tc complexes and ^{99m}Tc-sodium pertechnetate. A TSK SWXL3000 analytical column with a TSK SWXL guard column (Supelco, Bellefonte) (running at 1.0 ml/min with 100 m*M* sodium dihydrogen phosphate, 100 m*M* sodium sulphate, 0.05% sodium azide, pH 7.2, as the mobile phase) was used for this purpose. The eluate from the column was monitored by a flow-through UV absorbance detector at 280 nm and by radioactivity detection using an in-line NaI(Tl) crystal with associated electronics.

Biochemical quality control of the MAb-170 kits included SDS-PAGE and isoelectric focusing (IEF). SDS-PAGE was performed using a discontinuous system with a 10% resolving and a 4% stacking gel (100 × 80 × 0.75 mm) in a Mighty Small II Slab Gel Electrophoresis Unit (Hoefer, San Francisco, CA). The samples were prepared by boiling in the appropriate buffer for 5 min and running under both reducing (R-SDS-PAGE) and nonreducing (NR-SDS-PAGE) conditions at 20 mA for approximately 90 min. The gel was then stained with Coomassie Brilliant Blue R-150 for 30 min and then destained with a solution of methanol:acetic acid:water (25:10:65). The stained protein bands were then scanned using a 1312 Gel Scanner (Isco, Lincoln, NE) and an HP3390A Integrator (Hewlett-Packard, Palo Alto, CA) to obtain the relative percentage of each band. Isoelectric focusing was performed using a precast agarose IEF gel, pH 3–10 (Isogel, Mandel Scientific, Edmonton, Canada). The samples were applied together with broad pI standards and run under constant power at 5 W for the first 15 min, 15 W for the next 50 min and 25 W for the last 15 min. The IEF gel was then fixed under a trichloroacetic acid/sulphosalicylic acid/methanol/water solution and then stained with Coomassie Brilliant Blue R-250. The gel was finally destained with methanol/acetic acid/water and allowed to air dry. The pI range of the samples was determined from the pI standard calibration line and the number of major bands was observed visually.

The immunoreactivity of the MAb-170 kits was determined by an anti-idiotype radioimmunoassay. Polyclonal rabbit anti-MAb-170 (0.5 μg, Biomira Inc.) was coated on polystyrene tubes overnight at room temperature and washed to remove unbound antibody. Three sets of tubes containing a series of increasing identical amounts of the photoactivated MAb-170, the SnR MAb-170 and a reference MAb-170 were incubated with an aliquot of ^{125}I-MAb-170. After a 2-hr incubation period, the sets of tubes were washed again and the bound ^{125}I radioactivity and total ^{125}I input standard were measured by gamma scintillation counting. Graphs of the percent bound (and percent inhibition) versus the amount of MAb-170 added were prepared and the concentration at 50% inhibition (IC-50) was determined. The immunoreactivity index was calculated from the IC-50 values for the reference MAb-170 divided by the IC-50 values for the photoactivated MAb-170 or SnR MAb-170. Index values close to 1.0 represent equivalent immunoreactivity by this assay. Immunoreactivity for MAb-174 and MAb-c174 were measured using a similar (MAb-174 specific) anti-idiotype immunoassay. MAb-B80, MAb-B43.13 and MAb-B27.1 were assayed for immnoreactivity using standardized specific antigen-based inhibition immunoassays. MAb-425 was tested as previously reported (*28*).

In Vitro Stability Studies

The stability of photoactivated and SnR ^{99m}Tc MAb-170 against human serum was studied at 20 μg/ml MAb in human serum. The samples were incubated at 37°C and analyzed by SE-HPLC at 6 hr. Cysteine challenge was carried out by mixing equal volumes of a freshly prepared cysteine solution with a 1-mg/ml ^{99m}Tc MAb-170 solution to give a MAb-to-cysteine ratio of 1:50. The mixture was then incubated at room temperature for 5 hr and analyzed by SE-HPLC. Single analysis from two experiments was performed for each system and reported as the mean plus or minus the standard deviation.

Biodistribution Studies

Conventional Balb/c mice were injected intravenously via the tail vein with 20 μg (20 μCi) of photoactivated ^{99m}Tc MAb-170 or with 20 μg (20 μCi) of SnR ^{99m}Tc MAb-170. At 3 and 24 hr after injection, the mice (four animals per group) were killed by exsanguination under carbon dioxide anesthesia. The blood and other organs of interest were collected, weighed and analyzed in a programmable gamma counter. Dose standards containing a known dilution of the injectate were also prepared and counted as a measure of the injected dose. The percent of injected dose per gram of tissue was calculated from the decay-corrected data. The individual tissue data were analyzed using an unpaired two-tailed t-test for statistical significance judged at the 5% confidence level.

RESULTS

Preparation and Radiolabeling of MAb Kits

The procedures used to prepare these MAb kits involve photoactivation (and SnR) on an individual vial basis, although it is convenient to prepare numerous vials simultaneously. The preparations can be stored as the frozen liquid until required for radiolabeling with ^{99m}Tc-pertechnetate. The total time required for SnR is considerably longer, requiring 2 days incubation for MAb-170 compared to the less than 1 hr needed for an eight-vial photoactivation batch.

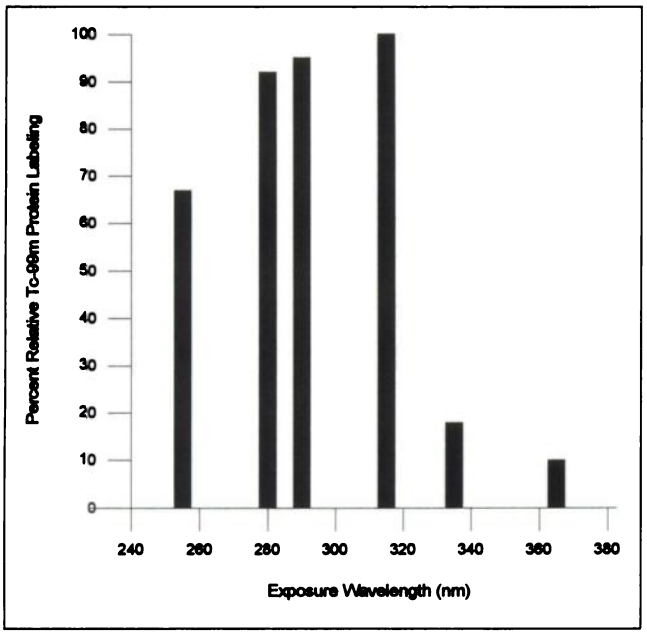

FIGURE 1. Relative radiolabeling yield of photoactivated MAb-170 with ^{99m}Tc as a function of wavelength of UV light exposure (normalized for exposure intensity).

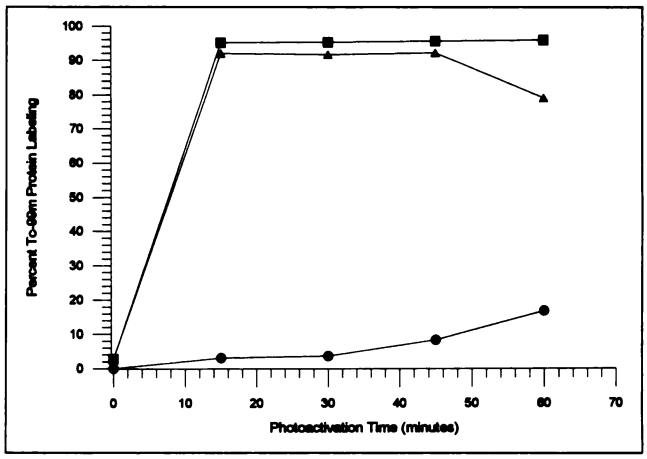

FIGURE 2. Radiolabeling yield of photoactivated MAb-170 components with ^{99m}Tc as a function of the duration of the photoactivation process. Total protein (-■-); polymeric MAb-170 (-●-); monomeric MAb-170 (-▲-).

It is convenient to prepare a solution containing the necessary ingredients for final product radiolabeling prior to photoactivation, and selected combinations of MAb, buffers, pH, stannous complexes and stannous concentrations were selected for evaluation, as shown in Table 1. The volume ratios used in this study (up to 60:1) ensures that the reaction solution content is largely contributed by the MAb matrix, and that even small amounts of stannous (and ^{99m}Tc) complexing agents play a role in the final radiolabeling yields. The effects of Tris, acetate and phosphate buffers with stannous phosphate at 20–25 μg/vial results in comparable radiolabeling yields. When stannous pyrophosphate is substituted for stannous phosphate, the yields are at least as good; the yields for EDTA are poor. Attempts to modify the radiolabeling yield and increase product stability by raising the stannous content produced similar or slightly reduced values. Therefore, further characterization of the photoactivation process was carried out using the sodium phosphate/stannous phosphate formulation with approximately 25 μg stannous ion per milligram of MAb.

The relative radiolabeling yield of MAb-170 as a function of wavelength for our narrow-band pass filter study is shown in Figure 1. The results demonstrate a clear preference for wavelengths between 280–313 nm, with 254 nm somewhat less effective and 334 and 365 nm exposure resulting in significantly reduced radiolabeling with ^{99m}Tc. We therefore used 300-nm (nominal) lamps as the source of UV light for photoactivation. The spectral emissions of these lamps are not monochromatic, with a range of 250–350 nm, but about 90% of the spectral energy is emitted at 300 ± 30 nm with the centroid at approximately 300 nm. The use of glass vials provides convenience but requires longer exposure times due to their UV light absorption characteristics compared to quartz tubes, especially at the shorter wavelengths. The other critical factor is related to exposure time (at a fixed intensity, vial configuration and wavelength) as shown in Figure 2. The protein labeling for MAb-170 with ^{99m}Tc rises extremely fast to approximately 95% after 15 min and remains relatively constant. The most notable detriment to further exposure past 15 min (well after optimum radiolabeling has occurred) is the significant increase in high molecular weight forms (polymers or aggregates) observed as radiochemical impurities. This is significantly influenced by the initial level of protein polymers such that those present at the time of exposure seem to catalyze further aggregation within the product.

The data for several murine IgG1 (kappa) MAbs, a murine IgG2a (kappa) MAb and a chimeric MAb (ch174) incorporating a human IgG_3 constant region are also shown (Table 2) with similar high yields, indicating that subclass/species changes should not significantly affect attainable yields. The variable parameters of exposure time and protein concentration demonstrate that a range of conditions exist under which radiolabeling may be achieved.

The radiolabeling procedure of the thawed photoactivated MAb can accommodate specific activities in the range of 740–1480 MBq/mg protein, which is sufficient for clinical studies with most whole MAbs even when imaging at 24 hr after injection.

Quality Control

The radiochemical and biochemical chromatograms of a pre- and postphotoactivated ^{99m}Tc MAb-170 kit is shown in Figure 3. These profiles were generated using a high-resolution SE-HPLC column commonly used for MAb analysis and separates the major radioactive and UV-absorbing impurities on this basis. The presence of dimeric and polymeric forms (<4%) of the MAb and low molecular weight protein (<1%) can be observed from the absorption profile

TABLE 2
Technetium-99m Radiolabeling of Photoactivated Monoclonal Antibodies

MAb	Subclass	Concentration (mg/ml)	Reaction duration (min) With photoactivation	Reaction duration (min) Without photoactivation	Percent ^{99m}Tc radiolabeled protein
MAb-B80	mIgG1	5.0	60	—	91.8
			—	120	0.7
MAb-174	mIgG1	1.8	5	—	97.5
			—	5	3.2
MAb ch174	hIgG3	1.0	10	—	97.0
			—	na	na
MAb-B43	mIgG1	6.0	60	—	88.2
			—	120	6.1
MAb-B27.1	mIgG1	5.0	30	—	86.5
			—	120	4.9
MAb-425	mIgG2a	5.0	30	—	89.4
			—	15	15.0

na = not available.

and do not significantly increase after photoactivation. Polymeric forms exist primarily as dimers with higher oligomers generated only at the longest exposure times. The radiochromatogram shows the corresponding ^{99m}Tc associated with these peaks (<5% for the polymeric forms and <2% for the low molecular weight forms, respectively). Additional radiolabeled species are observed eluting later representing very small amounts of [^{99m}Tc]pertechnetate and ^{99m}Tc complexes. The recovery of total radioactivity from the column was greater than 97% as confirmed by a peroxide column washing procedure (*29*). Total protein labeling was >95% and monomeric MAb-170 was greater than 90%. For ^{99m}Tc SnR MAb-170, these values were approximately 92% and 84%, respectively.

Additional biochemical data are presented in Table 3 using classical standardized methodologies. Photoactivated MAb-170 retains a large proportion of its composition as intact whole MAb (85%), even under denaturing conditions (NR-SDS-PAGE), and under reducing/denaturing conditions is almost completely converted to the expected heavy and light chains. This is distinct from the SnR MAb-170 in which NR-SDS-PAGE shows considerable antibody fragmentation using this analysis. The IEF values are consistent with those of native MAb-170 for both methods. The immunoreactivity index values of photoactivated MAb-170 and SnR MAb-170 are 0.9, indicating their inhibitory ability to the binding of ^{125}I-MAb-170 to the "antigen mimic" anti-idiotype antibody is at least 90% of the reference MAb-170 using this assay format. The ability of these MAb-170 preparations to react specifically with target adenocarcinoma tumor tissue by immunohistochemical analysis was also retained (data not shown). All other MAb biochemical results demonstrated less than 6% HMW (aggregate) material and adequate retention of immunoreactivity.

In Vitro Stability Studies

The stability of ^{99m}Tc-MAb-170 prepared by photoactivation and SnR was evaluated by the simple incubation of specific concentrations in the presence of either normal human serum or cysteine. As shown in Figure 4, both methods show a relatively small and comparable amount (10%–13%) of translocation to cysteine, while there is no observed change in the protein binding distribution in serum under these conditions.

Biodistribution Studies

Figure 5 presents the data generated from a 20-μg injection of ^{99m}Tc-photoactivated MAb-170 and from a similar group of mice injected with 20 μg ^{99m}Tc SnR MAb-170. The percent injected dose per gram of organ values for all tissues were not significantly different ($p < 0.05$) using an unpaired t-test at both the 3-hr and 24-hr time points.

DISCUSSION

The comparison of indirect versus direct radiolabeling of MAbs with ^{99m}Tc requires considerably more evaluation, especially at the clinical level where the significance of apparent in vitro differences in behaviour can be judged. Certainly, the lack of availability of bifunctional chelates, the necessity of unbound chelate purification and an efficient method to prevent nonspecific binding have to be overcome. Nonetheless, the conceptually attractive properties of high label stability and potential for ^{188}Re/^{186}Re substitution for therapy may offset these disadvantages. All of the current direct radiolabeling methods utilizing MAb modification require chemical reducing agents to cleave disulphide bonds. Some of these agents are potentially hazardous for in vivo use and must be rigorously purified from the MAb after treatment and may require two-step labeling procedures. Often control of the extent of reduction is difficult due to their strong reducing potential.

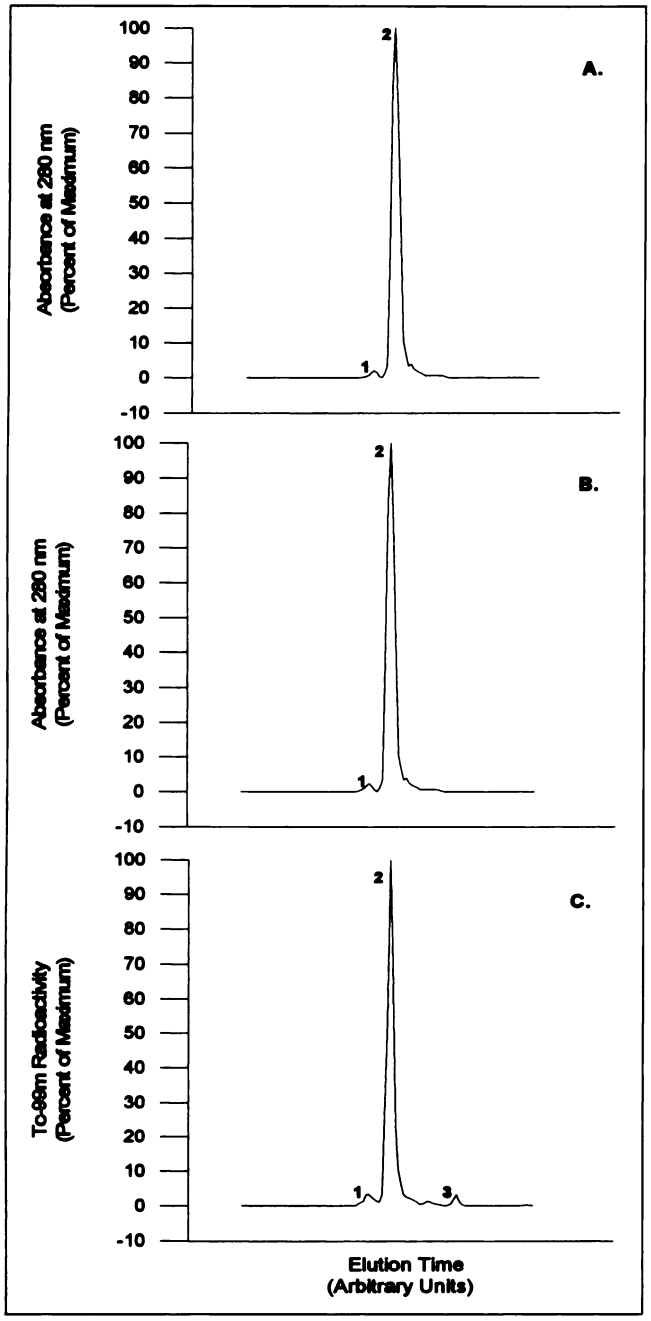

FIGURE 3. Biochemical and radiochemical quality control of MAb-170 by SE-HPLC. (A) Absorption (280 nm) chromatogram before photoactivation; peak 1 = high molecular weight protein (MAb polymers); peak 2 = monomeric MAb. (B) Absorption (280 nm) chromatogram after photoactivation; peak 1 = high molecular weight protein (MAb polymers); peak 2 = monomeric MAb. (C) Technetium-99m MAb-170 radiochromatogram after photoactivation; peak 1 = ^{99m}Tc high molecular weight protein (MAb polymers); peak 2 = ^{99m}Tc monomeric MAb; peak 3 = ^{99m}Tc complexes.

We observed that the slow SnR method does not always provide high radiolabeling yields, necessitating either intermediate product purification or final ^{99m}Tc product purification before use.

Our direct labeling process may be better understood on the basis of work performed in the early 1970s when UV exposure was used as a probe in the analysis of enzyme function. For one enzyme, papain, the exposure reportedly resulted in increased activity and thus it was photoactivated (*30*). The majority of enzymes showed progressive inactivity with UV exposure and the mechanism of such response has been attributed to the disruption of critical amino acids, most often cystine bridges (*31*). It is not surprising that the initial photodynamic effects of light interaction with proteins occurs primarily via energy absorption in aromatic amino acids, since their absorption characteristics are well established. In fact, for a generalized IgG molecule, it is estimated that approximately 10% and 80% of the total absorption may be attributed to its tyrosine and tryptophan content, respectively. The major sequelae of this absorption event is the production of a tryptophan and tyrosine radical cation with the associated hydrated electron and/or its aqueous radical counterparts. The nature and degree of this reaction depends on the hydrophilic/hydrophobic environment and both the primary and tertiary structure of the protein (*32*). Because cystine is an efficient electron acceptor it is extremely susceptible to cleavage to the corresponding two cysteine residues, thus creating free sulphydryl groups. It is also probable that an excited aromatic system is formed, which can transfer its energy via the peptide chain to a susceptible disulphide structure (*33*). Direct absorption of UV energy by cystine can occur, but this process is much more efficient at lower wavelengths (higher energies) where significant side reactions are also prevalent. Therefore, these conditions are less suitable for this purpose. The primary mechanism of photoactivation of MAbs is the indirect generation of sulphydryl groups which, under the described conditions for MAb-170, results in the formation of 1–3 sulphydryl groups per molecule as measured by Ellman's method (*34*).

The composition of the photoactivated solution emphasizes some important considerations for attaining high radiochemical yields. The lack of a selected MAb buffer influence at these pH values (Table 1) is understandable given the physical nature of the photoactivation process, such that relatively non-UV absorbing compounds such as Tris, acetate and phosphate would not be expected to play a major role. Highly aromatic buffer systems, however, by nature of their intense UV-absorbing properties, may alter the process. This relative independence of buffer (and pH) implies that a variety of MAb buffer solutions should be compatible with photoactivation and can therefore be chosen with some freedom to possibly enhance MAb stability. The presence of even small amounts of various stannous ion complexes (phosphate, pyrophosphate, EDTA) were evaluated in light of the critical role of stannous ion in pertechnetate reduction and the role of reduced ^{99m}Tc ligand formation necessary for ^{99m}Tc-MAb complex formation. It is apparent that stannous phosphate or stannous pyrophosphate in a phosphate environment produces successful radiolabeling, whereas stannous EDTA does not. Our data suggest that this is due to the formation of a highly stable ^{99m}Tc complex with EDTA which does not translo-

TABLE 3
Biochemical and Immunochemical Quality Control of Technetium-99m-MAb-170

Parameter	Method	Unit	Results	
			Photoactivation	SnR
Biochemical purity	R-SDS-PAGE	% H + L chains	100	100
	NR-SDS-PAGE	% IgG region	~85	~55
	IEF	No. major bands	4	4
		pI range	6.7–7.0	6.7–7.0
	SE-HPLC	% Monomeric MAb	>95	>95
Immunochemical reactivity	Anti-idiotype-RIA	IC-50 Ref/IC-50 Sample	0.9	0.9

SnR = stannous ion reduction.

cate to the MAb binding sites to a large extent during this time period. The amount of stannous ion was evaluated over a small range in this study, but it appears that amounts in the 20–40-μg/mg of MAb range are appropriate.

A primary consideration in the development of appropriate conditions for photoactivation is an assessment of UV wavelength. Our study utilized five narrow bandpass filters to select regions of the spectra for exposure and comparison of radiolabeling yields. The study design was hindered by the availability of only three lamp sources of various power emission and their lack of monochromatic properties. Our normalization process of estimating intensity at each wavelength for the appropriate lamps and varying exposure time to compensate for exposure intensity is reasonable but assumes no kinetic phenomenon occurs over the variable exposure duration at each wavelength. Nonetheless, the generated data are extremely realistic if the photoactivation as a consequence of protein absorption is considered. Therefore, the photoactivation effect for ^{99m}Tc radiolabeling is centered around the maximum ab-

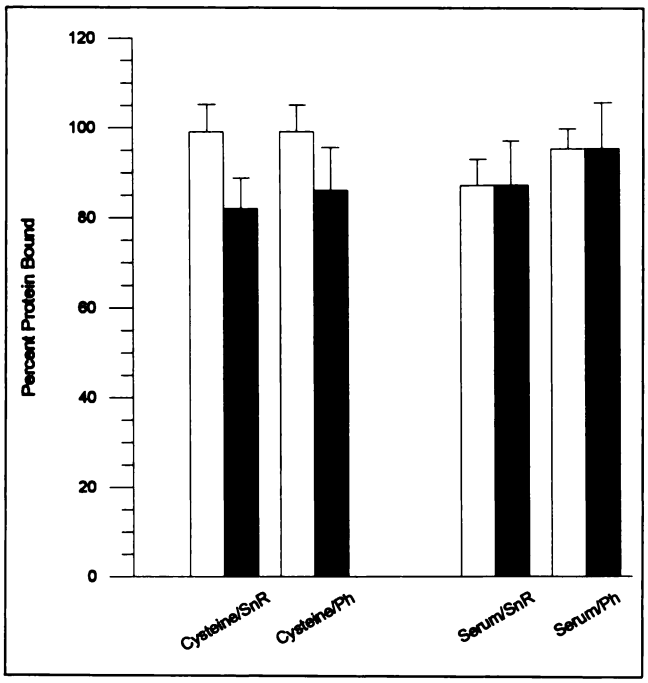

FIGURE 4. Comparison of the stability of ^{99m}Tc MAb-170 prepared by photoactivation (Ph) and stannous ion reduction (SnR) in the presence of cysteine (1:50 ratio for 5 hr at 1.0 mg/ml of MAb) and normal human serum (for 6 hr at 20 μg/ml of MAb). Control (□); cysteine/serum (■).

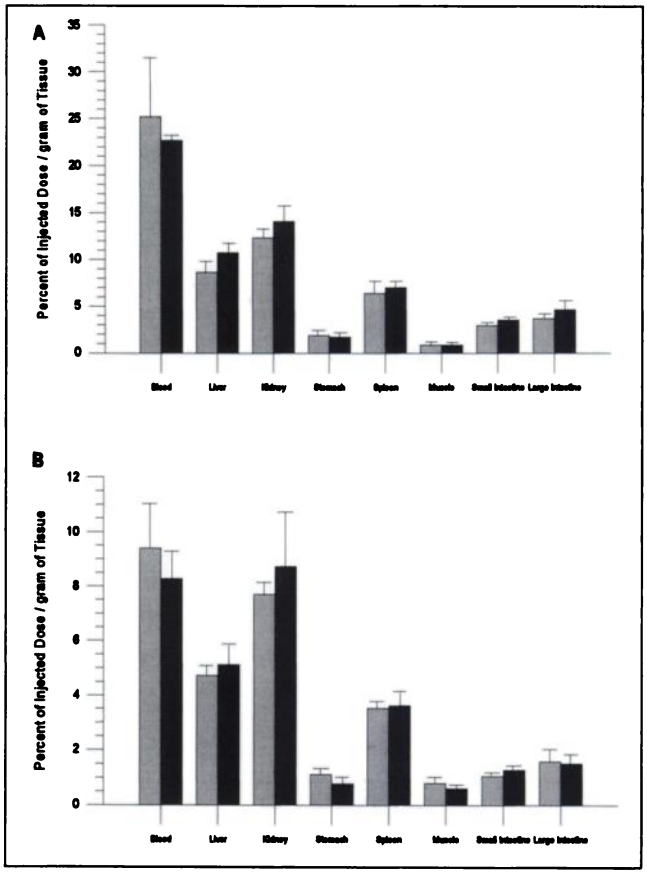

FIGURE 5. Comparison of the biodistribution pattern of ^{99m}Tc MAb-170 prepared by photoactivation and stannous ion reduction (SnR) in conventional mice at (A) 3 hr and (B) 24 hr after injection. Photoactivated ^{99m}Tc-MAb-170 (▨); SnR ^{99m}Tc-MAb-170 (■).

sorption of proteins, being highest around 280 nm. Based on this study, the use of 300-nm lamps (3.9 W each) were standardized for further photoactivation process development.

Various MAbs have been radiolabeled using the photoactivation process (Table 2) and it is also applicable to other disulphide containing proteins such as HSA (*35*). The results indicate that the technique is generally applicable to MAbs and exposure times normally in the order of 15–60 min produce high protein labeling yields even under these nonoptimized conditions for various protein concentrations. The data also show the variability of MAb response to UV exposure and highlights the need for individualized MAb photoactivation process development. As with any method, immunochemical and biochemical quality control must also be assessed prior to finalization of the reaction conditions. We have not observed any instance where satisfactory photoactivation could not be accomplished prior to significant deterioration of these criteria. The inclusion of control (nonexposed) conditions and their associated radiochemical yield values (Table 2) are proof of the requirements for photoactivation as the primary determinant of radiolabeling and the lack of substantial associated nonspecific ^{99m}Tc binding, which can be a problem in other direct methods (*36*). These features are related to the choice of buffer and stannous source (phosphate) so that minimal chemical reduction of disulphide bonds occurs under these conditions.

The resulting MAb-170 product (Table 2) and the other MAbs (data not shown) show excellent retention of their biochemical and immunochemical properties. For other direct chemical reduction processes, the MAb may exist primarily as dissociated heavy and light chains under denaturing conditions (*37,38*), as was evident in this study for SnR MAb-170. In distinction, photoactivated MAb-170 was observed to be primarily as intact whole MAb in the NR-SDS-PAGE analysis. Reduced antibodies retain their structure under native conditions due to the presence of noncovalent interactions (*39,40*), but if the interchain disulfides are cleaved, the heavy and light chains are readily observed under nonreoxidizing denaturing analysis such as used here. The significance of these analytical findings is not clear in that it may be a consequence of sample preparation for this test (*38*). Since both our preparations contain stannous ion, one may expect a similar level of in situ reduction during sample processing and thus it appears the results are not an artifact of the method. Therefore, the SDS-PAGE data probably represent the degree to which these bonds have been broken during the modification process. The relevance is further obscured in that under native conditions in vitro and in the blood in vivo, only intact MAb is observed (*41,42*). Perhaps more importantly, we have shown that the biodistribution of these preparations are comparable in spite of the differences in this analysis. Similarly, it has been reported that the biodistribution of radioiodinated MAb Mu-9 is unchanged after the reduction of up to three disulphide bonds producing six sulphydryl groups (*43*). We have not as yet ascertained whether a difference in their individual metabolic profile exits. The most likely observed effect of excessive photoactivation is the production of high molecular weight MAb polymers (aggregates) which is a well known phenomenon for UV exposed immunoglobulins (*44*) and can also be a problem for chemically reduced MAbs. As shown in Figure 2, the control of the appearance of aggregates provides a predefined endpoint for the process as a limit to relative high molecular weight impurities acceptable can be set. Fortunately, we have not observed a case where excessive aggregation is a problem before satisfactory radiolabeling occurs except when the original polymeric content of the MAb was high. Other potential consequences of UV exposure such as significant alteration of induced fluorescence and amino acid-specific changes generally occur after more intense exposure. The most critical functional assessment of MAb-170 integrity is observed in its immunoreactivity index values (Table 3), which are essentially the same as the control MAb. This is confirmed by its excellent tumor-localizing properties in a clinical setting (data not shown).

The conduct of serum and cysteine challenge experiments are useful to determine the degree of ^{99m}Tc MAb stability under in vitro conditions and provide potentially relevant information for the comparison of radiolabeling methods. We have demonstrated that the relative stability of photoactivated MAb-170 is comparable to that of SnR MAb-170 (Fig. 4). It is difficult to compare these results with others in the literature since the conditions employed are different. These data, therefore, are most relevant to the stated comparison between SnR MAb-170 and photoactivated MAb-170 as performed. In spite of these concerns, such studies are useful indicators of the relative radiochemical quality with some physiological relevance in that immediate and complete loss of ^{99m}Tc to serum proteins and/or serum sulphydryl groups would be expected to be undesirable. As previously demonstrated, there is some correlation of these assays with the in vivo behavior of ^{99m}Tc MAbs, the clinical significance of which requires further evaluation (*42*) since some degree of in vivo instability may be desirable to improve blood and or tissue clearance from nontarget sites which facilitates imaging. These assays also have some relevance to the nature of the ^{99m}Tc MAb bond in that we would expect a ^{99m}Tc to protein sulphydryl bond to be relatively sensitive to cysteine concentrations. Further proof of the nature of this bond can be realized from inhibition experiments whereby incubation of the photoactivated MAb (or SnR MAb) with sulphydryl-specific reagents such as N-ethylmaleimide and iodoacetamide completely inhibit ^{99m}Tc binding.

The biodistribution pattern of photoactivated MAb-170 in comparison to SnR MAb-170 is shown in Figure 5. The tissue content of ^{99m}Tc was remarkably similar for both preparations confirming the in vitro quality control and stability comparisons already discussed. Both preparations show the highest radioactivity in the blood which ap-

proaches that of the kidney at 24 hr. The high renal levels are a common finding for directly labeled ^{99m}Tc MAbs and indicative of their suggested metabolic handling by these organs (*40,41*). Other organs such as the stomach show little accumulation which provides evidence for the lack of significant pertechnetate as a radiochemical impurity in the preparations and/or in vivo oxidation of the product. The similar uptake of each preparation in the small intestine suggest the handling of a postulated biliary metabolite are also comparable. Since these are nontumored animals, no definite comparison on tumor uptake between the preparations is available. Given the similarity in their whole-body distribution (and radiochemical, immunochemical and biochemical status), it is likely this key biological factor would also be similar.

Based on the foregoing discussion, the photoactivation process has several unique features in comparison to current methodologies. To provide a single-vial radiolabeling kit, the only reagent required for addition to the MAb is a source of stannous ion. The nature and amount of this reagent may require some adjustments during the optimization of process conditions primarily due to the variations in the content and pH of the MAb media. Numerous buffers are compatible with photoactivation (in distinction from some of the chemical methods) which allows for flexibility in the choice of MAb buffer to facilitate optimum stability of the protein and final product processing, such as lyophilization. Additional properties of stannous ion stabilization and weak complexation of reduced ^{99m}Tc are desirable and available in the phosphate buffer/stannous phosphate system used here and numerous other systems should be equally comparable, such as demonstrated for stannous pyrophosphate. This simplicity of solution composition and the lack of any purification procedures (such as excess reducing reagent removal and ^{99m}Tc impurity removal) make the total product solution preparation an extremely uncomplicated and rapid process. In fact, the final purified MAb as a sterile nonpyrogenic solution in an injection vial becomes the reaction, radiolabeling and injection vessel such that MAb solution transfers are not required.

CONCLUSION

We have demonstrated the utility of the photoactivation process under individual vial conditions. The current apparatus allows for eight unit-dose vial exposures simultaneously, thereby permitting kit production batches for optimization studies and small-scale testing. This process can also easily be used in a radiopharmacy setting to prepare individual patient doses provided that daily control of the UV exposure conditions are maintained. Larger numbers of individual vials can be obtained by changing the exposure configuration if lot uniformity can be maintained. Other variations using slightly larger solution volumes and subsequent aliquoting into unit-dose vials are possible but re-evaluation of the exposure conditions must be performed and the general low penetration of the UV light into a bulk solution make this form of scale-up more difficult.

ACKNOWLEDGMENTS

The authors thank the following individuals for their contributions to this work: A. Abdul-Wajid, G. Boniface, A. Borowski, M. Denkiewcz, C. Erickson, S. Fromont, L. Gadowski, L. Golberg, A. Hertel, G. Hör, C. Yee, C. Legge, C. Lo, S. Luciak, L. Martin, A. McEwan, S. Muddukrishna, G. Murphy, S. Overgaard, M. Poglod, G. Stancyzk-Bresinska, V. Somayaji and C. Sykes. This work was presented in part at the 40th Annual Meeting of the Society of Nuclear Medicine, Toronto, Canada, June 8–11, 1993. Financial support was provided by Biomira Research Inc. and a grant from Industry Canada.

REFERENCES

1. Pressman D, Hall RF, Foote FW. The in vivo localization of anti-Wagner osteogenic sarcoma antibodies. *Cancer* 1953;6:619–622.
2. Granowska M, Britton KE. Radiolabeled monoclonal antibodies in oncology. II. Clinical applications in oncology. *Nucl Med Commun* 1991;12:83–98.
3. Chatal J-F, Peltier P, Bardies M, et al. Does immunoscintigraphy serve clinical needs effectively? Is there a future for radioimmunotherapy? *Eur J Nucl Med* 1992;17:205–213.
4. Baum RP, Cox PH, Hor G, Burragi GL, eds. *Clinical use of antibodies*. Dordrecht: Kluwer Academic Publishers; 1991.
5. Rhodes BA, Zamora PD, Newall KD. Technetium-99m labeling of murine monoclonal antibody fragments. *J Nucl Med* 1986;27:689–693.
6. Schwarz A, Steinstrasser A. A novel approach to ^{99m}Tc-labeled monoclonal antibodies [Abstract]. *J Nucl Med* 1987;28:721.
7. Mather SJ, Ellison D. Reduction-mediated technetium-99m labeling of monoclonal antibodies. *J Nucl Med* 1990;31:692–697.
8. Hnatowich, DJ, Virzi F, Rusckowski M, Winnard P. An alternative method for directly labeling antibodies with technetium-99m [Abstract]. *J Nucl Biol Med* 1993;37:170.
9. Koprivova H, Pelegrin A, Mach J-P. A modified method of direct antibody labeling with technetium-99m [Abstract]. *Eur J Nucl Med* 1993;20:999.
10. Pauwells EJ, Welling MM, Feitsma RI, Atsma DE, Niewenhuizen W. The labeling of proteins and LDL with ^{99m}Tc: a new direct method of employing KBH_4 and stannous chloride. *Nucl Med Biol* 1993;20:825–833.
11. Thakur ML, DeFulvio JD. Technetium-99m-labeled monoclonal antibodies for immunoscintigraphy. Simplified preparation and evaluation. *J Immunol Meth* 1991;137:217–224.
12. Hnatowich DJ, Virzi F, Winnard P Jr, Fogarassi M, Rusckowski M. Investigations of ascorbate for direct labeling of antibodies with technetium-99m. *J Nucl Med* 1994;35:127–134.
13. Blok D, Feitsma RI, Wasser MN, Niewenhuizen W, Pauwells EK. A method for protein labeling with Tc-99m. *Nucl Med Biol* 1989;16:11–16.
14. Wong DW, Mishkin F, Lee T. A rapid method of labeling human plasma proteins with Tc-99m-pertechnetate at pH 7.4. *Int J Appl Radiat Isot* 1978; 29:251–253.
15. Kanellos J, Pietersz GA, McKenzie IF, Bonnyman J, Baldas J. Coupling of the 99m-technetium-nitrido group to monoclonal antibody and the use of the complexes for the detection of tumors in mice. *J Natl Cancer Inst* 1986;77: 431–439.
16. Childs RL, Hnatowich DJ. Optimum conditions for labeling of DTPA-coupled antibodies with technetium-99m. *J Nucl Med* 1985;26:293–299.
17. Linder KE, Wen MD, Sharkey RM, et al. Preparation and characterization of the antibody B72.3 with Tc-99m BATO isothiocyanates [Abstract]. *J Nucl Med* 1990;(suppl)31:747.
18. Arano Y, Yokayama A, Magata Y, et al. Synthesis and evaluation of a new bifunctional chelating agent for Tc-99m labeling proteins. *Int J Nucl Biol Med* 1986;12:425–430.
19. Abrams MJ, Juweid M, tanKate CI, et al. Technetium-99m human polyclonal IgG radiolabeled via the hydrazino nicotinimide derivative for imaging focal sites of infection in rats. *J Nucl Med* 1990;31:2022–2028.
20. Joiris E, Bastin B, Thornbach JR. A new method for labelling monoclonal antibodies and their fragments with Tc-99m [Abstract]. *J Nucl Med Allied Sci* 1989;33:290.
21. Franz I, Volkert WA, Barefield EK. The production of ^{99m}Tc-labeled con-

jugated antibodies using a cyclam-based bifunctional chelating agent. *Nucl Med Biol* 1987;14:569–572.
22. Eisenhut M, Missfeldt M, Lehmann WD, Karas M. Synthesis of a bis(aminoethanethiol) ligand with an activated ester group for protein conjugation and Tc-99m labeling. *J Lab Compd Radiopharm* 1991;29:1283–1291.
23. Liang FH, Virzi F, Hnatowich DJ. Serum stability and non-specific binding of ^{99m}Tc-labeled diaminodithiol for protein labeling. *Nucl Med Biol* 1987; 14:555–561.
24. Kasina S, Rao TN, Srinivasan A, et al. Development and biologic evaluation of a kit for preformed chelate technetium-99m radiolabeling of an antibody Fab fragment using a diamide dimercaptide chelating agent. *J Nucl Med* 1991;32:1445–1451.
25. Rhodes BA. Direct labeling of proteins with Tc-99m. *Nucl Med Biol* 1991; 18:667–676.
26. Griffiths GL, Goldenberg DM, Jones AL, Hansen HJ. Radiolabeling of monoclonal antibodies and fragments with technetium and rhenium. *Bioconj Chem* 1992;3:91–99.
27. Delmon-Miongeon LI, Mahmood A, Davison A, Jones AG. Strategies for labeling monoclonal antibodies and antibody-like molecules with technetium-99m. *J Nucl Biol Med* 1991;35:47–59.
28. Hertel A, Serpp C, Baum RP, et al. Imaging of brain tumors with thallium, somatostatin analogue and anti-EGF antibody for evaluation of receptors and metabolism [Abstract]. *Eur J Nucl Med* 1993;20:962.
29. Muddukrishna SN, Jorge P, Machner W, Sykes TR, Noujaim AA. An improved method for analysis of radiolabeled antibody using size exclusion HPLC. *Appl Radiat Isot* 1994;45:1009–1019.
30. Dose K, Risi S. The action of U.V. light of various wavelengths on papain. *Photochem Photobiol* 1972;15:43–50.
31. Grossweiner LI, Kaluskar AG, Baugher JF. Flash photolysis of enzymes. *Int J Radiat Biol* 1976;29:1–16.
32. Pigault C, Gerard D. Influence of the localization of tryptophanyl residues in proteins on their photosensitivity. *Photochem Photobiol* 1984;40:291–296.
33. Wickens DG, Norden AG, Lunec J, Dormandy TL. Fluorescence changes in human gamma-globulin induced by free radical activity. *Biochim Biophys Acta* 1983;742:607–616.
34. Ellman GL. A colorometric method for determining low concentrations of mercaptans. *Arch Biochem Biophys* 1958;74:443–450.
35. Sykes TR, Woo TK, Qi P, Baum RP, Noujaim AA. Photolabeling of proteins with Tc-99m [Abstract]. *Proceedings of the Fifth European Symposium on Radiopharmacy Radiopharmaceuticals* 1993:15.
36. Paik CH, Phan NB, Hong JS, et al. The labeling of high affinity sites of antibodies with ^{99m}Tc. *Int J Nucl Med Biol* 1985;12:3–8.
37. Sakahara H, Saga T, Endo K, et al. *In vivo* instability of reduction-mediated ^{99m}Tc-labeled monoclonal antibody. *Nucl Med Biol* 1993;20:617–623.
38. Pimm MV, Pajput RS, Frier M, Gribben SJ. Anomalies in the reduction-mediated technetium-99m-labeling of monoclonal antibodies. *Eur J Nucl Med* 1991;18:973–976.
39. Palmer JL, Nisonoff A, Van Holde KE. Dissociation of rabbit gamma-globulin into subunits by reduction and acidification. *Proc Natl Acad Sci USA* 1963;50:314–321.
40. Sutton J, Alder JR, Easterbrook-Smith SD. The effect of cleavage of the inter-chain disulphide bonds of rabbit IgG on its ability to bind Clq. *Biochim Biophys Acta* 1984;787:39–44.
41. Sykes TR, Woo TK, Wong VC, et al. Preliminary studies on the metabolism of Tc-99m MAb-170. *Proceedings of the Seventh International Symposium on Radiopharmacology* 1991:34.
42. Hnatowich DJ, Mardirossian G, Rusckowski M, Forgarasi M, Virzi F, Winnard P. Directly and indirectly labeled antibodies: a comparison of in vitro and animal in vivo properties. *J Nucl Med* 1993;34:109–119.
43. Griffiths GL, Goldenberg DM, Knapp FF Jr., Callahan AP, Cheng C-H, Hansen HJ. Direct radiolabeling of monoclonal antibodies with generator-produced rhenium-188 for radioimmunotherapy: labeling and animal biodistribution studies. *Cancer Res* 1991;51:4599–4002.
44. Lunec J, Blake DR, McCleary SJ, Brailsford S, Bacon PA. Self-perpetuating mechanisms of immunoglobulin G aggregation in rheumatoid inflammation. *J Clin Invest* 1985;76:2084–2090.