TECHNICAL NOTE

Reduction-Mediated Technetium-99m Labeling of Monoclonal Antibodies

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A simple and generally applicable method for labeling antibodies with technetium-99m (^{99m}Tc) is described. Following reduction of intrinsic disulphide bonds, the antibody is labeled with ^{99m}Tc in the presence of a weak competing ligand methylene diphosphonate. High labeling efficiencies (>97%), in a final labeling step taking only a few minutes, can be routinely obtained with high in-vitro stability over 24 hr. No effect upon antibody reactivity is seen.

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Despite its general use for routine nuclear medicine studies, technetium-99m (^{99m}Tc) has not achieved widespread use as a radiolabel for monoclonal antibodies in immunoscintigraphic studies. This stems from two reasons: First, uncertainty as to whether the short half-life of technetium is appropriate for an agent with such slow pharmacokinetics and second, the lack of an acceptable, generally applicable radiolabeling technique.

A number of methods have been proposed for labeling proteins in general, and antibodies in particular. These range from methods which bind the technetium directly to the substituent amino-acid side chains of the protein, to more recent techniques for linking the radionuclide to a synthetic chelator conjugated to the protein—the 'bifunctional chelate' approach.

While the former method has achieved widespread use for labeling human serum albumin (HSA) for use in blood-pool studies, it is generally considered to provide inadequate stability for the more demanding antibody studies. The bifunctional chelate approach, while generally successful when labeling with indium-111 (^{111}In), seems to be less appropriate for ^{99m}Tc. When antibody-chelator conjugates are labeled in this manner, the radiolabel may bypass the chelator and bind directly to the protein with resulting poor stability (*1*). An alternative approach, which seeks to attach a prelabeled technetium chelate to the antibody, looks promising, and such methods are currently under development.

Proposals have been made for improving the stability of direct labeling methods. For example, Paik (*2*) has suggested that treatment with stannous chloride exposes high affinity labeling sites within the protein structure. Rhodes (*3*) has described a process of lengthy 'pretinning' for antibody labeling.

This communication describes results obtained using a similar approach. The work stems from an approach described by Schwarz (*4*), which was subsequently developed within Behring/Hoechst, FDR.

A general schema is shown in Figure 1. Disulphide bridges within the antibody molecule are cleaved by the use of the reductant 2-mercaptoethanol. Following a subsequent purification, the resulting reduced antibody is stored appropriately until required for use. Labeling is performed via Sn^{++} reduction of pertechnetate in the presence of an excess of a low-affinity chelating ligand. These conditions are conveniently provided by the use of a conventional radiopharmaceutical bone-scanning kit.

MATERIALS AND METHODS

Monoclonal Antibodies

Three antibodies were used for development of the labeling method: H17E2 (*5*), an antibody against human placental alkaline phosphatase, PR1A3 (*6*), an antibody used in the study of colorectal cancer, and SM3, an antibody that recognizes an epitope on stripped epithelial mucin (*7*). All are of the IgG1 isotype.

Antibody Reduction

The antibody, in neutral PBS, was first concentrated to ~10 mg/ml by ultrafiltration (Centricon, Amicon, MA). The antibody was then reduced by reaction with a molar excess of 2-mercaptoethanol ranging from 100:1 to 2000:1 (2-ME:Ab) at room temperature for 30 min. When possible, the undiluted reductant was added directly to the stirred antibody solution. When the required volumes of 2-ME were very small (<2 μl), a 1:10 dilution in neutral PBS was performed. The reduced antibody was then purified on a 2×6 cm G-50 Sephadex column (Pharmacia, Uppsala, Sweden). The number of resulting free sulphydryl groups was assayed with Ellmans reagent; 50 μl of sample was mixed with 50 μl of 5,5′-dithio-

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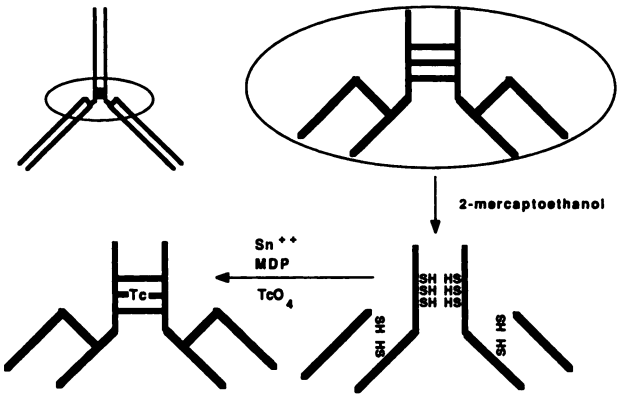

FIGURE 1
General schema showing outline of labeling procedure.

bis-(2-nitrobenzoic acid) 2 mg/ml (Ellmans reagent) and diluted to 1 ml with 0.1 *M* phosphate buffer pH 8. The mixture was incubated at room temperature for 15 min and coloration measured in a UV/vis spectrophotometer at 412 nm. The number of thiols was obtained by comparison with a standard curve obtained by the assay of a series of cysteine standards ranging from 0.1 to 0.0125 m*M*.

Radiolabeling

The ability of the reduced antibody to label with ^{99m}Tc was assessed as follows:

1. An MDP bone-scanning kit (Amerscan, Amersham, Int'l, Amersham, UK) containing 5 mg medronate, 0.34 mg stannous fluoride and 2 mg p-aminobenzoic acid was reconstituted with 5 ml of 0.9% sodium chloride injection.
2. 200 μl of the solution was added to 250 μg of antibody followed by 200 MBq of pertechnetate eluted from a ^{99}Mo/^{99m}Tc generator (Mallinckrodt, Petten, The Netherlands).
3. Labeling efficiency was measured by instant thin-layer chromatography (ITLC) (Gelman Sciences, Northampton, UK) developed in 0.9% sodium chloride solution.

Using antibody reduced at a ratio of 1000:1, the effect of varying the labeling conditions was assessed. Varying aliquots, taken from four different radiopharmaceutical kits (Amerscan MDP, Amersham; Technescan PYP, Mallinckrodt, St. Louis, MO; Osteolite MDP, Dupont, N. Billerica, MA; and Glucoscan Gluconate, Dupont). Labeling efficiencies were again measured by ITLC.

Radiochemical purity and in-vitro stability were measured by a combination of ITLC, high pressure liquid chromatography (HPLC), and cellulose acetate electrophoresis.

Size-Exclusion HPLC

A Beckmann HPLC system using a Dupont GF-250 gel-filtration column with UV 254 nm and radioactive flow detectors was used. 0.1 *M* phosphate pH 7 was used as mobile phase.

Cellulose Acetate Electrophoresis

Cellulose diacetate 78×150 mm strips (Electrofor, Shandon Southern Products, Runcorn, UK) were soaked in electrophoresis buffer (5 m*M* citrate/0.09 *M* phosphate pH 7.4) containing 0.5% Tween 20 and then were laid across the electrodes of a Shandon flat-bed electrophoresis tank. Samples for analysis were applied using a Phastgel sample applicator (Pharmacia, Uppsala, Sweden) and a current of 4 mA/strip was run for 1 hr. The electrophoresis strips were then dried and autoradiographed on x-ray film (Fuji RX, Fuji Photo Film Co., Tokyo, Japan) overnight.

Antigen-Binding Studies

The effect on the immunoreactivity of antibody PR1A3 of reduction at ratios of 10, 100 and 1000:1 was measured by ELISA on LS174T cells using an amplified fluorescence assay system developed by Durbin (*8*). Exponential curves were fitted to the ELISA-obtained curves (Cricket Graph, Cricket Software, Malvern, PA). Intercept values at 50% reduction in fluorescence intensity were estimated from the fitted curves.

Reduced antibody was compared with nonreduced antibody in its ability to compete with radioiodinated antibody in an antigen binding assay. Antibody SM3 was iodinated with ^{125}I using the iodogen method to a specific activity of 1 mCi/mg and diluted with phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA) to a concentration of 1 μg/ml. Increasing amounts of SM3 reduced to a ratio of 1000:1 and unreduced SM3 at cold:hot ratios up to 1000:1 were added to 25-μl aliquots. The antibody mixtures were pipetted into a multiwell plate containing 4 ng of partially stripped epithelial mucin blocked with 5% BSA in PBS. After 2 hr incubation, the supernatants were removed, and the wells were washed four times and then counted in a gamma counter.

Animal Biodistribution Studies

Following the standardized labeling protocol described in Table 1, 5 μg of antibody PR1A3 labeled with 370 KBq of ^{99m}Tc were injected i.p. into normal mice. At time intervals of 2, 5, 24, 28, and 48 hr postinjection, groups of three mice were killed, imaged on a gamma camera (Ohio Nuclear, Solon, Ohio), and major organs were dissected and counted in a well counter (LKB ultrogamma).

RESULTS

Figure 2 shows the influence of the reduction conditions on the number of free sulphydryl groups detected by the thiol assay. It can be seen that the apparent number of -SH groups is much too high. Mouse IgG contains only 6 interchain and 12 intrachain disulphide bonds giving an absolute maximum of 36 -SH groups per antibody molecule (*9*). A considerable proportion of the measured thiols are certainly due to the presence of contaminating mercaptoethanol which has survived the gel-filtration step. However, as expected, increasing the ratio of mercaptoethanol to antibody in the reaction mixture does increase the number of apparent -SH groups per antibody and also, as seen in Figure 3, the eventual labeling efficiency when the reduced antibody preparations were labeled with ^{99m}Tc.

At this stage in the development work, a more efficient purification of the reduced antibody was performed by gel-filtration on Superose-6 FPLC (Pharmacia, Uppsala, Sweden). Thiol assay following FPLC

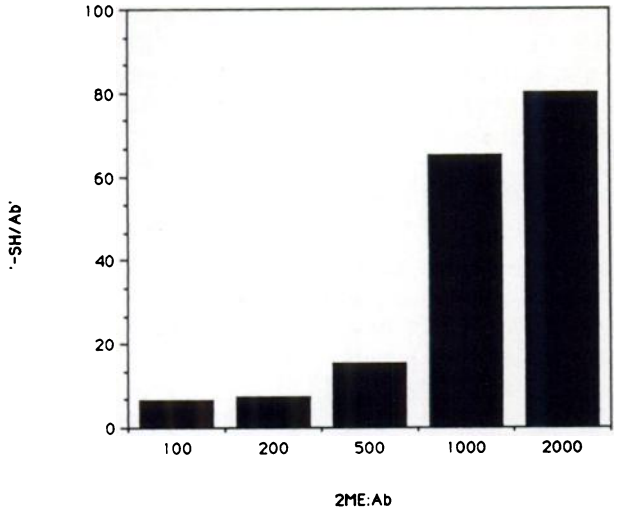

FIGURE 2
Effect of reduction conditions upon apparent number of resulting thiol groups per antibody molecule.

purification showed an average of <1 thiol group per antibody and poor subsequent labeling was obtained.

In previous (as yet unpublished) work in this laboratory, antibodies have been thiolated using 2-iminothiolane, in order to provide a point of attachment for thiol-reactive bifunctional chelating agents. These conjugates are routinely purified by FPLC and, while oxidation of the thiols at neutral pH can be shown to occur, this is generally slow. The reoxidation of reduced disulphides however, is likely to be somewhat quicker, given the close proximity of adjacent thiol groups, and it is possible that, in the absence of small amounts of 2-mercaptoethanol, this occurs too rapidly for labeling to be performed.

More work needs to be performed to define the mechanism of labeling and in particular, the role of the

TABLE 1
Standard Protocol for Labeling Antibody with Technetium-99m

1. By ultrafiltration, concentrate antibody to 10 mg/ml.
2. To stirred solution of antibody add sufficient 2-mercaptoethanol to provide a molar ratio of 1000:1/2-ME:antibody.
3. Incubate at room temperature for 30 min with continuous rotation.
4. Purify reduced antibody by gel filtration of Sephadex-G50 using PBS as mobile phase.
5. Collate antibody fractions and divide into 0.5-mg aliquots. Freeze immediately at −20° C.

Label antibody as follows:

6. Thaw frozen antibody aliquot.
7. Reconstitute Amerscan MDP kit with 5 ml of 0.9% saline injection.
8. Add 50 μl of MDP solution to antibody aliquot and mix well.
9. Add required amount of [^{99m}Tc]pertechnetate ~700 MBq to antibody/MDP mixture. Wait 10 min.
10. Assess labeling efficiency by chromatography using ITLC developed in 0.9% saline. (Should be >95%)
11. If necessary, the labeled antibody can be further purified by gel-filtration on Sephadex G-50 prior to injection.

Labeled antibody is stable for some hours after preparation.

reductant. However, in practical terms, it appears that the contaminating mercaptoethanol plays an essential role, possibly by preventing reoxidation of free thiols or conceivably by contributing to the subsequent chelation of the technetium.

Since these first experiments suggested that a reducing ratio of 1000:1 was required in order to produce high labeling efficiencies, this ratio was then used in a further series of experiments to determine: (a) the best choice of labeling kit and (b) the optimum amount of Sn^{++} and competing ligand to be added. In order to facilitate the labeling procedure for clinical studies, no attempt was made to alter the Sn^{++}: ligand ratio in these

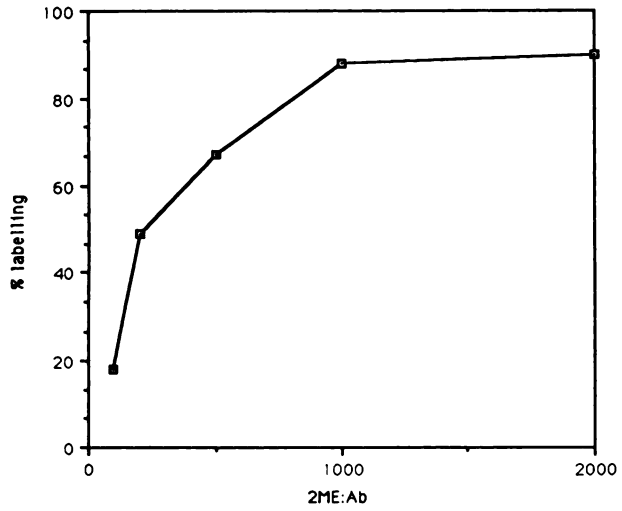

FIGURE 3
Effect of reduction conditions upon antibody labeling efficiency.

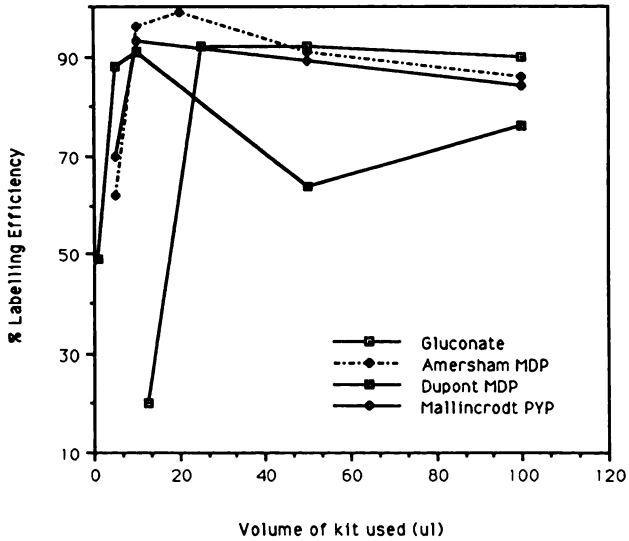

FIGURE 4
Effect of using different radiopharmaceutical kits for labeling procedure.

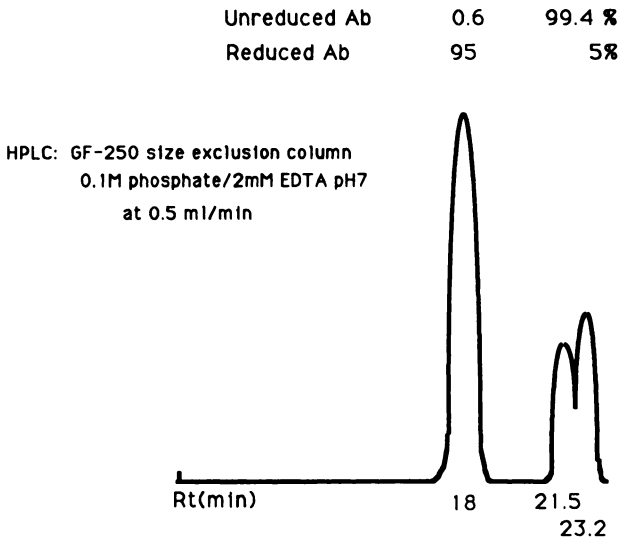

FIGURE 5
Diagram of radioactive detector profile seen on HPLC analysis of antibody reaction mixture. When reduced antibody is labeled, 95% of the activity elutes at a retention time identical to the native antibody. Approximately 5% of the activity elutes at retention times seen with low molecular weight (<1000) compounds.

kits, however, it seems likely that a further improvement could be achieved, if this extra variable was introduced.

Similar results were obtained using all kits although best results were obtained using the Amerscan MDP kit (see Fig. 4). These results should not be seen necessarily as a head-to head comparison of the utility of each kit, since the amounts of tin and ligand in each kit is variable. It does indicate, however, that a variety of radiopharmaceutical kits may be used for this purpose. In each case, the same pattern of results can be seen; when small amounts of the kit were used, low labeling efficiencies were obtained since insufficient Sn^{++} was present to fully reduce the pertechnetate. Increasing the amount of added kit beyond the optimum reduces antibody labeling efficiency presumably due to competition from the increasing amount of ligand present.

HPLC gel-filtration of the labeled antibody shows the majority of the activity to be associated with a peak of identical retention time to that of the native antibody (Fig. 5). Two minor peaks with retention times corresponding to small molecular weight species are sometimes seen. Interestingly, despite the relatively severe reduction step, no peaks corresponding to possible antibody fragments are seen in either the UV or the radioactive traces.

Cellulose acetate electrophoresis (Fig. 6) shows the presence of a major constituent having a migration identical to that of native antibody. No activity is seen at the point of application on the electrophoresis strip indicating the absence of any reduced technetium colloids.

When the labeling procedure was performed using nonreduced antibody, only a small proportion (0.6% measured by HPLC) binds to the antibody. It seems likely, therefore, that the majority of the labeling is mediated through the exposed -SH groups and not with other amino-acid side chains.

Stability of the labeled antibody was measured by ITLC and HPLC up to 5 hr and at 24 hr after labeling. Radiochemical purity remained essentially unchanged for the first five hours after preparation. Stability was also measured in the presence of increasing amounts of DTPA. Molar ratios of up to 100:1 DTPA:ab had no discernible effect upon radiochemical purity. At 24 hr, the percentage of radioactivity associated with the protein had fallen from a mean of 99% to 91% (Fig. 7).

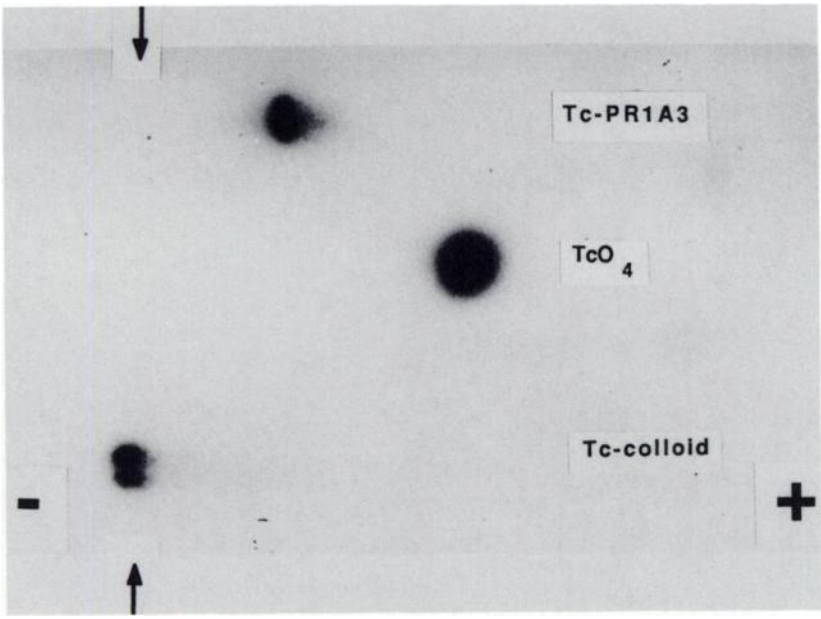

FIGURE 6
Autoradiograph of cellulose acetate electrophoresis of ^{99m}Tc-PR1A3 (upper track). Lower tracks show reference compounds [^{99m}Tc]-pertechnetate and ^{99m}Tc-colloid. Arrows show point of application.

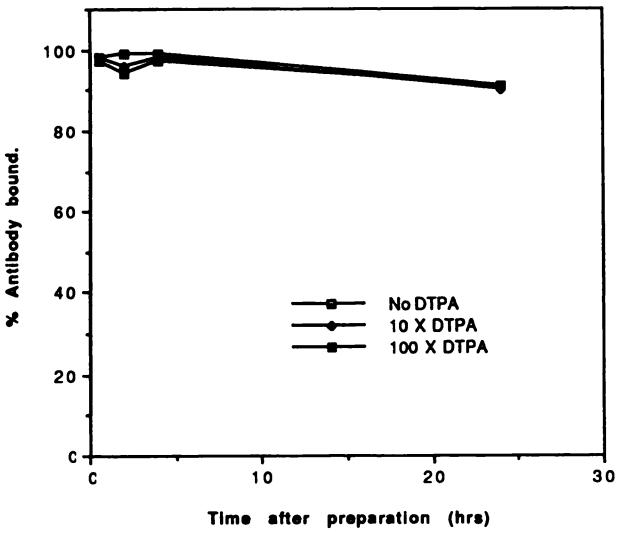

FIGURE 7
Graph showing ITLC determinations of radiochemical stability of ^{99m}Tc-PR1A3 in the absence and presence of DTPA.

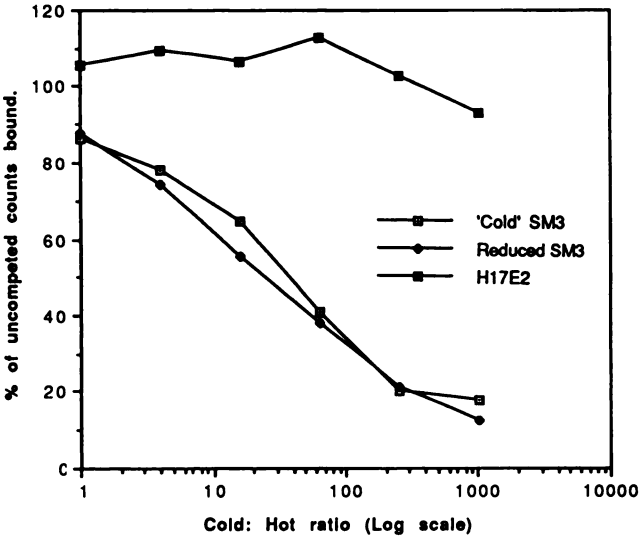

FIGURE 9
Graph showing RIA of ^{125}I-SM3 antibody in competition with unreduced SM3, reduced SM3 (1000:1 ratio), and nonspecific antibody H17E2.

Immunoreactivity of the reduced antibody was measured by both ELISA and a direct radioimmunoassay. The cell line LS174T expresses the antigen recognized by PR1A3 only weakly so that a particularly sensitive assay is required in order to provide an adequate signal of antibody binding. The assay used combines a system of amplification by bridging B-galactosidase/anti-B-galactosidase complexes to the test antibody via rabbit anti-mouse immunoglobulin and low background by the use of a fluorogenic substrate at a pH specific for the bacterial form of the enzyme. Results from the assay of PR1A3 reduced at mercaptoethanol:antibody ratios of 10, 100 and 1000:1 are shown in Figure 8. The reduced antibodies actually produce an enhanced signal when compared to the unreduced control antibody, however no significant difference is seen when the concentrations of antibody giving a 50% reduction in maximum fluorescence are compared, indicating that the reduction process has little, if any, effect upon the immunoreactivity of the antibody.

In a competitive RIA, reduced and nonreduced antibody were equally able to compete with radioiodinated antibody for the bound antigen (Fig. 9).

Gamma camera scans and dissection studies in normal mice injected with ^{99m}Tc-labeled antibody show no accumulation of the radionuclide in any organ (Fig. 10).

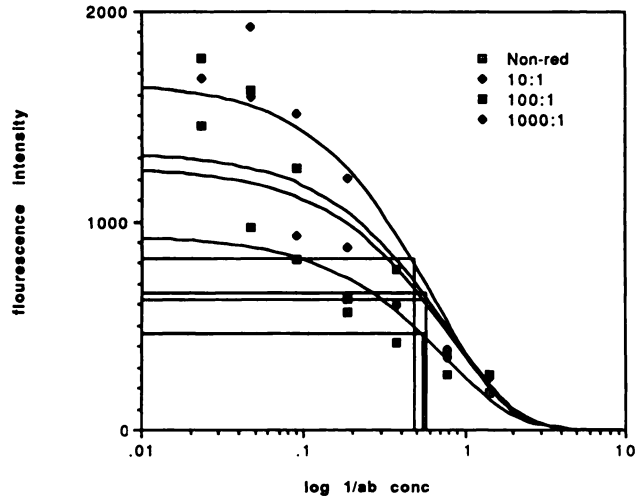

FIGURE 8
Graph showing fluorescent ELISA profiles of nonreduced PR1A3 and PR1A3 reduced at ratios of 10, 100 and 1000:1. Dotted lines indicate the antibody dilution corresponding to a 50% drop in maximum fluorescence intensity.

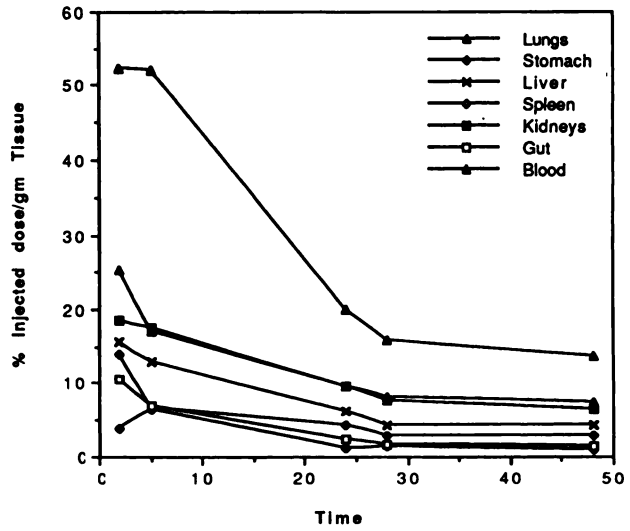

FIGURE 10
Graph showing biodistribution of ^{99m}Tc-PR1A3 at 2, 5, 24, 28, and 48 hr postinjection.

DISCUSSION

In any antibody labeling procedure, the aim is to produce a label with high stability using a procedure with minimal effect upon the immunoreactivity of the antibody. When using radionuclides with short half-lives, such as ^{99m}Tc, it is also desirable that the labeling procedure be rapid, in order to minimize losses due to radioactive decay, and simple, so that the technique may be widely applicable.

The method described above fulfills all these requirements. The procedure is simple and has now been used in this laboratory to successfully label six different monoclonal antibodies. The final labeling step is rapid and results in high labeling efficiencies, which negate the need for post-labeling purification. The method is particularly suitable for the development of 'kits,' which further simplify the process and permit the use of these radiopharmaceuticals in centers with limited radiopharmaceutical expertise.

In theoretical terms, the ideal method for labeling antibodies is probably one based on the conjugation of a pre-labeled technetium chelate to the antibody molecule (*10*). Such an approach gives maximum control over the labeling process and radiochemical purity and should also be applicable to a wide variety of protein molecules. However, such methods, in their present stage of development have a number of disadvantages, notably the lengthy preparation times, and the high level of chemical expertise required (*11*).

Labeling methods based on labeling the pre-formed antibody-chelator conjugate suffer from the potential disadvantage that the technetium can avoid the chelator and combine directly with other parts of the antibody structure. This does not appear to happen when using the procedure described herein. When unreduced antibodies are used, only a minute fraction of the radiolabel attaches to the antibody. The labeling, therefore, appears to be thiol-specific.

A number of antibodies labeled in this manner have now undergone successful clinical evaluation in the detection of colorectal and ovarian cancer. These results will be the subject of a future publication.

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