^{99m}Tc Direct radiolabeling of PR81, a new anti-MUC1 monoclonal antibody for radioimmunoscintigraphy

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ABSTRACT

Introduction: *Monoclonal antibodies labeled mainly with ^{99m}Tc are being widely used as imaging agents in nuclear medicine. Recently PR81 was introduced as a new murine anti-MUC1 MAb against human breast carcinoma. This antibody reacts with the tandem repeat of 20-mer peptide of protein $^{-8}$ M^{-1}. Due to high specific reactivity, this antibody was shown to have high diagnostic potential in nuclear medicine. As the first step towards the use of an antibody for imaging purposes it has to be labeled with a radioisotope. In this study we have investigated a method for optimum labeling of this MAb with ^{99m}Tc.*

Materials and Methods: *The Ab reduction was performed with 2-mercaptoethanol (2-ME) at a molar ratio of 2000:1 (2-ME:MAb) and reduced Ab was labeled with 99mTc* via *stannous tartrate as a transchelator. The labeling efficiency was determined by ITLC. The integrity of reduced MAb was checked by means of SDS-PAGE and gel filtration chromatography (FPLC) on Superose 12 HR 10/30 (purity>99%). The amount of radiocolloids were measured by cellulose nitrate electrophoresis.* In vitro *stability of labeled product was checked in time intervals over 24 hrs by ITLC. Radioimmunoassay was used to test immunoreactivity of the labeled MAb. Biodistribution of the radiolabeled MAb was studied in normal mice at 4 and 24 hrs post-injection.*

Results: invitro *less than %2 radiocolloids were found. There was no Ab fragmentation due to reduction procedure. Both labeled and unlabeled MAbs were able to compete for binding to the MUC1. Biodistribution studies in normal mice showed that there was no significant accumulation in any organ.*

Conclusion: *Because of no significant loss of immunoreactivity of MAb due to labeling procedure, high* in vitro *stability and no accumulation in vital organs, 99m-Tc-PR81 may be a promising candidate for radioimmunoscintigraphy studies of breast cancer.*

Key Words: *MUC1; Monoclonal antibody; PR81; Technetium-99m-radiolabeling*

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Introduction

Antibodies were introduced to nuclear medicine more than two decades ago (1). Since then many antibodies have been used and examined for diagnostic and therapeutic purposes. Radioimmunoscintigraphy (RIS) using monoclonal antibodies (MAbs) is now an important and well established diagnostic procedure in nuclear medicine (2).

Recently a new murine anti-MUC1 MAb against human breast carcinoma has been introduced. The new monoclonal antibody was assigned PR81 (3). This antibody was found to react with the membrane extracts of several breast cancerous tissues and the cell surface of some MUC1 positive cell lines. Due to high specific reactivity, it may be a promising agent in diagnosis and therapy of breast cancer that is the second highest cause of mortality among females in western countries (4).

To investigate the application of the PR81 in nuclear medicine the series of experiments were performed. At the first step, we investigated the efficient method for labeling of this MAb with ^{99m}Tc. ^{99m}Tc is the most common radioisotope used in nuclear medicine that replaced most radionuclides owing to its nuclear properties and its availability from a generator (5).

There are two general methods for labeling of an antibody with ^{99m}Tc. In the direct methods the reduced antibody directly reacts with ^{99m}Tc (6-10). In the indirect methods a bifunctional chelating agent is used to conjugate the antibody to ^{99m}Tc (11-13). Direct methods are efficient and adaptable to a kit type of radiolabeling procedure. Nevertheless, the use of a reducing agent may affect immunoreactivity of the reduced antibody and the labeled antibody could be unstable. These characteristics have to be evaluated.

The direct method for radiolabeling of reduced MAbs with ^{99m}Tc in presence of stannous tartrate was developed and reported by Griffiths et al (14). This method uses the reduction of disulfide bridges of the molecule by adding an excess of reducing agent (2-mercaptoethanol, 2-ME). After a separation step in order to eliminate the excess of 2-ME, the reduced antibody is labeled with ^{99m}Tc via Sn^{+2} reduction of pertechnetate, using stannous tartrate as a weak competing ligand. Based on the direct method referred above, the present report describes the preparation of a ^{99m}Tc-labeled MAb for immunoscintigraphic applications. The effects on immunoreactivity and stability of labeled antibody are reported.

Materials and Methods

Antibody

The antibody PR81 is an anti-MUC1 murine MAb of IgG1 class and subclass, containing kappa light chain. PR81 exhibited good and ^{8} M^{-1}) towards TSA-P1-24 and A-P1-15, which are mainly shared in the hydrophilic sequence of PDTRPAP. This antibody was provided by M. Paknejad, et al (3).

Radiolabeling

A direct reduction-mediated technique was used to label the PR81 with ^{99m}Tc. Antibody reduction was investigated using 2-mercaptpethanol ([2-ME] MERCK) at a molar ratio ranging from 500:1 to 3000:1 (2-ME:antibody). After a 30-min incubation at room temperature, excess thiol was removed using a sephadex G25 (Sigma) desalting column -equilibrated with phosphate-buffered saline (PBS), PH 7.4. The protein concentration was determined by Folin phenol method (15) and the reduced antibody was stored

in 0.1-mg fractions at -20°C until it was required.

^{99m}Tc was obtained from the decay of ^{99}Mo on an aluminum oxide column in a bench-top generator(The Atomic Energy Organization of Iran, Tehran, Iran).The ^{99m}Tc was eluted from the generator in sterile NaCl (0.9% w/v)at room temperature in the form of sodium pertechnetate solution.

The ability of the reduced MAb to be labeled with ^{99m}Tc was assessed as follows. Aliquots of 5

radiolabeling. A solution of stannous tartrate was prepared in acetate-buffered saline at the appropriate pH (range, 3.9-5.2) such that the molar ratio of sodium potassium tartrate (Sigma) to stannous chloride (Sigma) was ranging from 7:1 to 10:1.Stannous tartrate was added to an aliquot of reduced antibody to a final ratio ranging from 1000 to 2000 stannous ions to one molecule of antibody. The required amount of ^{99m}Tc-sodium pertechnetate was then added (20 ^{99m}

allowed to proceed at room temperature while stirred.

Quality Control

The labeling efficiency was determined by instant thin-layer chromatography (ITLC) on Whatman 3MM paper and 0.9% saline. The

from the bottom of the strip. The strip was then placed in a solvent (NaCl, 0.9% w/v) that allowed free ^{99m}Tc to migrate with the solvent front while ^{99m}Tc-PR81 complex remained at the origin. After being developed and dried, the strip was cut at one-third the distance between the origin and the solvent front. Each part was counted separately in a γ-counter (EG & G, ORTEC, Model 4001M) and the first part was considered as ^{99m}Tc-PR81 complex.

Radiocolloids were determined by cellulose

cellulose nitrate (Sartorious) were soaked in electrophoresis buffer (5 mM citrare, 0.09 M phosphate [pH 7.4] containing 0.5% Tween-20) and then were laid in a flat-bed electrophoresis tank (gel electrophoresis apparatus GNA-100,

current of 20 mA was run for 2 hrs. Strips were then dried, cut and counted separately in a γ-counter.

Immunoreactivity Test

The immunoreactivity of PR81 labeled with ^{99m}Tc was tested by radioimmunoassay (17, 18). Briefly, 96-well microtitre plates (Orange,

night at

room temperature. The plates were rinsed with PBS /Tween 20(0/05%) and incubated with 200

for 1 h. The wells were then washed with PBS/Tween 20 and the samples containing the ^{99m}Tc-labeled antibody were added at a

for comparative purposes. The plates were incubated at 37˚C for 1 h, washed with PBS/Tween 20 and the radioactivity of each well was measured in a γ-counter.

Stability

The stability of the labeled antibody was assessed in time intervals up to 24 hrs at room temperature like the procedure described above by ITLC.

Integrity

Samples were analyzed by gel filtration chromatography (FPLC System, Pharmacia,

Uppsala, Sweden) and SDS-PAGE (Phast

each sample was analyzed on Superose 12 HR 10/30 high performance gel filtration (Pharmacia, Uppsala, Sweden)) at 0.5 ml/min flow rate and monitored by UV detector. Fractions of 0.5 ml were collected and counted in a γ-counter.

solution were applied on SDS-PAGE under non reducing conditions. The gel was stained by Coomassie blue dye, and each band was cut and counted separately in a γ-counter.

Biodistribution

Normal BALB/c mice (20-25gr) were used for biodistribution assessment. The ^{99m}Tc-PR81

post injection, groups of 5 mice were sacrificed by ether anesthesia. The liver, spleen, thyroid, bladder, kidney, brain, small and large intestine, sternum, bone, skin, lung, heart, stomach, a sample of muscle (free of fat) were dissected and weighed. Blood and urine samples were also collected and weighed. Activities of all samples were measured in a γ-counter and organ activity was expressed as a mean percentage of injected doses per grams of tissue.

Results

Radiolabeling

Paper chromatography showed that the molar ratios of 2000:1 (2-ME:MAb), 8:1 (Sodium tartrate to stannous chloride) and 1500:1 (stannous ions to antibody) are proper for labeling efficiency of more than 96%.

As the table 1 shows, labeling efficiency in the procedure is dependent on the concentration

or higher is needed to obtain optimum labeling efficiencies of more than 90%. Results of cellulose nitrate electrophoresis showed that radiocolloids were less than 2% of total radioactivity.

Immunoreactivity Test

Labeling procedure includes a reduction step of antibody with 2-ME to generate thiol groups that may affect its immunoreactivity. Immunoreactivity of the ^{99m}Tc-PR81 was measured by direct radioimmunoassay. The results show that the labeled MAb retained its immunoreactivity. On the other hand both

Table 1. The effect of the concentration of the reduced antibody on the labeling efficiency.

Antibody concentration (μg/ml)	Labeling Efficiency (%)
150	
120	
100	
80	
50	
20	
5	

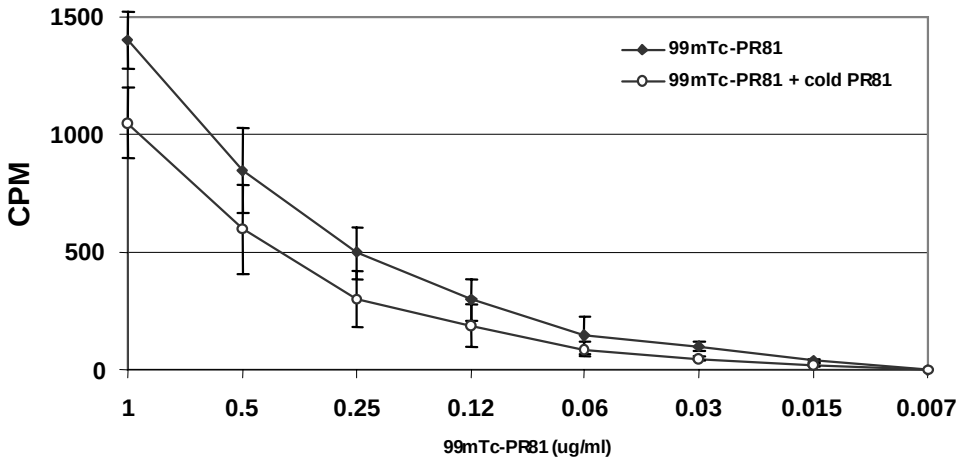

Figure 1. The immunoreactivity of the ^{99m}Tc-PR81 as measured by radioimmunoassay. The results are expressed in CPM (count per minute) of the radiolabeled PR81 bound to the MUC1 and of the binding of radiolabeled PR81 in competition with 'cold' PR81.

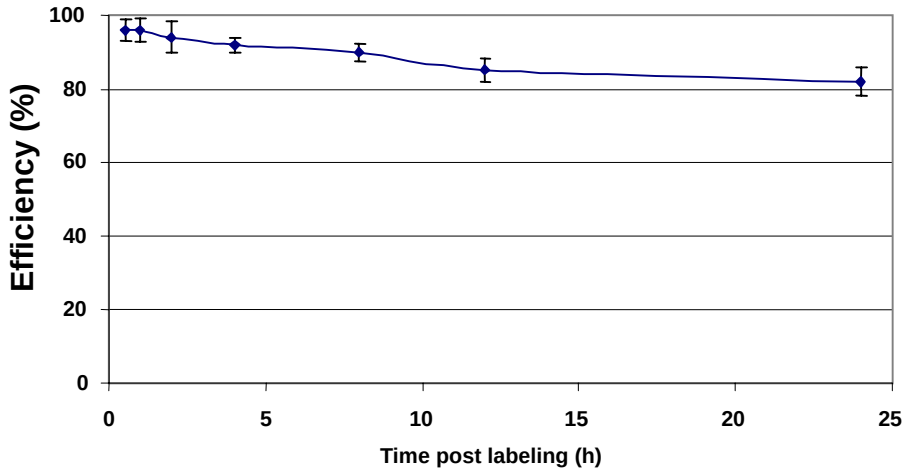

Figure 2. The stability of the ^{99m}Tc-PR81 complex as measured by ITLC during 24 hrs after complex production.

labeled and unlabeled PR81 are able to compete for binding to the MUC1 (Figure 1).

Stability

The stability of ^{99m}Tc-PR81 complex was analyzed by ITLC over a period of 24 hrs after labeling. Figure 2 illustrates the high stability of the antibody complex during storage at room temperature. Nearly 30 min. after labeling all 2.8). Subsequent ITLC analyses showed that the ^{99m}Tc was dissociated from the antibody very slightly when stored at room temperature. The proportion of activity remaining bound to the

Integrity

Reduced MAb was analyzed on Superose 12

HR 10/30. Only one peak was observed with purity greater than 99.0% for reduction condition of 2000:1 (2-ME:MAb). Retention volumes from 10.5 to 10.65 ml for reduced antibody proved that no fragmentation occurred during reduction step (Figure 3). Retention volume for non reduced control antibody was 10.5 ml. These results were confirmed using SDS-PAGE with only one migration band present (Figure 4).

Biodistribution

Results of the antibody biodistribution in mouse expressed as percentage of injected doses per gram of tissue are presented in figure 5.There was a slight precipitation of ^{99m}Tc-PR81 in k

hrs.These levels were reduced in kidneys

hrs post injection, showing that there was no significant accumulation of radioactive material in vital organs.

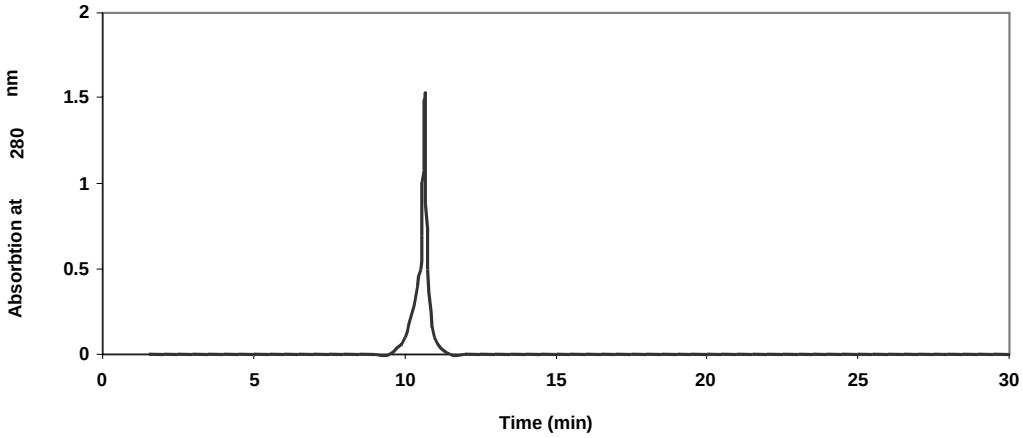

Figure 3. Integrity of reduced PR81 analyzed on Superose 12 HR 10/30, FPLC.

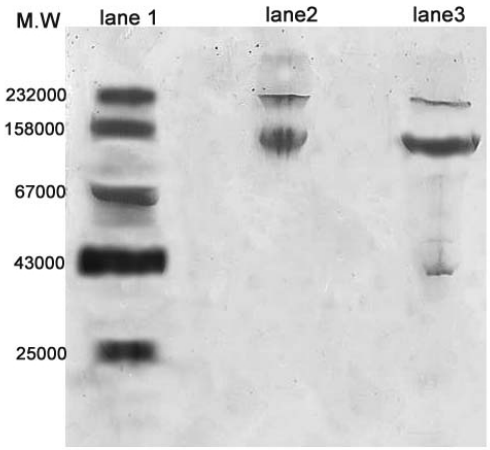

Figure 4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie blue staining of molecular weight standards (lane 1), Intact PR81 (lane 2), ^{99m}Tc-PR81 (lane 3).

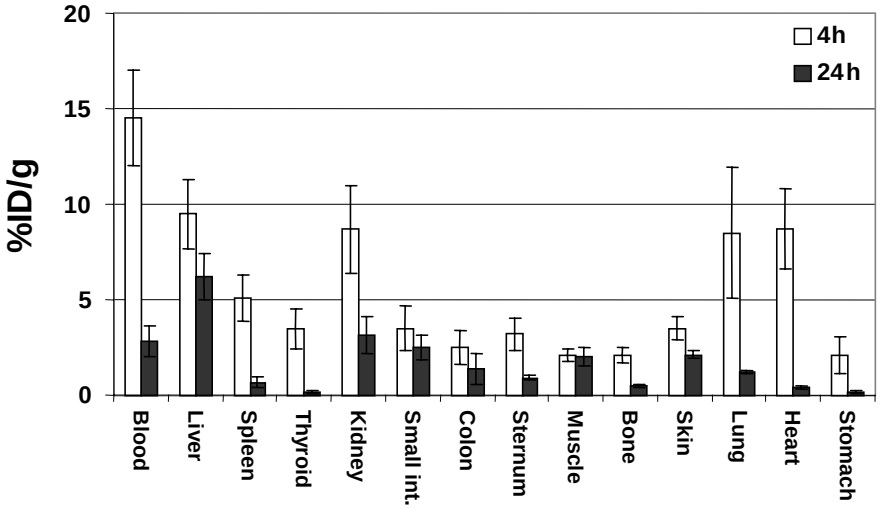

Figure 5. Organ biodistribution of ^{99m}Tc-PR81 in BALB/c mice performed at 4 and 24 hrs post-injection and expressed as percentage of injected dose per gram of tissue (%ID/g) as described in Materials and Methods. `

Discussion

The radioimmunoscintigraphy (RIS) was found widespread clinical application in tumor diagnosis. Tumors of diameter 0.7 cm^3 to 2.0 cm^3 can be detected by this technique. A very important feature of RIS is that it can be assistance in the diagnosis of metastases and therefore it can continue to patient management concerning clinical decisions (10, 19). Furthermore, RIS can be explored as a scouting procedure to radioimmunotherapy (RIT), since the behavior of a novel product can be well characterized before its application in RIT (20).

Human epithelial mucin, MUC1, is commonly expressed in adenocarsinoma including 80% of breast cancers and carries many of the associate antigenic determinants recognized by MAbs (21). Numerous MAbs against MUC1 have been reported by immunizing BALB/c mice with various preparation of antigen such as breast cancer tissue homogenate, cell lines, mixture of human cell line, human milk fat globulin (HMFG) and synthetic MUC1 peptide based on tandem repeat sequences (22). However, antibodies generated against a MUC1 tandem repeat do not always react with natural MUC1 present on the surface of malignant cells. This may indicate that the importance of glycans in the configuration of the epitopes (23). PR81 was essentially raised against homogenized breast cancerous tissues without any enzymatic treatment. Therefore, the three-dimensional configuration of natural antigen expressed on the tumor cells remained intact (3).

It was previously found that IgG antibodies $^{-8}$) are optimal for both imaging and therapy (24). In this way, PR81, which exhibited high affinity towards two $^{-8}$ M^{-1}), may be suitable for *in vivo* tumor targeting and imaging and labeling it with a radioisotope is the first step towards the use of this antibody in imaging studies.

In any antibody labeling procedure, the most important factors taken into account are as follows (16, 18): (1) the overall yield of pure labeled species; (2) the simplicity,

reproducibility and the cost of the method applied; (3) the immunoreactivity of the labeled derivative; and (4) the stability of the radioactive species obtained. 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 the activity loss via physical decay. The method used in this work fulfills all these requirements. The final labeling step is rapid and results in high labeling efficiency and negates the need for post-labeling purification. This method is based on the generation of sulphydryl groups in murine MAb by a controlled reduction with 2-ME that is very well documented and found to be quite suitable for clinical studies (25-29).

High labeling efficiency was obtained with a molar ratios of 2000:1 (2-ME:ab), 8:1 (Sodium tartrate to stannous chloride) and 1500:1 (stannous ions to antibody). Similar results were reported by Griffiths et al (12) and Simms et al (30) in other monoclonal antibodies. However higher labeling efficiency was obtained in our study. The high radiolabeling efficiency

remove free $^{99m}TcO_4^-$ or radiochemical impurity. Concentration of reduced antibody was found as a parameter to take into account to obtain good or higher were needed for labeling efficiency of more than 90%. The MAb PR81 retained its immunoreactivity after reduction with 2-ME at a molar ratio of 2000:1, and no fragmentation was found by SDS-PAGE and gel filtration chromatography (FPLC).

Conclusion

In conclusion these results show that, using Griffiths method for radiolabeling MAb PR81, labeling yield higher than 96% will be reached. Due to no significant loss of immunoreactivity, high *in vitro* stability and no significant accumulation in vital organs, the ^{99m}Tc-PR81 is regarded as a promising candidate for radioimmunoscintigraphy of breast cancer. Further studies are now underway to investigate the breast tumor localization and reaction of this complex in other MUC1-expressing cancers.

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