DIRECT ^{99m}Tc LABELING OF MONOCLONAL ANTIBODIES: EVALUATION OF REDUCING AGENTS AND HPLC ANALYSIS

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SUMMARY

Human IgG was used as a model antibody and the results for direct ^{99m}Tc labeling of IgG using 2-mercaptoethanol(2-ME), dithiothreitol(DTT) and ascorbic acid (AA) as reducing agents were evaluated. DTT was chosen as reducing agent and an intact monoclonal antibody 3H11 was labeled with ^{99m}Tc with a labeling yield of more than 98% while its Fab fragment was labeled with ^{99m}Tc in less than 35% yield. The ^{99m}Tc labeled antibodies were analysed by HPLC on a size exclusion column with an eluent of 0.1mol/L PBS(pH 7.0), and the in vitro stability of ^{99m}Tc-3H11 was investigated. HPLC analysis revealed that ^{99m}Tc-3H11 Fab is not in a singular structure, and the procedure for direct ^{99m}Tc labeling of antibody's Fab Fragment should be further investigated.

Keywords: ^{99m}Tc , IgG, Monoclonal antibody, Labeling method, Stability, HPLC

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CCC 0362–4803/98/010037–09$17.50

Received 19 June 1997
Revised 20 August 1997

INTRODUCTION

In recent years much effort has been invested in developing radiolabeled monoclonal antibodies (McAb) for diagnostic and therapeutic applications[1]. Among the radionuclides currently used in nuclear medicine, ^{99m}Tc is the most suitable element for imaging applications, due to its excellent nuclear characteristics (E=140KeV, $T_{1/2}$=6.02h) and its availability from a generator. Several methods have been investigated to label McAbs with ^{99m}Tc. These can be divided into two groups: indirect and direct labeling methods. Compared with indirect procedures in which bifunctional chelating agents such as DTPA, cyclam and diamido dithio ligands are conjugated for covalent bonding with ^{99m}Tc[2-4], the direct labeling method is appealing because it avoids McAb conjugation, the reduction is easily controlled, the immunospecificity is less likely to decrease, and importantly, it can be utilized to design a one-step radiopharmaceutical "kit".

Some agents have been used to reduce antibodies in direct labeling procedures of antibodies with ^{99m}Tc[5-9]. In this paper, 2-ME, DTT and AA were used to reduce a model antibody, human IgG and the results of the labeling efficiency and in vitro stability of the ^{99m}Tc labeled IgG were evaluated. Then, an anti-human gastric cancer McAb 3H11 and its Fab fragment were labeled with ^{99m}Tc using one of those reducing agents, and the ^{99m}Tc labeled antibodies were analysed by HPLC on a size exclusion column.

EXPERIMENTAL

Materials and apparatus

Human IgG was obtained from Chengdu Institute of Biological Products (Chengdu), and antigastric cancer monoclonal antibody 3H11 and its Fab fragment from Beijing Institute for Cancer Research (Beijing).

a ^{99m}Mo-^{99m}Tc generator was purchased from China Institute of Atomic Energy (Beijing) with a radioactivity of 1.11×10^{10}Bq; MDP kit containing 0.21mg

$SnCl_2$ was given by Nuclear Power Institute of China (Chengdu) and was dissolved in 1.0mL of 0.1mol/L PBS(pH 7.0). 2-ME was purchased from Sigma Chemical Co.; All the other chemical reagents were of A.P grade and redistilled water was used.

HPLC system 334 (Beckman Co., USA) consisted of a model 421A controller, two model 110B pumps, a model 163 variable wavelength detector, a model 427 integrator and a model 170 radioisotopes detector.

Separating and analytical methods

The reduced and radiolabeled antibodies were separated by a Sephadex G_{50} column (ϕ1.2cm×16cm) with an eluent of 0.1mol/L PBS (pH 7.0).

The radiopurity or labeling efficiency of the ^{99m}Tc labeled antibodies were determined using a Sephadex G_{50} column as described above or by HPLC on a size exclusion column (TSK 4000SW, 13μ, ϕ75mm×300mm) with 0.1mol/L PBS(pH 7.0) as the mobile phase at a flow rate of 1.0mL/min. The effluent was monitored with a UV detector at 225nm and assayed with a radioactivity detector.

The protein concentration of antibodies or radiolabeled antibodies was also measured by the HPLC procedure.

Reduction of human IgG.

To the test tubes containing 20mg of human IgG, 9.32μL of 2-ME ($M_{2\text{-ME/IgG}}=1000:1$), 115.7$\mu$L of 130mg/mL DTT ($M_{DTT/IgG}=1000:1$) and 320 μL of 500mg/mL AA ($M_{AA/IgG}=5000:1$) were added, respectively. The mixtures were diluted to 1.0mL by 0.1mol/L PBS(pH 7.0) and incubated at room temperature(RT) for 30min. The reduced IgG was isolated, and the purified IgG was dispensed into 1.0mg aliquots and kept frozen at -20℃ for use.

^{99m}Tc labeling of the reduced IgG

10μL of 0.05mol/L EDTA and 40μL of MDP solution followed by (1.0-5.0)×10^7Bq of the freshly eluted $^{99m}TcO_4^-$ were added to 1.0mg of the reduced

IgG. The mixture was incubated for 60min at RT with occasional shaking. Aliquots were removed periodically and the labeling efficiency was examined. The radiolabeled IgG was isolated for in vitro stability experiments.

In vitro stability experiments

This was investigated by subjecting ^{99m}Tc-IgG to a solution of PBS and other reagents for more than 24h. To the test tubes charged with 0.5mL of (a) 0.1mol/L PBS (pH 7.0), (b) 0.05mol/L EDTA, (c)0.05mol/L DTPA and (d) 0.01mol/L cysteine was added 0.5mL of PBS eluate containing (1) ^{99m}Tc-IgG (2.72×10^{6}Bq, 144.5μg, with 2-ME as reducing agent) or (2) ^{99m}Tc-IgG (4.05×10^{6}Bq, 117.5μg, with DTT as reducing agent). Aliquots were removed at 12 and 24h, respectively, and the radiochemical purity was measured.

Labeling 3H11 and its Fab fragment with ^{99m}Tc

DTT was chosen as reducing agent. McAb 3H11 and its Fab fragment were reduced, radiolabeled, analysed or isolated by the same procedure as IgG described above. The in vitro stability of the isolated ^{99m}Tc-3H11 (5.05×10^{6}Bq, 27.2μg) was also investigated by the same procedure as ^{99m}Tc-IgG.

RESULTS

HPLC analysis

This HPLC system was equipped with a UV-detector as well as a radiodetector. The results showed that the chromatographic peak areas and protein concentrations of antibodies have a good linear relation with a correlation coefficient of 0.9992. Obviously, the protein concentration and labeling efficiency or radiopurity of the ^{99m}Tc labeled antibodies can be determined simultaneiously by the HPLC procedure.

HPLC profiles of ^{99m}Tc labeled 3H11 McAb before and after isolation by Sephadex G_{50} column are described in Fig. 1. By means of size exclusion column and PBS eluent, it was shown that the UV-peak of 3H11 McAb corresponds to the radio-peak of ^{99m}Tc-3H11 with a retention time of 9.20 ± 0.05min and completely separates with the free ^{99m}Tc(Fig. 1a), and the isolated ^{99m}Tc-3H11 has

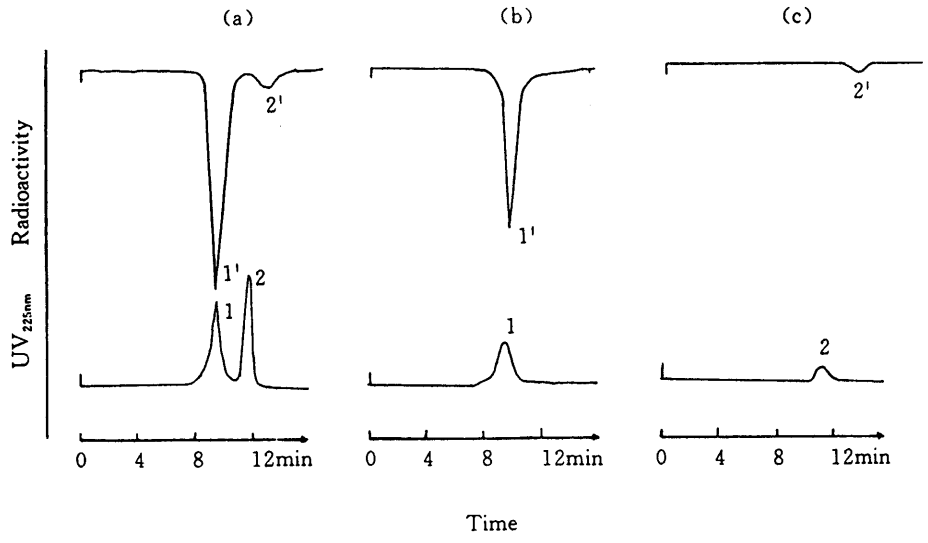

Fig. 1 HPLC profiles of the ^{99m}Tc labeled McAb 3H11
(a)reaction mixture (b)the isolated ^{99m}Tc-3H11 (c)free ^{99m}Tc
1. 3H11 McAb 2. EDTA 1'. ^{99m}Tc-3H11 2'. free ^{99m}Tc

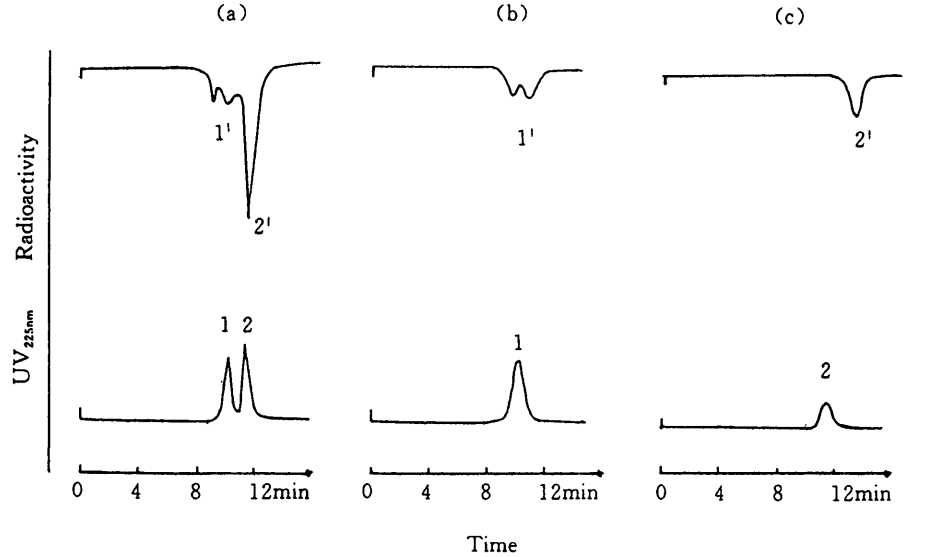

Fig. 2 HPLC profiles of the ^{99m}Tc labeled 3H11 Fab fragment
(a)reaction mixture (b)the isolated ^{99m}Tc-3H11 Fab (c)free ^{99m}Tc
1. 3H11 Fab 2. EDTA 1'. ^{99m}Tc-3H11 Fab 2'. free ^{99m}Tc

only a UV-peak and a corresponding radio-peak (Fig. 1b) while the free ^{99m}Tc has no UV-peak or radio-peak at the same retention time as that of 3H11 McAb or ^{99m}Tc-3H11(Fig. 1c). HPLC profiles of the ^{99m}Tc labeled human IgG was almost the same as that of the ^{99m}Tc labeled 3H11 McAb. Unfortunately, HPLC analysis of the ^{99m}Tc labeled 3H11 Fab fragment was somewhat different from that of the ^{99m}Tc labeled 3H11 McAb in that the isolated ^{99m}Tc-3H11 Fab fragment had split radio-peaks even though it had only a UV-peak with a retention time of 9.15±0.05min (Fig. 2), implying that ^{99m}Tc-3H11 Fab is not in a singular structure and may not be suitable for further biological or clinical applications. This may be correlated to the unsatisfactory labeling efficiency of the ^{99m}Tc labeled 3H11 Fab fragment.

Evaluation of reducing agents

The labeling yields of ^{99m}Tc labeled human IgG using 2-ME, DTT and AA as reducing agents are summarized in Table 1. As can be seen, the labeling yields of ^{99m}Tc-IgG with 2ME or DTT reducing agent were more than 98.0% at 30-60min, whereas a labeling efficiency of less than 25% was observed when AA was used to reduce human IgG. Both 2-ME and DTT were suitable for ^{99m}Tc labeling of human IgG and incubation time of 30min was enough.

Table 1 Labeling efficiency of the ^{99m}Tc labeled human IgG with 2-ME, DTT and AA as reducing agent($n=4, \bar{X}\pm SD$)

Reducing agent	Labeling efficiency (%)		
	10min	30min	60min
2-ME	85.0±1.2	98.5±2.0	98.2±1.5
DTT	85.9±1.3	99.0±1.4	98.9±1.0
AA	5.2±2.1	13.3±2.4	24.7±1.6

The radiopurity change of ^{99m}Tc-IgG obtained by using DTT and 2-ME as reducing agents when challenged with several reagents in vitro are given in Table 2. The results show that ^{99m}Tc-IgG was stable when challenged with PBS, EDTA or DTPA solution, but there was a rapid loss of ^{99m}Tc from IgG in the presence of cysteine. Comparatively, ^{99m}Tc-IgG obtained by using DTT as reducing agent appeared to be more stable.

According to these results, DTT was chosen to reduce 3H11 McAb and its Fab fragment.

Table 2 The radiochemical purity change of ^{99m}Tc-IgG or ^{99m}Tc-3H11 when challenged with reagents in vitro ($n=4, \bar{X}\pm SD$)

	Reducing agent	Time(h)	Percentage of ^{99m}Tc remaining bound to IgG(%)			
			PBS	EDTA	DTPA	Cysteine
^{99m}Tc-IgG	2-ME	12	96.1±2.2	95.8±1.6	97.0±2.4	17.4±1.2
		24	94.0±1.6	93.8±1.9	95.9±2.1	10.5±1.7
	DTT	12	97.5±1.8	97.9±1.2	97.8±2.0	20.1±1.4
		24	96.8±1.4	95.5±1.3	97.0±1.6	11.0±1.8
^{99m}Tc-3H11	DTT	12	96.8±1.4	96.5±2.1	97.0±2.3	16.4±1.1
		24	95.5±2.0	94.6±1.8	95.8±1.6	10.7±0.9

^{99m}Tc labeling of 3H11 McAb and its Fab fragment

With DTT as reducing agent, the reduced 3H11 was easily labeled with ^{99m}Tc with a labeling yield of more than 98.0% while only a labeling efficiency of less than 35% was measured for ^{99m}Tc labeling of the reduced 3H11 Fab fragment, suggesting that the procedure for direct ^{99m}Tc labeling of antibodies Fab fragment should be further investigated.

In vitro stability of ^{99m}Tc-3H11

Like ^{99m}Tc-IgG, ^{99m}Tc-3H11 remained stable in vitro when challenged with PBS, EDTA or DTPA, but there was a rapid loss of ^{99m}Tc from 3H11 in the presence of cysteine(Table 2).

DISCUSSION

Sephedex G_{50} Column can be used to isolate the radiolabeled antibodies and determine the radiochemical purity, but it takes considerable time. Paper chromatography was used to examine the radiochemical purity in many experiments. Unfortunately, Xinhua No. 1 paper (China) with several mobile phases were used in our laboratory without satisfactory results. In this experiment, by size

exclusion HPLC with a mobile phase of PBS, the protein concentration and radiochemical purity of the ^{99m}Tc labeled antibodies wae determined rapidly and simultaneously. This HPLC procedure is possibly suitable for analysis of antibodies labeled with other radionuclides such as ^{186}Re, ^{111}In or ^{90}Y.

2-ME and DTT were used to reduce human IgG and intact monoclonal antibody 3H11, and labeling yields of more than 98% were achieved. After evaluating the results for direct ^{99m}Tc labeling of IgG or IgM antibodies with several reducing agents including 2-ME, DTT and AA in trace level experiment, Thakur mL[9] recommended that AA is the preferred reducing agent to make "one-step" kit because the best labeling efficiency and immunoreactivity were obtained when AA was used as reducing agent. Unfortunately, there were few reports available concerning the direct ^{99m}Tc labeling of antibodies with AA as reducing agent. In other experiments, AA was used as antixidizing agent of the reduced antibodies or stablizer of the ^{99m}Tc labeled antibodies[10,11]. In this study, the labeling efficiency of less than 30% for direct ^{99m}Tc labeling of human IgG was measured at either trace level or in normal level experiments with a reducing agent of AA according to a $M_{AA/IgG}$ ratio of 5000:1 recommended by Thakur ML[9], implying that the direct ^{99m}Tc labeling of antibodies with AA as reducing agent is not easily executed.

^{99m}Tc direct labeling method of antibodies was based on the generation of stable ^{99m}Tc-SH bond at high affinity sites. The concentration of -SH groups of the reduced antibodies correlated well with the labeling efficiency of antibodies with ^{99m}Tc. Generally, the concentration of -SH groups of antibodies' fragments was much less than that of intact antibodies[12]. This is one of the reasons why the labeling efficiency for direct ^{99m}Tc labeling of 3H11 Fab fragment was much less than that of intact antibody 3H11 using the same procedure. Additionally, there was a rapid loss of ^{99m}Tc from antibodies in the presence of challenging reagents containing -SH groups such as cysteine.

ACKNOWLEDGEMENTS

This work was supported by the Foundation of Radiophysical and Technological Opening Laboratory of State Education Committee of China.

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