PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Direct technetium-99m labeling of anti-hepatoma monoclonal antibody fragment: a radioimmunoconjugate for hepatocellular carcinoma imaging

Hui Jie Bian[1], Zhi Nan Chen[1] and Jing Lan Deng[2]

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Abstract

AIM To directly radiolabel an anti-hepatoma mAb fragment HAb18 F(ab')$_2$ with ^{99m}Tc by stannous-reduced method, and assess the stability, biodistribution and *radioimmun* oimaging (R II).

METHODS Immunoreactive fraction was determined according to Lin dmo's method. Ellman's reagent was used to determine the number of thiols in the reduced F(ab')$_2$. Labeling efficiency and homogeneity were measured by paper chromatography, sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and autora diography. Challenge assay involved the incubation of aliquots of labeled antibo dy in ethylenediaminetetraacetate (EDTA) and L-cysteine (L-cys) solutions with different molar ratio at 37℃ for 1 h, respectively. Investigations *in vivo* utilized nude mice bearing human hepatocellular carcinoma (HHCC) xenografts with gamma camera imaging and tissue biodistribution studies at regular intervals.

RESULTS The labeling procedure was finished within 1.5 h compared with the "pretinning" method which would take at least 21 h. *In vitro* studies demonstrated that the radiolabeled mAb fragment was homogen eous and retained its immunoreactivity. Challenge studies indicated that ^{99m}Tc-labeled HAb18 F(ab')$_2$ in EDTA is more stable than in L-cys. Imaging and biodistribution showed a significant tumor uptake at 24 h post-injection of ^{99m}Tc-labeled HAb18 F(ab')$_2$. The blood, kidney, liver and tumor uptakes at 24 h were 0.56 ± 0.09, 56.45 ± 11.36, 1.43 ± 0.27 and 6.57 ± 3.01 (%ID/g), respectively.

CONCLUSION ^{99m}Tc-HAb18 F(ab')$_2$ conjugate prepare d by this direct method appears to be an effective way to detect hepatoma in nude mice model.

INTRODUCTION

The introduction of mAbs as targeting devices in nuclear medicine is well develo ped and many different antibodies which labeled with a variety of isotopes have been reported in cancer diagnosis. It seemed that ^{99m}Tc is the most popular radionuclide for nuclear medicine imaging because of its favorable physi cal characteristics, low cost, and ready availability. ^{99m}Tc labeled mAb fragments should be superior to other big molecule radioimmunoconjugates for u se in tumor R II. A number of methods have been proposed for ^{99m}Tc labe ling proteins, and mAbs in particular. In general, these methodologies can be d ivided into two categories: indirect and direct methods[1]. In indirect method the protein was modified with a technetium binding ligand and then reacte d with a technetium complex. Several bifunctional chelating agents have been syn thesized and used, such as diethylenetriaminepenta acetic acid (DTPA)[2] , diamide dimercaptide N_2S_2 ligands, and hydrazino nicotinamide analog [3]. Although it is said that the indirect method can lead to loss of immuno reactivity. Joiris *et al*. have tested that the derivatization of antibody or fragment by iminothiolane does not split the protein and keeps the immunoreact ivity [4]. By direct method, ^{99m}Tc metal ion binds directly to endogenous donor groups on the antibody. The method is simple to perform and co mpatible with practical clinical use. However, direct labeling of mAbs with ^{99m}Tc was reported to be unstable due to non-specific binding (low and high-affinity)[5,6], but some reports suggest an improved labeling of proteins with ^{99m}Tc.

[1]Cell Engineering Research Center, Basic Medical Department, Fourth Military Medical University, Xi'an 710032, Shaanxi Province, China
[2]Department of Clinical Nuclear Medicine, Xijing Hospital, Xi'an 710033, Shaanxi Province, China

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Correspondence to: Prof. Zhi Nan Chen, Cell Engineering Research Center, Basic Medical Department, Fourth Military Medical University, Xi'an 710032, Shaanxi Province, China
Tel. 0086-29-3374545, Fax. 0086-29-3293906
Email. Chcerc 1@fmmu.edu.cn

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In the Schwarz and Steinstrasser procedure, as modified by Mather and Ellison[7], disulide bridges in the mAb are reduc ed with 2-mercaptoethanol (2-ME). After purification, the resulting reduced an tibody can be stored frozen until required for use. Labeling is accomplished by addition of stannous ion from a bone- scanning kit and pertechnetate. In addit ion to using regular reducing agents, such as 2-ME, stannous ions[8], b orohydride[9], ascorbic acid[10], dithionite[11], or gl utathione[12] to generate sulphydryl groups, other peculiar approaches also appeared recently. Direct ^{99m}Tc labeling of mAbs were finis hed by reduction of antibodies using photoactivation and insoluble macromolecula r Sn (II) complex[13,14]. With the development of direct method, there ha ve been a few reports of successful use of this technique in colorectal, breast, and ovarian cancer imaging[15-17].

In this report, we describe a direct method for radiolabeling anti-hepatoma mon oclonal antibody fragment HAb 18F (ab')$_2$ with ^{99m}Tc. The stability and homogeneity of ^{99m}Tc-HAb18 F(ab')$_2$ were evaluated. The biodis tribution and tumor localization in nude mice bearing a HHCC xenograft were studied.

MATERIALS AND METHODS

Monoclonal antibody

The mAb HAb18 is of murine IgG$_1$ isotype and was developed by our laboratory[18]. F(ab')$_2$ fragment of HAb18 was generated by papain digestion with a molecular weight of -96 000 dalton[19].

Tumors

Hepatocellular carcinoma grown in Balb/c mice was used as a prototype tumo r model. Approximately 10^7 HHCC cells obtained from Shanghai Cell Institute of Chinese Academy of Sciences were implanted in the left thigh of the animals and the tumors were allowed to grow for 8-10 days to approximately 1cm in diameter.

Antibody reduction

The antibody concentrated to 8 g/L in neutral PBS was reduced by reaction with a molar excess of stannous/glucoheptonate (Sn/GH) ranging from 10: 1 to 50:1 (Sn/GH: MAb) at 37℃ for 15 min-30 min. The Sn/GH with a mass ratio of 1:100 was dissolved in 50mM acetate-buffered saline (ABS), pH 5.3 purged with nitrogen. The reduced antibody was isolated from reductant t hro ugh a PD-10 column (Pharmacia) equilibrated with 0.05 mol/L ABS. The number of resulting free sulphydryl groups was assayed with Ellman's reagent 5, 5'dithio-bis (2-nitrobenzoic acid), (DTNB, Sigma Chemical Co., USA) [20]. One hundred μL of sample was mixed with 20 μL of 0.01 mol/L-DTNB and diluted to 3 mL with 0.05 mol/L Tris-HCl buffer pH 8.4. The mixture was incubated at room te mperature for 15 min and coloration measured with an UV/VIS spectrophotome ter at 412 nm. The number of thiols was obtained by comparison with a seri es of L-cysteine (L-cys) standards ranging from 0.312 mg/L to 10 mg/L.

The integrity of the reduced F(ab')$_2$ was determined by non-reduced SDS-PAGE with 100 g/L gel using Vertical Gel Electrophoresis System (Bio-Rad). The gel was stained with Coomassie brilliant blue R250. Control experiments were run using unreduced mAb F(ab')$_2$.

Radiolabeling

For labeling, 160 μg of reduced HAb18 F(ab')$_2$ was mixed with a 10 μL-20 μL of diluted Sn/GH solution (0.2 g/L), and pertechnetium solution (0. 2 mL, 74MBq), (Chinese Academy of Atomic Energy) was injected into the mixture. The Sn/ GH solution was freshly prepared each time by dissolving 100 mg GH and 1mg $SnCl_2 \cdot 2H_2O$ in 5 mL of saline purged with nitrogen. The reaction mixture was incubated for 0.5 h-1 h at 37℃ before it was analyzed by Whatman 3MM paper chromatography which was then developed in acetone or 100 g/L trichloroacetic acid (TCA). R-f values for acetone are: mAb 0.0, ^{99m}Tc-GH 0.0, and $^{99m}TcO_4$-0.9-1.0. R-f values for 100 g/L TCA are: mAb 0.0, ^{99m}TcGH 0, and $^{99m}TcO_4$- 0.7. Labeled mAb was differentiated from ^{99m}Tc colloid by the method of Thrall *et al*[21]. The same strips impregnated with 10g/L-20g/L human serum albumin before development with 5:2:1, water: ethanol: 5N NH_4OH. Colloid remained on the bottom of the strip while mAb-bound isotope migrated with the solvent front.

The integrity of the labeled F(ab')$_2$ was assayed using the same non-reduced SDS-PAGE as described above. The gel was autoradiograghied on x-ray film before stained with Coomassie brilliant blue R250.

mmunoreactivity assessment

The *in vitro* immunoreactivity of the radiolabeled HAb18 F(ab')$_2$ was evalu ated by a live cell assay[22]. Briefly, HHCC cells 5×10^9/ L were centrifuged (1 000r/min) for 5 min and washed twice with 1% bovine serum albumin (BSA) in PBS, then 5 serial 1:2 dilutions were made in 10 g/L BSA in Eppendorf tubes precoated with BSA. Radiolabeled HAb18 F (ab')$_2$ at a concentration of 40 ng/mL in 10 g/L BSA was added using a volume equal to half the volume of cell suspension. The total volume of cell-binding assay solution was 0.3 mL. After incubation for 2 h at 37℃, the total as well as the cell-bound radioactivity were counted in a gamma counter.

In Vitro stability studies

The stability was analyzed by using two different challenging agents, EDTA and L-cys. An aliquot of 50 μL ^{99m}Tc-HAb 18 F(ab')$_2$ solution was incubated with EDTA or L-cys at 37℃ for 1 h. The molar ratio of mAb to challenging agent was at a maximum of 10 000:1. Dissociation ratio was analyzed on paper chromatography.

Biodistribution and imaging

Balb/c mice bearing HHCC were divided into three groups. Each group consisted of three animals and each animal received approximately 15 μg antibody with about 7.4MBq through a lateral tail vein. At time intervals of 4, 10 and 24 h postinjection, three groups of mice were killed , and imaged on a SPECT (Starcam 3 000, UK). Data were collected 100 000 co unts per image and peak energy settings at the 140 ke V (20%) window for ^{99m}Tc. The blood and other organs of interest were collected. Tissues were washed, blotted, weighed and counted in a gamma counter. For each mouse, data are expressed as percent of injected dose per gram of tissue (%ID/g) after physical decay corrected.

RESULTS

Figure 1 represents the calibration curve for the determination of sulphydryl groups using L-cys standards over a range of 0.312 to 10 mg/L, by plotting optical density at 412 nm versus L-cys standard concentrations after subtraction of the background due to Ellman's reagent. Linear regression was used and correlation coefficient 0.999 was obtained. Table 1 shows the influence of the reduction conditions on the number of free sulphydryl groups detected by this thiol assay. As expected, increasing the molar ratio of Sn/GH to antibody in the reaction mixture does increase the number of apparent -SH groups per antibody, and increase the labeling efficiency correspondingly, which results in the labeling efficiency at a maximum of 84.2%. The free $^{99m}TcO_4^-$ and colloid amounts determined by Whatman 3MM paper using different developing systems were also showed in Table 1. In control expe ri ments, labeling efficiency was 2% when unreduced HAb18 F(ab')$_2$ was used. SD S-PAGE by both staining and autoradiography showed that the radioactivity co-m igrated with the proteins and that there were almost no protein fragments prese nt within the 60:1 of molar ratio of Sn/GH to mAb (Figure 2). However , another SDS-PAGE in Figure 3 illustrates that fragmentation occurred during t he reduction procedure when the molar ratio of Sn/GH to mAb was at 500:1.

As shown in Figure 4, the immunoreactive fraction, 0.84 was determined by plott ing the inverse of the bound fraction compared with the inverse of the cell conc entration, which is based on the assumption that the total antigen concentration (cell concentration) is a good enough approximation for the free antig en concentration.

Challenging with EDTA did not remove ^{99m}Tc from the labeling conjugat e remarkably, while L-cys at a molar ratio of 625:1 remove approximately one-tenth of the label (Figure 5).

Biodistribution of radioactivity in blood and excised tissues are displayed in Table 2. The preparation localized at the tumors was more than at any organ examined at both 10 h and 24 h after injection, except the kidneys. Th e lower radioactivity in blood at 24 h suggested fast blood clearance. The imaging results in Figure 6 showed significant tumor uptake at 24 h post-i njection.

Table 1 Effect of molar ratio (Sn/GH: mAb) on quantity of-SH, fre e $^{99m}TcO_4^-$ and colloid and labeling efficiency (%, *n* = 3)

Molar ratio (Sn/GH: mAb)	SH groups /mAb	$^{99m}TcO_4^-$	colloid	labeling efficiency
Control	0 ± 0	62.1 ± 4.5	1.1 ± 1.2	2.0 ± 0.9
10:1	0.43 ± 0.04	3.0 ± 1.4	1.4 ± 1.14	4.6 ± 3.8
20:1	1.25 ± 0.10	2.9 ± 1.1	1.4 ± 0.77	2.8 ± 5.1
30:1	2.46 ± 0.08	2.0 ± 0.9	3.2 ± 1.47	8.6 ± 3.2
40:1	3.34 ± 0.09	1.8 ± 1.2	2.8 ± 1.38	4.2 ± 2.8
50:1	3.61 ± 0.12	2.1 ± 0.8	3.6 ± 1.58	4.4 ± 3.4

Table 2 Biodistribution of ^{99m}Tc-HAb 18F(ab')$_2$ in nude mice bearing hepatoma (x ± s, %ID/g)

Organ	Time after injection (h)		
	4	10	24
Blood	2.21 ± 0.24	1.45 ± 0.15	0.56 ± 0.09
Kidney	72.38 ± 4.37	70.47 ± 15.23	56.45 ± 11.36
Liver	1.82 ± 0.48	1.59 ± 0.31	1.43 ± 0.27
Lung	1.62 ± 0.34	1.40 ± 0.17	0.75 ± 0.21
Stomach	1.37 ± 0.39	1.05 ± 0.28	0.50 ± 0.29
Spleen	2.35 ± 0.81	2.11 ± 0.75	1.82 ± 0.85
Large intestine	1.16 ± 0.34	1.42 ± 0.39	0.94 ± 0.32
Small intestine	0.97 ± 0.31	0.95 ± 0.18	0.62 ± 0.24
Heart	2.04 ± 0.55	1.83 ± 0.48	1.17 ± 0.42
Muscle	1.15 ± 0.20	0.77 ± 0.28	0.51 ± 0.25
Brain	0.18 ± 0.02	0.07 ± 0.04	0.02 ± 0.01
Tumor	5.14 ± 2.26	5.84 ± 2.98	6.57 ± 3.01

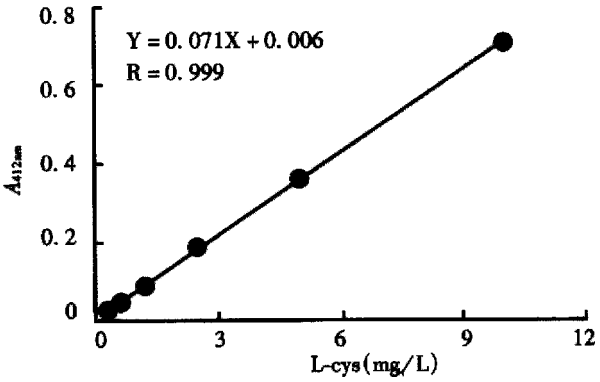

Figure 1 L-cysteine standard curve for sulphydryl d etermination using Ellman reaction.

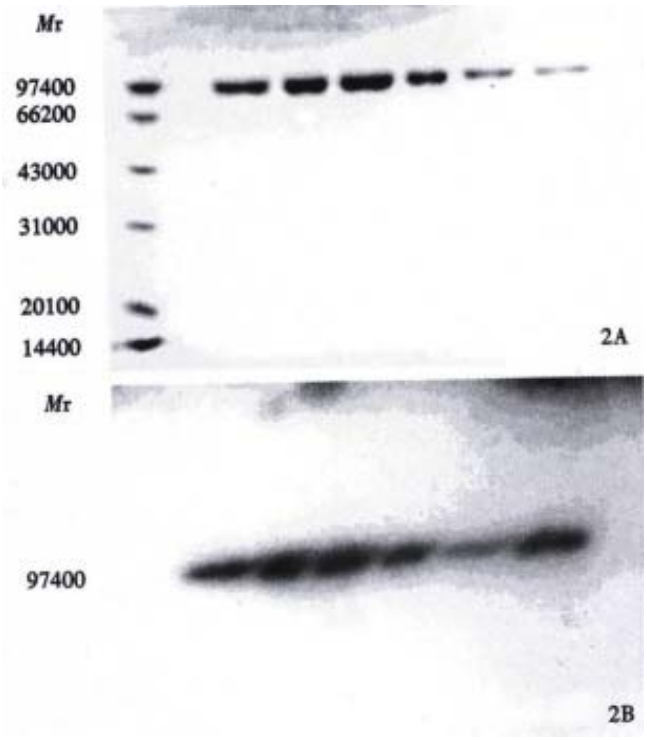

Figure 2 Effect of reduction on integrity of ^{99m}Tc-labeled HAb18 F(ab')$_2$ as monitored by SDS-PAGE. Vertical lanes re present molar ratios of Sn/?GH to HAb18 F(ab')$_2$: 1, 10:1; 2, 20:1; 3, 30:1; 4, 40: 1; 5, 50:1; 6, 60:1. (A)Coomassie brilliant blue R250 staining. Molecular weight (kD) is indicated at the left. (B) autoradiography.

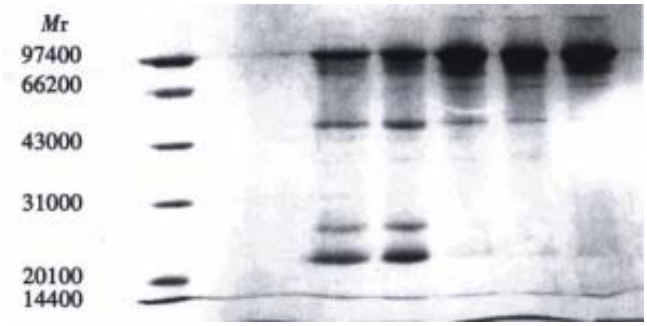

Figure 3 Effect of reduction on integrity of HAb18 F(ab')$_2$ as monitored by SDS-PAGE. Molecular weights (kD) are indicated at the left. Vertical lanes represent molar ratios of Sn/GH to HAb18 F(ab')$_2$: 1, 1000:1; 2, 500:1; 3, 50:1; 4, 10:1; 5, unreduced F(ab')$_2$.

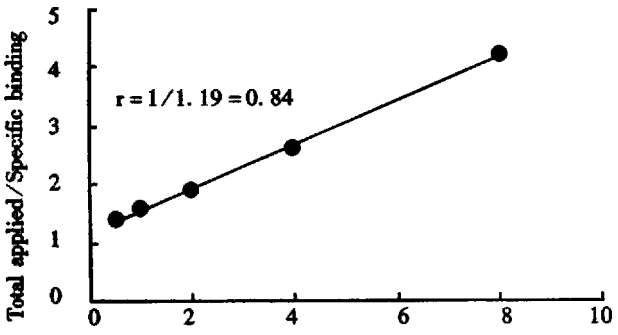

Figure 4 Binding assay for the determination of the immunoreactive fraction of ^{99m}Tc-labeled HAb18 F(ab')$_2$.

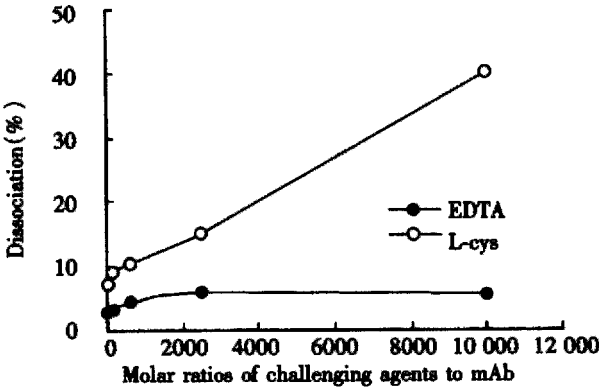

Figure 5 Dissociation of ^{99m}Tc-labeled HAb18 F(ab')$_2$ with increasing molar ratio of EDTA to mAb (●) and L-cys to mAb (○).

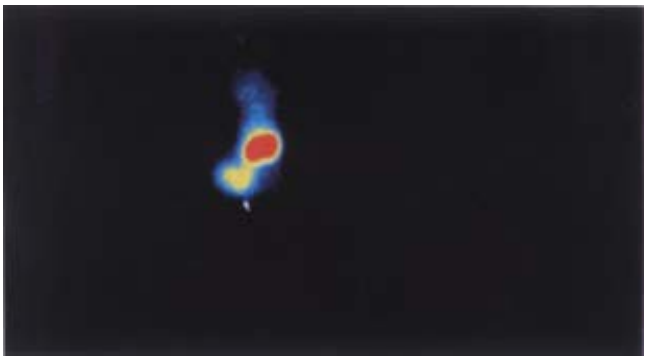

Figure 6 Images of nude mice bearing human hepatocel lular carcinoma with ^{99m}Tc-HAb18 F(ab')$_2$ at 24 h.

DISCUSSION

Great efforts have been made to develop a method that can be used for the direct labeling of mAbs with ^{99m}Tc[16]. Earlier studies involved the incubation of mAbs with stannous phthalate tartrate solution for up to 21 h at room temperature, which was named "pretinning" method. Clinical success with this method has been claimed by the author[23].

One aim of our study was to further evaluate the role of stannous as a reducing agent in the direct labeling of mAb F(ab')^a-2 with ^{99m}Tc. The difference between the "pretinning" method and this method is that we use GH instead of phthalate-tartrate as transfer ligand and stabilizer to avoid Sn or Tc-c olloid formation. To do this, we investigated the effect of the quantity of Sn/ GH on the labeling time and efficiency. When the molar ratio of Sn/GH to mAb F (ab')$_2$ was constant, we found that there was no obvious difference on the number of-SH between the reduction time of 20 min and 30 min or even longer[24]. The whole labeling process can be accomplished within 1.5h. Hnatowich *et al.* reported that labeling efficiency in the case of the stannous ion-reduced a ntibodies was generally in excess of 70%[12], however, in our method mol ar ratio of Sn/GH to mAb was an important parameter to obtain good labeling re sults, and molar ratio of 40:1 or higher were needed to get labeling

effici ency of more than 80% (Table 1). The low percentage of free $^{99m}TcO_4$ and radiocolloid in each sample implied that pH 5.3 and GH are the opti mal pH value and transfer ligand. Under this condition, the labeled mAb HAb18 F(ab')$_2$ keeps its immunoreactivity. Autoradiography of SDS-PAGE had only one m igration of component identical to that of native HAb18 F(ab')$_2$ determination by staini ng with Coomassie brilliant blue R250 (Figure 2), which demonstrated that S n/GH reduction is mild and does not destroy interchain bridges in mAbs. Labeli ng efficiency of 2% in control experiments using unreduced HAb18 F(ab')$_2$ indicated that there was no exchange with the low affinity sites and also demonstrated tha t reduction of disulfides is a necessary initial step in ^{99m}£Ôc direct labeling of antibodies. The bond between^{a2}SH and Tc is stronger than that of N -Tc or O-Tc which was verified by the challenge assay of ^{99m}Tc-HAb18 F(ab')^2 in the presence of EDTA. We found that EDTA even at a molar rat io of 10 000:1 failed to remove a significant amount of ^{99m}Tc, this is in agreement with the results of Rhodes *et al*[8]. But L-cys at 625¡Ã1 remove one-tenth of the label (Figure 5). Despite such insta bility of the label, there was no *in vivo* evidence of release of pertechneta te due to no thyroid imaging observed in the whole imaging process (Figure 7) . Tumor localization of ^{99m}Tc-HAb18 F(ab')$_2$ was successfully dem onstrated in a human tumor/nude mouse xenograft model. Biodistribution and ima ging results showed the highest tumor uptake at 24 h post-injection. Where as kidney levels were foun d to be higher in the whole process. Accumulation of radioactivity in the kidney may be the result of retention of this metallic radionuclide by the kidney pro ximal tubule[25], the possible release of ^{99m}Tc-labeled cysteine and gluta thione[26]stemming from the adioimmunoconjugate catabolism, and the relative amount of ^{99m}Tc-GH. A technique has been used in patients to block renal tubule uptake of ^{99m}Tc-anti-CEA Fab' fragments by amin o acid infusion[27].

In conclusion, a radioimmunoimaging conjugate for hepatoma detection was prepare d by direct labeling mAb HAb18 F(ab')2-with ^{99m}Tc using stannous/g lucoheptonate as reducing agent. Although the labeling efficiency is not satisf actory to some degree, it has several advantapes: simple, easy and quick, besides, the labeled mAb fragment retains its immunoreactivity. Biodistribution and imaging studies reveal that this conjugate is useful for the detection of hepato ma.

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