67Ga-labeled Antibodies for Immunoscintigraphy and Evaluation of Tumor Targeting of Drug-Antibody Conjugates in Mice

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ABSTRACT

To assess the in vivo behavior of cytotoxic agents linked to antibodies, deferoxamine, known to form stable chelates with 67Ga, was conjugated with monoclonal antibodies using three different methods. One method used a homocoupling reagent, glutaraldehyde, whereas two other methods used heterocoupling reagents, N-succinimidyl-3-(2-pyridyldithio)propionate and succinimidyl-6-maleimidohexanooate, linking deferoxamine to antibodies through alkylamine, disulfide, and thioether bonds, respectively. Antibodies were efficiently labeled with 67Ga through chelation with deferoxamine without losing antigen-binding capability. 67Ga-labeled antibodies clearly visualized transplanted tumors in nude mice. However, the biodistribution of radioactivity was markedly different with the coupling methods used for the conjugation of deferoxamine and antibodies. High nonspecific uptake in the liver and spleen was observed with 67Ga-labeled antibodies prepared by the glutaraldehyde method. 67Ga-labeled antibodies linked by thioether bonds demonstrated in vivo stability and the highest tumor/liver ratio, whereas 67Ga-labeled antibodies conjugated with disulfide bonds were rapidly cleared from the circulation. These results indicate that antibody conjugates linked by thioether bonds are a better choice for drug targeting and that 67Ga-labeled antitumor monoclonal antibodies are useful not only for the immunoscintigraphy but also for the quantitative assessment and visualization of the biodistribution of drug-antibody conjugates.

INTRODUCTION

Recently, MoAbs1 that recognize tumor-associated antigen are used to carry drugs, toxins, or radionuclides to cancer tissues. These antibody conjugates could serve as potential new cancer diagnostic and therapeutic agents. 111I- or 125I-labeled antitumor monoclonal antibodies are widely used for the diagnosis of cancer (1–6). Toxins and antineoplastic drugs can also be conjugated with MoAbs using several coupling reagents for the treatment of cancer (7–18). Retention of immunoreactivity, stability during transport, and tumor accumulation of MoAb conjugates are required for effective therapy. However, few studies have been done on the effect of the method of coupling cytotoxic agents and MoAbs on the in vivo biodistribution and tumor targeting of the agent.

Recently we have demonstrated that MoAbs are efficiently labeled with radiogallium through chelation with DFO (19). DFO forms a stable chelate with 67Ga, a suitable radionuclide for scintigraphic imaging. In addition, DFO contains an amino group of high reactivity regarded as an available functional group for the coupling reaction with MoAbs. In the present study, three coupling methods were used for the conjugation of DFO and MoAb, resulting in different biodistributions of 67Ga-labeled MoAb according to the chemical reaction used. Since similar chemical reactions have also been used for the coupling of antineoplastic drugs and MoAbs (7–18), these results would be of use for the quantitative assessment of the biodistribution of antibody conjugates and visualizing tumor targeting.

MATERIALS AND METHODS

Tumor Model

KT005 closed human osteosarcoma was maintained by serial s.c. transplantation in athymic nude mice. Tumors of 0.5 to 1.0 g in size at 2 to 4 wk posttransplantation of 1- to 2-mm3 tissues were used for the in vivo study. To study in vitro immunoreactivity of antibody-conjugates, single cell suspensions were also obtained from xenografted tumors as described previously (20).

Monoclonal Antibody

MoAb OST7 (IgG1) was generated by a standard hybridoma technique using osteosarcoma tissues freshly resected from an untreated patient (21). OST7 was derived from the ascitic fluid obtained from hybridoma-injected BALB/c mice and purified by Protein A affinity chromatography (Bio-Rad, Richmond, CA).

An isotype-matched MoAb 56C, specific for human choriocarcinogonadotropin, was also used as a control MoAb.

Antibody Conjugation with DFO

The conjugation of DFO with MoAb was performed as previously reported (22). We have used glutaraldehyde, pyridyl disulfide, and maleimide methods for the conjugation of an amino group in DFO and that in MoAb as shown in Fig. 1. The molar ratio of DFO to MoAb within the conjugates was adjusted to about 1 for all preparations so as to maintain the immunoreactivity of antibody conjugates (22).

Glutaraldehyde Method. DFO was conjugated with MoAb using glutaraldehyde which is thought to link directly through Schiff’s base formation between amino groups of MoAb and DFO (Fig. 1, left). Briefly, a 60 mm solution of DFO (2 ml) in 0.1 m borate-buffered saline (pH 8.6) was mixed to 80 @l of glutaraldehyde (25%) and stirred for 4 min at 4°C. Thirty @l of this reaction mixture were mixed with 2.5 ml of MoAb solution (5 mg/ml) in 0.1 m PBS (pH 7.5) and stirred for 1 h at 4°C. Then, 150 @g of sodium borohydride in distilled water (100 @g) were added and stirred another 15 min at 4°C. DFO-MoAb conjugates were separated from unreacted DFO using Sephadex G-50 column chromatography.

Pyridyl Disulfide Method. The coupling of DFO and MoAb through disulfide bonds was introduced using SPDP (Pharmacia, Uppsala, Sweden). This method consisted of three parts: the introduction of 2-pyridyl disulfide into both MoAb and DFO using SPDP; the reduction of 2-pyridyl disulfide residues coupled to MoAb by diethyldithitol; and the conjugation of a thiol group of MoAb and 2-pyridyl disulfide of DFO by a thiol-disulfide exchange reaction. DFO is thought to link with MoAb through disulfide bonds (Fig. 1, center). (a) Briefly, for DFO-2-pyridyl disulfide, 200 @l of 40 mm SPDP in absolute ethanol were incubated with 500 @l of 45 mm DFO in 10 mm borate-buffered saline (pH 8.6) with mixing for 30 min at room temperature. After the addition of a tracer amount of 67Ga chloride, the mixture was resolved on a Sephadex G-10 column equilibrated with 0.1 m PBS (pH 7.5). (b) For thiolated MoAb, 15 @l of 20 mm SPDP in absolute ethanol were added to 1 ml of MoAb (5 mg/ml) in 0.1 m PBS with mixing for 30
The percentage of bound radioactivity was determined as described method(22). The specific activity of ⁶⁷Ga-labeled MoAb was about 5
and paper chromatography. MoAb was ⁶⁷Ga-labeled by the chloramine

type gamma counter. The immunoreactivity of radiolabeled MoAb was
determined by Sephadex G-50 gel chromatography, electrophoresis,
MoAb (10 ng per tube), increasing concentrations of unlabeled MoAb
(50 μl), and 1 × 10⁶ cells (100 μl) were incubated for 2 h at 4°C, and
the percentage of bound radioactivity was determined as described
above.

In Vitro Stability

In vitro stability of MoAb conjugates labeled with ⁶⁷Ga was examined
by incubating in 10 mM Tris/HCl buffer, pH 7.5, fresh human serum,
and murine liver homogenates. Normal murine liver was homogenized
in 10 mM Tris/HCl, pH 7.5, using a Potter-Elvehjem-type homogenizer
and centrifuged at 800 × g for 10 min at 4°C. Forty μl of radiolabeled
MoAb were added to 960 μl of Tris/HCl buffer, fresh human serum,
or 800 × g supernatant of liver homogenates and placed in a shaking
water bath adjusted to 37°C. Small aliquots were removed at the start
and after 1, 3, 6, 12, and 24 h of incubation for analysis by gel and
paper chromatography. Gel chromatography was performed on a Sephdex
G-50 column eluted with 0.05 M PBS, which separated labeled
protein from ⁶⁷Ga-DFO chelates and ⁶⁷Ga. For paper chromatography,
the solvent system was a mixture of normal butyl alcohol, normal propyl
alcohol, and water (9:6:5), and solid phase was Toyo 51B filter
paper. ⁶⁷Ga-DFO-MoAb was found at Rf = 0, and ⁶⁷Ga-DFO was found
at Rf = 0.4. All experiments were performed at least twice using
different preparations of ⁶⁷Ga-labeled MoAb, and good agreement in
the results was obtained.

Biodistribution

For biodistribution studies, KT005-bearing nude mice were given
injections of radiolabeled OST7 at a dose of 20 μg of MoAb/mouse.
The mice were killed 24 and 48 h after the injection. Organs were
removed and weighed, and the radioactivity was counted. In each of
three series (glutaraldehyde, maleimide, and pyridyl-disulfide methods),
two mice were put into a metabolic cage, and their urinary radioactivity
was collected over 48 h. Free ⁶⁷Ga chloride and ⁶⁷Ga-labeled isotype-matched irrelevant
MoAb 56C were also used for control studies. Biodistribution data
were shown as the percentage of the injected dose per gram of tissue
normalized to a 20-g mouse.

Imaging Study

Animal images were made using a gamma camera (Searle, Chicago,
IL) equipped with a pinhole collimator following i.v. administration of
50 μCi of ⁶⁷Ga-labeled OST7. Images were taken at 3, 24, and 48 h
after the injection.

RESULTS

In Vitro Study. All DFO-MoAb conjugates prepared by three
different methods efficiently formed chelates with ⁶⁷Ga. The labeling
efficiency (radiochemical yield) was more than 90% with good reproducibility [96.4 ± 2.1% (means ± SD) for the

Fig. 1. Scheme for the preparation of DFO-MoAb conjugates using three methods. Left, glutaraldehyde method; center, pyridyl disulfide method; right, maleimide
method. Ab, antibody; DTT, dithiothreitol.
glutaraldehyde method, 95.8 ± 1.7% for the maleimide method, and 93.0 ± 2.6% for the pyridyl-disulfide method. Fig. 2 shows the binding of labeled MoAb to KTO05 cells as a function of cell number. There was no significant difference among the bound percentage in 67Ga-labeled OST7 prepared by the three different methods and 125I-labeled OST7. The immunoreactive fraction of each labeled OST7 was considered to be between 50 and 60%. Although nonspecific MoAb 56C was also labeled with 67Ga with similar efficiency, its binding to KTO05 cells was less than 5.1%. Fig. 3 shows the result of competitive immunoassay. Unlabeled OST7 almost completely inhibited the binding of labeled OST7 to KTO05, and there was no significant difference in the inhibitory curves of 67Ga-labeled OST7 obtained by the three different methods and 125I-labeled OST7.

67Ga-labeled MoAb was stable in 0.1 M Tris/HCl buffer, pH 7.5, and free 67Ga or 67Ga-DFO chelates were not detected in 67Ga-labeled OST7 linked with alkylamine and thioether bonds in the presence of fresh serum or liver homogenates. However, 67Ga-labeled OST7 linked with disulfide bonds was unstable when incubated with fresh serum and liver homogenates at 37°C (Fig. 4). Using gel and paper chromatography, about 10% of 67Ga was released from MoAb conjugates when incubated with serum, and up to 30% of radioactivity was dissociated from MoAb in liver homogenates within 1 h (Fig. 5). This liberation of 67Ga was dependent on the incubation temperature, and no release of 67Ga was observed when the incubation temperature was 4°C (data not shown).

In Vivo Studies. Table 1 shows the results of the biodistribution studies of 67Ga-labeled OST7 in KTO05-bearing athymic mice. 67Ga-labeled OST7 prepared by the glutaraldehyde method was localized in tumors at 20% or more of the injected dose per gram at 24 and 48 h after injection, and its blood clearance was as slow as that of radioiodinated MoAb (22). However, its uptake in the liver was high. MoAb conjugates linked with thioether bonds showed a blood radioactivity and tumor uptake level similar to those obtained with the glutaraldehyde method.

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Fig. 2. Reactivity of radiolabeled OST7 to KTO05 cells in vitro. Various numbers of KTO05 cells were incubated with 67Ga-labeled OST7 prepared by the glutaraldehyde method (O), 67Ga-labeled OST7 prepared by the pyridyl disulfide method (•), 67Ga-labeled OST7 prepared by the maleimide method (△), 125I-labeled OST7 (A) or 67Ga-labeled 56C prepared by the maleimide method (□). The percentage of radioactivity bound to cells was plotted against cell number.

Fig. 3. Competitive immunoassay of radiolabeled OST7 to KTO05 cells. Increasing concentrations of unlabeled OST7 and 1 × 10⁶ KTO05 cells were incubated with 67Ga-labeled OST7 prepared by the glutaraldehyde method (O), 67Ga-labeled OST7 prepared by the pyridyl disulfide method (•), 67Ga-labeled OST7 prepared by the maleimide method (△), or 125I-labeled OST7 (A). The percentage of radioactivity bound to cells was plotted against the unlabeled OST7 concentration added. After incubation at 4°C for 2 h, the radioactivity associated with cells was measured. The binding to cells in 50 µg of unlabeled MoAb was considered as nonspecific binding and subtracted from the data.

Fig. 4. Effect of serum and liver homogenates on the in vitro stability of 67Ga-labeled MoAb. 67Ga-labeled MoAbs were incubated with 0.1 M Tris/HCl buffer, fresh human serum, or liver homogenates at 37°C for 1 h and analyzed by paper chromatography. A to D, 67Ga-labeled MoAb prepared by the pyridyl disulfide method. A, no treatment; B, incubated with 0.1 M Tris/HCl buffer alone; C, after incubation with fresh serum; D, after incubation with liver homogenates. E, 67Ga-labeled MoAb prepared by the glutaraldehyde method, after incubation with liver homogenates; F, 67Ga-labeled MoAb prepared by the maleimide method, after incubation with liver homogenates. 67Ga attached to MoAb, free 67Ga chloride remained at an applied point (Rf = 0), and 67Ga-DFO chelate complexes migrated to Rf = 0.4. The form of the radioactivity at Rf = 0.1 detected in D was unknown. Samples were also analyzed by gel chromatography, and the percentage bound to MoAb was the same as that of paper chromatography.

Fig. 5. Time course of the in vitro stability of 67Ga-labeled MoAb in fresh serum and liver homogenates. 67Ga-labeled MoAb prepared by the glutaraldehyde method (O), 67Ga-labeled MoAb prepared by the pyridyl disulfide method (•), and 67Ga-labeled MoAb by the maleimide method (△) were incubated up to 24 h with serum (left) or murine liver homogenates (right). Small aliquots (3 samples at each point) were taken and analyzed by paper chromatography. Points, percentage of radioactivity at Rf = 0; bars, SD.
Data are expressed as the percentage of injected dose per gram of tissue.

### Table 1: Tissue distribution of 

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Glutaraldehyde method</th>
<th></th>
<th>Maleimide method</th>
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<th>Pyridyl disulfide method</th>
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<td></td>
<td>24 h</td>
<td>48 h</td>
<td>24 h</td>
<td>48 h</td>
<td>24 h</td>
<td>48 h</td>
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<tr>
<td>Blood</td>
<td>11.32 ± 1.34*</td>
<td>7.97 ± 1.31</td>
<td>12.15 ± 1.56</td>
<td>7.49 ± 1.37</td>
<td>5.90 ± 1.50</td>
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<td>Liver</td>
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<td>7.84 ± 2.83</td>
<td>4.86 ± 0.37</td>
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<td>2.36 ± 0.33</td>
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<td>Kidney</td>
<td>4.51 ± 0.46</td>
<td>4.54 ± 0.50</td>
<td>4.33 ± 0.56</td>
<td>3.24 ± 0.36</td>
<td>2.65 ± 0.22</td>
<td>1.86 ± 0.21</td>
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<td>Intestine</td>
<td>1.62 ± 0.03</td>
<td>1.65 ± 0.45</td>
<td>1.95 ± 0.16</td>
<td>1.29 ± 0.13</td>
<td>1.04 ± 0.11</td>
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<td>Stomach</td>
<td>0.98 ± 0.03</td>
<td>1.11 ± 0.30</td>
<td>1.23 ± 0.16</td>
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<td>0.82 ± 0.17</td>
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<td>6.04 ± 1.48</td>
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<td>Lung</td>
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<td>4.72 ± 0.74</td>
<td>3.51 ± 0.69</td>
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<td>Muscle</td>
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<td>Bone</td>
<td>2.73 ± 0.38</td>
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<td>3.42 ± 0.31</td>
<td>3.35 ± 0.43</td>
<td>1.36 ± 0.24</td>
<td>1.19 ± 0.21</td>
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<td>Tumor</td>
<td>21.30 ± 3.19</td>
<td>28.45 ± 5.98</td>
<td>25.65 ± 3.58</td>
<td>24.46 ± 3.30</td>
<td>11.74 ± 1.52</td>
<td>9.92 ± 2.17</td>
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*Mean ± SD for 4 mice.

### Table 2: Tissue distribution of 

<table>
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<th>Tissue</th>
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<th>Pyridyl disulfide method</th>
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<tr>
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<td>4.13 ± 0.56</td>
<td>2.54 ± 0.35</td>
<td>1.92 ± 0.22</td>
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<tr>
<td>Stomach</td>
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<td>1.67 ± 0.43</td>
<td>0.65 ± 0.03</td>
<td>0.37 ± 0.01</td>
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<tr>
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<td>1.03 ± 0.27</td>
<td>0.35 ± 0.05</td>
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</table>

*Data for 2 mice.  
†Mean ± SD for 4 mice.

Fig. 6. Scintigrams of mice bearing KTOO5 injected with 

$^{67}$Ga-labeled OST7 prepared by the glutaraldehyde method. Arrowheads indicate transplanted tumor.

Deoxysugar and its uptake in the liver was low. In the case of $^{67}$Ga-labeled OST7 linked by disulfide bonds, blood clearance was very fast, and its net tumor uptake was less than 10% of the injected dose per gram at 48 h after injection. Tumor:blood ratios of $^{67}$Ga-labeled OST7 calculated for three antibody conjugates were similar, ranging from 3.1 to 3.4, and tumor:muscle ratios were between 32.0 and 34.0 at 48 h. Compared with tumor:blood and tumor:muscle ratios, tumor:liver ratios varied from 6.7 to 3.6 depending on the coupling method used. The liver:blood ratio was the lowest with $^{67}$Ga-labeled OST7 prepared by the maleimide method and the highest with conjugates prepared by the glutaraldehyde method. The cumulative urinary excretion of $^{67}$Ga collected for 24 and 48 h was 2.5% and 3.1% of the injected radioactivity with the glutaraldehyde method, 7.0% and 14.4% with the maleimide method, and 17.7% and 34.0% with the pyridyl disulfide method, respectively. By gel and paper chromatography, the radioactivity in the urine was found to be $^{67}$Ga-DFO chelates unattached to MoAb (data not shown).

Table 2 shows the results of biodistribution studies obtained with $^{67}$Ga-labeled irrelevant MoAb 56C and $^{67}$Ga chloride used as control experiments. MoAb conjugates prepared by either maleimide or pyridyl disulfide methods showed no specific localization in KTOO5 tumors. Rapid clearance from the circulation was also observed with $^{67}$Ga-labeled 56C linked by disulfide bonds. The localization index which shows the tumor targeting ratio or specific:nonspecific ratio was calculated by dividing the tumor:tissue ratio of OST7 by that of 56C. With
67Ga-labeled MoAbs with thioether bonds at 48 h, it was 6.0 for blood, 5.7 for liver, and 5.5 for muscle; and with antibody conjugates with disulfide bonds, it was 4.3, 6.5, and 5.5, respectively.

Figs. 6 to 8 show scintigrams of KTOO5 tumor-bearing athymic mice given an i.v. injection of 67Ga-labeled OST7. Scintigraphies confirmed the results of biodistribution studies. 67Ga-labeled OST7 prepared using three coupling methods clearly visualized KTOO5 tumors without using the subtraction technique. 67Ga-labeled OST7 linked by thioether bonds showed a high localization in tumors with low background radioactivity. 67Ga-labeled OST7 prepared by the glutaraldehyde method also visualized transplanted tumors clearly, but background imaging in the liver was high. Tumors were also visualized by 67Ga-labeled OST7 linked by disulfide bonds. However, it took a longer time than the other two preparations, reflecting the rapid blood clearance of 67Ga from the circulation.

DISCUSSION

This study demonstrated that (a) DFO-MoAb conjugates were efficiently labeled with 67Ga without losing reactivity to tumor cells, (b) 67Ga-labeled OST7 prepared using three different methods clearly visualized the KTOO5 tumor transplanted in nude mice, and (c) biodistribution of antibody-conjugates varied, although the same MoAb and the same tumor were used. Because the chemistries involved in conjugating DFO to MoAb are the same in conjugating toxins and drugs to MoAb in targeting of tumors, this study provides some information on the selection of the chemistry of drug-MoAb conjugation.

Drug-MoAb conjugates have been reported to impair the growth of tumors in nude mice and to serve as potential new cancer therapeutic agents (9, 14, 17, 18). Several techniques are available for the coupling of toxins or antineoplastic drugs to antitumor MoAbs. However, few studies have dealt with the stability or biodistribution of drug-MoAb conjugates because of the lack of proper techniques. One example is the work of Rowland et al. who utilized 3H, a β-emitter isotope (17).

We have used DFO as a bifunctional chelating agent, which binds to MoAb at one end and forms stable chelates with 67Ga at the other end. Three different methods were used for the preparation of DFO-MoAb conjugates. DFO was linked with MoAb by alkylamine, thioether, and disulfide bonds, which have been widely applied for the preparation of drug- or toxin-antibody conjugates for immunochemotherapy (7–18). 67Ga is a clinically useful isotope with a half-life of 3.3 days and suitable γ-emission for diagnostic imaging. When DFO is separated from MoAb conjugates, 67Ga-DFO chelate complexes are easily excreted in the urine (23). Using 67Ga, DFO, and antitumor MoAb, we can easily evaluate the in vitro and in vivo stability of the linkage between drug and MoAb and visualize the tumor targeting of drug-MoAb conjugates.

67Ga-labeled OST7 prepared by the glutaraldehyde method showed an intense accumulation in the transplanted tumor and prolonged blood retention, but a rather high uptake in the liver.
Gluutaraldehyde is a homocoupling reagent and causes high-molecular-weight polymer formation of MoAb (7, 22). Inter- and intra-cross-linkage of MoAb may result in a high accumulation in the liver. In contrast, SPDP and EMCS are heterocoupling reagents and do not make polymers of MoAb in their reactions (22). Radiolabels linked by disulfide bonds showed rapid clearance from the circulation and the lowest accumulation in the tumor and liver. Liver tissues contain many kinds of enzymes and abundant thiol, mainly glutathione (24), which may cause a cleavage of the disulfide linkage, resulting in instability and the fastest blood clearance of radioactivity. The difference in blood clearance of radioactivity influenced the absolute tumor uptake of 67Ga, indicating that some degree of blood retention of drug-MoAb conjugates would be of importance for delivering antineoplastic drugs to desired sites.

67Ga-labeled OST7 prepared by the maleimide method was localized in the tumor and showed a similar blood clearance rate and lower liver uptake when compared with the radiolabel prepared by the glutaraldehyde method. Although the tumor/nontumor ratio of radioactivity was similar to that of other coupling methods, MoAb conjugates linked by thioether bonds were considered to be optimal for in vivo use because of their in vivo stability and low uptake in the liver.

Other parameters such as tumor vascularization or blood flow are to be considered for the accessibility of MoAb to cancer cells. Moreover, after the interaction with specific surface antigens, the internalization and intracellular degradation will also be important in releasing an active form of drug. However, the present models have value in furthering our understanding of the stability and behavior of drug-MoAb conjugates both in vitro and in vivo. 67Ga-labeled MoAb will visualize the tumor targeting of MoAb conjugates which may be useful in predicting the outcome of immunochemotherapy.

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