Pharmacokinetics of $^{99m}$Tc(Sn)- and $^{131}$I-labeled Anti-Carcinoembryonic Antigen Monoclonal Antibody Fragments in Nude Mice

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ABSTRACT

The biodistribution, radioimmunoimaging, and high pressure liquid chromatography activity profiles of $^{99m}$Tc(Sn)- and $^{131}$I-labeled anti-carcinoembryonic antigen monoclonal antibody fragments were compared. Nude mice, bearing specific (colonic carcinoma, LS174T) and nonspecific (pancreatic carcinoma, MIA) xenografts were given injections of the respective radiolabeled antibody fragments and also of irrelevant $^{125}$I-labeled antibody fragments (MOPC-21). The animals were imaged at 24 h after being given injections, they were sacrificed, and biodistribution studies were performed. Results of the study showed high kidney uptake (48.6% injected dose (ID)/g ± 8.1% (SD)) and low tumor uptake (1.5% ID/g ± 0.6%) for $^{99m}$Tc(Sn)-labeled fragments and higher uptake (4.4% ID/g ± 0.6%) for $^{131}$I-labeled fragments, resulting in a higher localization index for the radiiodinated monoclonal antibody fragments. Imaging results showed good tumor visualization at 24 h after injection for the $^{131}$I-labeled fragments and poor tumor visualization with predominant kidney uptake for $^{99m}$Tc(Sn)-labeled fragments. After radiolabeling, high pressure liquid chromatography analysis indicated that $^{131}$I was primarily associated with Fab' fragments, whereas $^{99m}$Tc was mostly associated with Fab fragments.

INTRODUCTION

Radiolabeled MOAB$^1$ to tumor-associated antigens are currently being examined as potential diagnostic and therapeutic agents. Because of availability and suitable radionuclide properties, technetium-$^{99m}$m-labeled antibodies have been investigated as possible imaging agents. A number of methods, including direct and indirect radiolabeling, have been used to attach $^{99m}$Tc to MOAB. Direct labeling methods involve the reduction of technetium by stannous ion, either as stannous chloride ($^{99m}$Tc to MOAB. Direct labeling methods involve the reduction of technetium by stannous ion, either as stannous chloride (1-3) or stannous tartrate (4, 5), followed by complexing of reduced technetium with amino acid residues of monoclonal antibodies. Extensive studies, including human imaging trials (6-9), have been performed with monoclonal antibody and/or antibody fragments directly labeled with $^{99m}$Tc. However, few comparisons between $^{99m}$Tc labeling and other radiolabeling methods on the same MOAB have been performed. This paper compares the biodistribution and radioimmunoimaging of $^{99m}$Tc(Sn)- and $^{131}$I-labeled murine anti-CEA monoclonal antibody fragments in nude mice, each bearing specific and nonspecific xenografts.

MATERIALS AND METHODS

Monoclonal Antibodies. The IgG1 murine anti-CEA monoclonal antibody was provided by Abbott Laboratories (North Chicago, IL). Monoclonal antibody Fab' fragments, pretinned and native, as well as irrelevant antibody F(ab')$^2$ fragments were prepared by Summa Medical Corp. (Albuquerque, NM). The identity of the fragments was confirmed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10). Results revealed a single band when stained with Coomassie blue, corresponding to a molecular weight of approximately 120.

Radiolabeling of Monoclonal Antibodies. Radiolabeling with $^{131}$I was achieved using the chloramine-T method (11). In a typical reaction, between 1000-1200 µCi of Na$^{131}$I (New England Nuclear, North Billerica, MA) was added to 100 µg of anti-CEA fragments in 100 µl PBS (iodine/MOAB ratio = 1). Chloramine-T (5 µg) was then added and the antibody solution incubated at 0-2°C for approximately 10 min. The reaction was terminated by adding 5 µg sodium metabisulfite. The radiolabeled protein was purified using gel exclusion chromatography consisting of Bio-Gel P-10 (Bio-Rad Laboratories, Richmond, CA). After purification, free iodide levels were determined using Gelman instant thin layer chromatography-silica gel (Gelman Instrument Co., Ann Arbor, MI) with 0.9% NaCl solution as the solvent (12). Free iodide levels were less than 5% after final antibody purification.

Iodine-$^{125}$I radiolabeling of irrelevant MOAB fragments (MOPC-21) was accomplished by the chloramine-T method (11). Typically, about 500 µCi Na$^{125}$I (Amersham Corp., Arlington Heights, IL) was added to 100 µg of protein in 100 µl PBS. Chloramine-T was then added (10 µg) and the solution incubated at 0-2°C for 10 min. The reaction was terminated by adding 10 µg of sodium metabisulfite. Protein purification was achieved as outlined above. After purification, free iodide levels were determined as outlined and were less than 5%.

Technetium-$^{99m}$m labeling of pretinned anti-CEA MOAB fragments as well as pretinned irrelevant antibody fragments was accomplished according to the manufacturer’s instructions. Between 10 and 30 mCi of $^{99m}$Tc pertechnetate, in 0.5 ml saline, was added to the antibody reaction vial containing 200 µg of lyophilized MOAB(Sn). After pertechnetate addition, the solution was incubated for 45-60 min at room temperature. One ml of normal saline containing 1% human serum albumin was then gently added to the reaction vial. The radiolabeled antibody solution was then purified using gel exclusion chromatography (Filtech; Summa Medical Corp., Albuquerque, NM) consisting of Sephadex G-25 (Pharmacia Fine Chemicals, Piscataway, NJ) which had been treated with stannous phthalate and gentisate (3). After purification, the radiochemical purity was measured using instant thin layer chromatography-silica gel with 0.5% NaCl solution (12). Free pertechnetate levels were less than 10% after final purification.

Radioimmunoreactivity. Microtiter plates (96 well) were coated with purified CEA by incubating a 0.1-ml solution of the antigen (1.0 µg in 10 mm Tris, pH 7.4) in each well. After an overnight incubation at room temperature, the wells were emptied and washed twice with distilled water. The plates were then overcoated for 2 h at room temperature with a solution of bovine serum albumin (0.1%) in 0.1 M phosphate buffered saline (pH 7.4). The overcoated plates were stored at 0-2°C with 0.1 ml of overcoating buffer until needed. Immediately before use, the plates were emptied and washed twice with distilled water.

The radioimmunoreactive fraction, at infinite antigen excess, was measured using the procedure outlined by Lindmo et al. (13) with one notable exception. In the original procedure, antibody binding was determined with increasing antigen/antibody ratios. This was accomplished by increasing antigen levels while maintaining constant antibody levels. In the present study, increasing antigen/antibody ratios were obtained by varying the antibody levels while maintaining constant antigen levels. The specific radiolabeled MOAB fragments, ranging from 0.15-8.0 ng in 20 µl PBS, were added to microtiter plates containing fixed CEA to determine specific binding and to fixed RBC isotype suspension to determine nonspecific binding. The plates were rotated at room temperature for 120 min, washed 5 times with PBS,
and counted for radioactivity. In addition, the total activity added to each well was determined. For each specific antibody concentration, 4 replicate samples were obtained and statistical analysis performed. The data were then graphically expressed with total activity/specific binding on the ordinate and antibody/antigen on the abscissa. The immunoreactive fraction, at infinite antigen excess, was determined by linear extrapolation to the ordinate.

Animal Biodistribution and Imaging Studies. Athymic nude mice (BALB/c-nu/nu background) were given injections s.c. in the right flank of a suspension of LS174T colorectal carcinoma cells (2.5 x 10⁶ cells in 0.1 ml saline) and in the left flank with a suspension of MIA pancreatic carcinoma cells (5 x 10⁶ cells in 0.1 ml saline). Two to 3 weeks after injection, the CEA-expressing LS174T tumors had grown to about 0.5 g, while the MIA tumors, which did not express significant amounts of CEA, were about 0.2 g.

Athymic nude mice, each bearing s.c. xenografts as outlined, were given injections in the tail vein of 8–10 µg (in 0.1–0.3 ml PBS) of ¹³¹I-labeled (70–80 µCi) or ⁹⁹ᵐTc(Sn)-labeled (500–550 µCi) anti-CEA MOAB fragments and also injections of 8–10 µg of ¹²⁵I-labeled (35–50 µCi) of irrelevant fragments. Immediately after injection, whole body counts were obtained with a NaI(Tl) scintillation detector and multichannel analyzer. Posterior whole body images were acquired at 2 hr, 7 hr, and 24 h postinjection using a gamma scintillation camera with a pinhole collimator interfaced to a computer system. After imaging, 5 animals in each specific group were sacrificed, whole body counts acquired, and tissue biodistribution studies performed. No thyroid blocking agents were administered throughout the study.

In order to characterize the monoclonal antibody fragments, ¹³¹I- and ⁹⁹ᵐTc-labeled anti-CEA monoclonal antibody fragments were prepared and an aliquot of each removed and analyzed by HPLC. Several nude mice were then given injections of the specific radiolabeled anti-CEA fragments, as outlined above. Blood samples were withdrawn at 15 min postinjection, centrifuged, and the plasma fraction removed. Urine samples were also collected from these animals up to 6 h after initial injection. HPLC analysis was performed on the urine and plasma samples.

Nude mice were also given injections in the tail vein of 8–10 µg (0.15–0.20 ml in PBS) of ⁹⁹ᵐTc(Sn)-labeled irrelevant MOPC-21 antibody fragments (500–600 µCi). At 24 h postinjection, 3 animals were sacrificed and tissue biodistribution studies performed.

The biodistribution data were statistically summarized by calculating mean percentage of injected dose per g and SD for each specific tissue. In addition, the localization index (14, 15) and specific/nonspecific tumor ratios were also calculated from the data.

High Pressure Liquid Chromatography. High pressure liquid chromatography was performed using a Rainin isocratic HPLC system (Rainin Instrument Co., Woburn, MA), consisting of an Altex 110A pump and a NaI(Tl) gamma scintillation detector interfaced to a multichannel analyzer. Chromatographic separation was performed using a 300- x 7.5-mm Bio-Sil TSK-250 molecular weight sizing column and the corresponding Bio-Sil TSK guard column (Bio-Rad). After injection, samples were eluted in a 0.5 M Na₂SO₄/0.02 M NaH₂PO₄ buffer, pH 6.8, at a flow rate of 1.5 ml/min. The eluted radioactivity was monitored with the multichannel analyzer using multichannel scaling (1-s interval).

RESULTS

The results of the radioimmunoreactivity study are shown in Fig. 1. The immunoreactive fractions, at infinite antigen excess, for ⁹⁹ᵐTc- and ¹³¹I-labeled MOAB fragments were 0.53 and 0.57, respectively. These results agree with the results of other investigators (14) using ¹¹¹In- and ⁹⁹ᵐTc-coupled anti-CEA antibodies. Nonspecific binding on fixed RBC lysate microtiter plates was less than 5% throughout the study.

The results of the biodistribution study are summarized in Tables 1 and 2. No thyroid blocking agents were administered throughout these studies. From the biodistribution results in Table 1, significant differences in tissue activities of the radio-

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**Table 1** Biodistribution of radiolabeled monoclonal antibodies in athymic nude mice, each bearing a specific (LS174T colon carcinoma) and a nonspecific (MIA pancreatic carcinoma) tumor and obtained 24 h after injection.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>⁹⁹ᵐTc(Sn)-labeled anti-CEA</th>
<th>¹³¹I-labeled anti-CEA</th>
<th>⁹⁹ᵐTc(Sn)-labeled MOPC-21</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F(αβ')₂</td>
<td>F(αβ')₁</td>
<td>F(αβ')₃</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.19 ± 0.06</td>
<td>0.29 ± 0.07</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>Thyroid</td>
<td>0.33 ± 0.06</td>
<td>1.59 ± 0.79</td>
<td>0.19 ± 0.02</td>
</tr>
<tr>
<td>Blood</td>
<td>0.53 ± 0.10</td>
<td>1.39 ± 0.36</td>
<td>0.31 ± 0.05</td>
</tr>
<tr>
<td>Heart</td>
<td>0.40 ± 0.05</td>
<td>0.63 ± 0.19</td>
<td>0.19 ± 0.04</td>
</tr>
<tr>
<td>Lung</td>
<td>0.60 ± 0.21</td>
<td>1.27 ± 0.36</td>
<td>0.24 ± 0.03</td>
</tr>
<tr>
<td>Liver</td>
<td>1.85 ± 0.66</td>
<td>0.55 ± 0.18</td>
<td>1.27 ± 0.14</td>
</tr>
<tr>
<td>Spleen</td>
<td>1.12 ± 0.46</td>
<td>0.55 ± 0.23</td>
<td>0.98 ± 0.23</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.21 ± 0.08</td>
<td>3.88 ± 1.33</td>
<td>0.09 ± 0.06</td>
</tr>
<tr>
<td>Kidneys</td>
<td>48.62 ± 8.13</td>
<td>0.99 ± 0.25</td>
<td>18.20 ± 0.20</td>
</tr>
<tr>
<td>Bone</td>
<td>0.51 ± 0.38</td>
<td>0.29 ± 0.11</td>
<td>0.05 ± 0.04</td>
</tr>
<tr>
<td>Specific tumor</td>
<td>1.46 ± 0.61</td>
<td>4.36 ± 0.59</td>
<td>0.38 ± 0.05</td>
</tr>
<tr>
<td>Nonspecific tumor</td>
<td>0.51 ± 0.05</td>
<td>0.78 ± 0.25</td>
<td>0.35 ± 0.05</td>
</tr>
</tbody>
</table>

*Mean ± SD for 5 animals.

**Table 2** Localization index and specific/nonspecific tumor ratios of radiolabeled monoclonal antibody fragments.

<table>
<thead>
<tr>
<th>Ratio</th>
<th>⁹⁹ᵐTc(Sn)-labeled anti-CEA</th>
<th>¹³¹I-labeled anti-CEA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Localization index</td>
<td>1.47 ± 0.48</td>
<td>5.23 ± 2.05</td>
</tr>
<tr>
<td>Specific/nonspecific tumor</td>
<td>2.89 ± 1.19</td>
<td>5.85 ± 1.47</td>
</tr>
</tbody>
</table>

*Mean ± SD for 5 animals.
AND I31I-LABELLED ANTI-CEA MOAB IN NUDE MICE

Fig. 2. Posterior gamma scintillation images of 131I-labeled (A) and 99mTc(Sn)-labeled (B) anti-CEA antibody fragments in nude mice, each bearing a right flank specific (LS174T colon carcinoma) and a left flank nonspecific (MIA pancreatic carcinoma) xenograft, and obtained 24 h postinjection. For each radiolabeled antibody, the right image was intensified, blanking out major areas of activity. L, liver; S, stomach; K, kidneys; B, bladder; ST, specific tumor.

images are presented; the intensity of one of the 2 images is increased, blanking out major areas of activity, in order to visualize less intense areas of localization. As confirmed from the biodistribution data, predominant specific LS174T tumor was visualized for the 131I-labeled fragments at 24 h postinjection. Some soft tissue activity, especially stomach and liver, was also visualized, particularly after image intensification. For 99mTc-labeled anti-CEA antibody fragments (Fig. 2B), significant kidney activity was observed on the 24-h images. Specific LS174T tumor and bladder activity were visualized only after image intensification. Nonspecific MIA tumor localization, in the left flank area, was not visualized with either radiolabeled anti-CEA fragment.

The radiometric elution profiles of 131I- and 99mTc-labeled anti-CEA MOAB fragments, prior to and immediately after animals had been given injections, are shown in Fig. 3. For the radioiodinated fragments (Fig. 3, C and D), most of the radioactivity was associated with the F(ab')2 fragments (Fig. 3, C and D, peak b). Some minor radioactivity was also associated with the whole antibody (Fig. 3, C and D, peak a) and free iodine (Fig. 3, C and D, peak d). No significant differences in the elution profiles between in vitro and in vivo radioiodinated fragments were observed. The radiometric elution profile for 99mTc-labeled anti-CEA fragments are shown in Fig. 3, A and B. Most of the radioactivity was associated with Fab' fragments (Fig. 3, A and B, peak c), whereas minor activity was associated with F(ab')2 fragments (Fig. 3, A and B, peak b) and whole antibody (Fig. 3, A and B, peak a). The radiometric elution profile in mouse plasma (Fig. 3B), obtained 15 min after animals were given injections, was similar to the initial in vitro elution profile (Fig. 3A).

The HPLC elution profile of urine radioactive activity, obtained 6 h after injection of 131I- and 99mTc-labeled anti-CEA MOAB fragments, are shown in Fig. 4. For radioiodinated anti-CEA fragments, a single peak of activity was observed (Fig. 4B) corresponding to free iodine. For 99mTc-labeled fragments, several peaks were observed (Fig. 4A). The major radioactivity in urine samples corresponded to free 99mTc (Fig. 4A, peak f). A significant amount of urine radioactivity was also associated with an intermediate molecular weight component (Fig. 4A).
peaks e). Other radioactive elution peaks included whole antibody (Fig. 4A, peak a) and Fab’ fragments (Fig. 4A, peak c).

Whole body activity retention was acquired at 24 h postinjection. No significant differences between 99mTc(Sn)-labeled [30.2% ± 4.0% (SD) of injected dose for 5 animals] and 131I-labeled [26.1% ± 8.7% of injected dose for 5 animals] anti-CEA fragments were observed.

**DISCUSSION**

Significant differences in biodistribution and radioimmunoimaging were noted between 99mTc(Sn)- and 131I-labeled anti-CEA MOAB fragments, even though the fraction of immunoreactive radiolabeled antibody was very similar for both. Significantly higher tumor uptake, with minimal soft tissue activity, was observed for the radiodinated antibody fragments, resulting in a higher localization index, a higher specific/nonspecific tumor ratio, and better tumor images. HPLC analysis of the radiodinated MOAB fragments prior to and immediately after animals were given injections confirmed that most of the radioactivity was associated with F(ab’)_2 fragments. However, free iodine was observed in urine samples obtained 6 h after injection indicating in vivo dehalogenation. This was confirmed by the results of the biodistribution and imaging studies showing increased stomach activity.

The biodistribution and radioimmunoimaging data for 99mTc(Sn)-labeled anti-CEA MOAB fragments as well as irrelevant fragments showed very high kidney and relatively low liver activity. The specific tumor uptake was also low, resulting in a low localization index. Although the biodistribution data generally agree with other published data for 99mTc-labeled antibody fragments (16-18), the degree of kidney uptake was significantly higher in our study for both relevant and irrelevant antibodies and may be the result of the manufacturer’s prelabeled process and/or the radiolabeling procedure.

The question arises as to whether the significant kidney uptake can be attributed to a breakdown of antibody fragments from the divalent to the monovalent form during the prelabeled process and/or the radiolabeling procedure. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, which was performed on the prelabeled fragments, confirmed that the predominant fragments were divalent F(ab’)_2. After radiolabeling, HPLC analysis confirmed that the predominant radioactivity was associated with the monovalent Fab’ fragments. Other investigators have obtained similar results (18). These data strengthen the hypothesis that the prelabeled process may cause some reduction in disulfide bonds with the subsequent generation of monomeric fragments. This may be followed by the preferential labeling of monomers by 99mTc through free sulfhydryl groups (17-19). In addition, Tc labeling of sulfhydryl groups may prevent the reformation of disulfide bonds, thus preventing F(ab’)_2 formation.

Another possible explanation for significant kidney uptake includes immune complex formation of the radiolabeled monoclonal antibody with circulating CEA. We do not believe that kidney uptake is attributable to immune complex formation since high kidney uptake was also observed with the irrelevant 99mTc(Sn)-labeled MOAB (MOPC-21) (Table 1). In addition, other investigators have also reported significant kidney uptake with 99mTc(Sn)-labeled antibody fragments which recognize other antigens (18).

As mentioned previously, the immunoreactive fraction of the radiolabeled monoclonal antibody fragments was determined by maintaining constant antigen levels (1000 ng CEA) while varying antibody levels (from 0.15-8.0 ng). In the original procedure (13), the antigen/antibody ratio was determined by varying the antigen levels while maintaining constant antibody levels. We believe that the modified procedure as outlined is valid because antigen excess was maintained, even at the highest antibody levels.

For our experimental model, the data indicate that 131I-labeled anti-CEA fragments are more suitable for tumor visualization than the corresponding 99mTc(Sn) analogue. Realizing the disadvantages of 131I in radioimmunoimaging, we are currently evaluating the use of 125I-labeled anti-CEA fragments.

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