Tumor Imaging with $^{99m}$Tc-Labelled Antibody Fragments

Buck A. RHODES
Summa Medical Corporation
Albuquerque, New Mexico, USA

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1. Introduction

Although the labelling of proteins with $^{99m}$Tc was established in 1964 by McAfee, et al., this isotope has been avoided by most investigators for the labelling of antibodies for tumor localization. There are several reasons for this: 1) the half-life is only 6 hours while most previous radiolabelled antibody localization studies have shown that the required tumor-to-blood ratio needed for tumor visualization occurs after four days, i.e. after 16 half-lives; and 2) previous studies of the stability of the Tc-immunoglobulin bond have indicated that most methods do not result in a bond strength which is sufficient to prevent loss of radiolabel by mechanisms such as ligand exchange. In recent years two innovations have been made which permit these previous problems to be solved. First, it has been recognized that fragments of antibodies distribute throughout the body and clear the bloodstream much faster than whole immunoglobulin molecules, making it possible to use shorter-lived isotopes. Secondly, at least two methods have been described which result in strong bonding of the technetium to antibody protein. One of these methods is the "pretinning" method and the other is the coupling of the technetium to the antibody via a bifunctional chelate.

2. Radiolabelling Methodology

2.1 The pretinning method

A solution of the immunoglobulin is prepared in the presence of phthalate and tartrate ions such that the final pH is 5.65 and the protein concentration is between 1 and 10 mg/ml. It is very important to avoid the contamination of the solution with phosphate ions because these ions poison the reaction of the technetium to antibody protein. One of these methods is the "pretinning" method and the other is the coupling of the technetium to the antibody via a bifunctional chelate.
Table 1 The pretinning method

1. Prepare phthalate/tartrate solution: 2.82 g of potassium tartrate pulse 8.17 g of potassium hydrogen phthalate per l of nitrogen gas purged, sterile water.
2. Prepare stannous chloride in concentrated HCl: 2.256 g of stannous chloride dihydrate in 20 ml of concentrated hydrochloric acid.
3. Prepare pretinning solution: Add 10 ml of the stannous chloride in concentrated HCl to 1 l of the phthalate/tartrate solution and adjust the pH to 5.65 with 10 normal sodium hydroxide.
4. Prepare antibody solution: 1 mg of protein (purified fragments of mouse monoclonal antibody) per ml of 0.9% saline solution.
5. Mix antibody solution and pretinning solution in a ratio of 3 to 2 and purge with nitrogen.
6. Overlay solution with an equal volume of gas composed of two parts nitrogen and one part air. Seal vial.
7. Incubate for 21 hours at room temperature or until the stannous ion concentration is reduced to 20% of the total added tin.
8. The solution may be either freeze-dried for later use or labelled immediately with $^{99m}$Tc. The minimum amount of protein that can be labelled is 150 µg.
9. To radiolabel, add up to 1.85 MBq (50 mCi) of $^{99m}$Tc per 150 µg of protein. Incubate for 1 h. Add equal volume of 1% human serum albumin and pass through a Filtech (TM)* radiopharmaceutical filter column.

* Available from Summa Medical Corporation, Albuquerque, New Mexico, USA.

Immediately after pretinning or it is added to a sealed vial containing the freeze-dried, pretinned protein. The technetium is allowed to incubate with the pretinned protein for 69–90 minutes. Next 1 ml of 1% human serum albumin is added. This serves as a carrier protein which protects from radiolysis, losses due to manipulation, and also protects against oxidation. The labelled protein is then passed through a pre-washed Filtech radiopharmaceutical filter column into a sterile, empty vial. An additional 0.5 ml of sterile saline is passed through the filter and added to the vial. This is done to recover any labelled protein remaining in the void space of the filter column. Usually 60–90% of the radioactive technetium is recovered from this filtration process and the final solution is then free of significant radiocchemical impurities. The solution is also stable for several hours thereafter.

2.2 The bifunctional chelate method

Khaw, et al.6),7) has prepared the Fab fragments of affinity purified rabbit anti-cardiac myocin and coupled this protein to diethylene triamine penta-acetic acid (DTPA) using the carboxycarbonic anhydride of DTPA according to the procedure of Krejcarek and Tucker. Sodium pertechnetate is reduced with a 5 x $10^6$ excess sodium dithionite for no more than 10 minutes at room temperature. The reduced technetium is then added to an aliquot of the Fab-DTPA conjugate in 0.3 M phosphate buffered saline, pH 8.0. The labelled protein is then separated from the other reaction production by size exclusion chromatography on Sephadex-G-25 column preequilibrated with 1% serum albumin in lactated Ringer's solution. The peak from the void volume of the first chromatographic separation is then rechromatographed on a Sepharose-4B column to remove high molecular weight radioactive contaminants which localize in the liver.

2.3 Comparison of methods

In a previous report2) data was presented which showed that other methods reported in the literature, other than the two cited above, did not produce a bond between the protein and the technetium which would resist trans-
chelation. To measure this, the $^{99m}$Tc-labeled protein was reacted with Sephadex-G 25. Sephadex is able to bind reduced technetium; thus if the radiolabeled protein in vulnerable to transchelation, the $^{99m}$Tc was transferred onto the Sephadex. The Sephadex is a solid phase reagent thus it is easy to separate it from the solution containing the $^{99m}$Tc-protein. The ratio of radioactivity between the solid and the liquid phases, therefore, was used as a measure of transchelatable technetium. The result of this comparison is given in Table 2.

When all of the methods for labelling immunoglobulins are compared it becomes apparent that the key to forming a strong bond between the protein and the technetium is the use of a linking substance. In the case of the pretinning method the link is apparently inorganic, i.e. a stannic oxide linkage. In the case of the bifunctional chelate method the link is organic, i.e. an organic chelating moiety covalently linked to the protein. A limitation to this latter method which has not been observed with the pretinning method is that the number of linking groups per molecule of protein must be carefully limited. Palt, et al.9) has shown that the immunoreactivity of the immunoglobulin decreases with an increase in the number of linking groups per mole of protein, ideally this should be limited to no more than one to one. Thus with the use of organic linkers, the specific activity of the final product is also limited.

A second problem with the bifunctional chelate method is that the technetium must be reduced prior to its addition to the protein-chelate conjugate. If it is reduced in the presence of the protein-chelate conjugate, then it can bond either to the protein directly or to it via the chelate. In fact, Lanteigne and Hnatowich12) have shown that direct labelling takes precedence over labelling via the chelate. Therefore, if the reduction takes place in the presence of the protein multiple species will be formed possibly including transchelatable technetium. In any event, the higher avidity of the technetium for direct binding to the protein over the chelate suggests that the use of the bifunctional chelate is both unnecessary and may actually result in nothing more than damaging of the original protein.

### 3. Radiochemical Properties of Tc-Labelled Antibodies

#### 3.1 Immunoreactivity

To measure the immunoreactivity of a radiolabelled antibody or antibody fragment, the labelled antibody is usually reacted with the antigen attached to a solid phase matrix. When the antigen is available it can be bound to particles such a Sepharose-4B. This is the method we have used extensively with antibodies to human chorionic gonadotropin13). The antigen coupled to the Sepharose-4B particles are incubated with the radiolabelled antibody. After the incubation, the particles are separated from the solution and the radioactivity of each is measured. The percentage of immunoreactive radiotracer is calculated from these measurements. Care must be taken to assure that the amount of antigen on the particles is in excess of the added radiolabelled antibody.

When antigen is not available as a purified substance, then cells or membranes of cells which express the antigen may be used as the solid phase source of the antigen14),15). This method usually results in lower estimates of radioimmunochemical purity than does the method which uses the purified antigen bound to a solid matrix.

#### 3.2 Stability of the radiolabeled antibody

The method of Loberg and Fields16,17 can be used to measure the relative stability of the

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**Table 2: Estimation of relative transchelatable $^{99m}$Tc**

<table>
<thead>
<tr>
<th>Method</th>
<th>$[\text{Sn}^2+]$</th>
<th>$^{99m}$Tc Activity and concentration</th>
<th>$K^*$</th>
<th>Sephadex column yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ref. 10</td>
<td>$1.9 \times 10^{-3}$</td>
<td>200 mCi/20 ml</td>
<td>3.017</td>
<td>1.85%</td>
</tr>
<tr>
<td>Ref. 11</td>
<td>$2.7 \times 10^{-4}$</td>
<td>60 mCi/20 ml</td>
<td>1.376</td>
<td>32.47%</td>
</tr>
<tr>
<td>Ref. 4</td>
<td>$8.3 \times 10^{-3}$</td>
<td>200 mCi/20 ml</td>
<td>0.085</td>
<td>72.65%</td>
</tr>
</tbody>
</table>

* $K$: Activity of Sephadex compartment (solid phase)/activity of aqueous compartment (liquid phase).

$(10 \text{ mCi}=370 \text{ MBq})$
technetium bonded to the protein. Briefly, the labelled protein is incubated with a strong chelating agent and the percentage of the radioactivity transferred to the chelating agent is measured. When we applied this method to \(^{99m}Tc\)-antibodies labelled by the pretinning method, it was found that there was less than 5% transchelation. No transchelation took place until the concentration of chelate (DTPA) reached a molar excess of 4000. See Fig. 1. This test reveals that the bonding of the technetium to the protein is very strong. In other experiments\(^{17}\), we showed that the \(^{99m}Tc\) did not migrate from the labelled antibody to other proteins in the circulation.

4. Biological Properties

4.1 Tissue distribution in mice

The \(^{99m}Tc\)-labelled F(\(ab'\)\(^2\)) fragments of antibodies, when injected intravenously, are rapidly cleared from the bloodstream via the kidneys. Table 3 reports typical biodistribution data in Swiss-Webster mice.

The presence of circulating antigen decreases both blood clearance and kidney uptake and gives a slight increase in liver uptake. Circulating antigen, although it alters the biodistribution, does not change uptake at sites of fixed antigen\(^{17}\). Thus the experimental data in animals supports the clinical observation that circulating antigen does not interfere with uptake of the radiotracer by tumor\(^{18}\).

4.2 Pharmacokinetics in man

Lyster, et al.\(^{19}\) has demonstrated that the blood clearance of \(^{99}Tc\) labelled antibodies is faster in man than is \(^{111}\)In labelled antibodies. This permits more rapid imaging of the patient within 4 to 24 hours post injection. As was previously shown in animals the primary route of excretion for antibody fragments is the urine. Although some gastro-intestinal secretion was occasionally observed in a few patients. In studies of more than 100 patients, no adverse reactions were observed. The dose in this series was, however, kept to less than 150 \(\mu\)g/patient. Some patients received multiple doses with no ill effects. Skin test doses were used to demonstrate that the patients did not develop an allergic response to the antibody fragments.

4.3 Radiation dosimetry estimates

Calculations based on both animal and human biodistribution studies provide estimates of radiation dosimetry as shown in Table 4. These calculations reveal that the kidney is the critical organ with respect to radiation exposure.

5. Clinical Studies

5.1 Methods

The patients studied to date have received 370\(\sim\)740 MBq (10\(\sim\)20 mCi) of \(^{99}Tc\) labelled to 150 \(\mu\)g of F(\(ab'\)\(^2\)) murine monoclonal antibody reactive against the beta subunit of human chorionic gonadotropin. Dynamic blood pool
Table 3 Biodistribution of $^{99m}$Tc labeled F(ab')$_2$ fragments of murine monoclonal antibodies to hCG-beta. Values are reported at 0.5 and 5.0 h after i.v. injection in mice. Each value is the mean of 4 animals except lot 001 at 0.5 h—this is the mean of 3 mice.

<table>
<thead>
<tr>
<th>Organ</th>
<th>0.5 h</th>
<th></th>
<th>5.0 h</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>001</td>
<td>002</td>
<td>004</td>
<td>Lot TAK-MYB-</td>
</tr>
<tr>
<td>Liver</td>
<td>6.95±0.60</td>
<td>5.40±0.85</td>
<td>7.15±1.08</td>
<td>5.62±1.05</td>
</tr>
<tr>
<td>Kidneys</td>
<td>32.78±2.06</td>
<td>65.01±7.36</td>
<td>45.07±4.99</td>
<td>27.99±5.51</td>
</tr>
<tr>
<td>Blood</td>
<td>17.72±1.69</td>
<td>10.22±1.60</td>
<td>13.84±1.06</td>
<td>6.26±1.09</td>
</tr>
<tr>
<td>Spleen</td>
<td>4.96±0.52</td>
<td>4.13±0.43</td>
<td>4.23±0.55</td>
<td>2.65±0.28</td>
</tr>
<tr>
<td>Stomach</td>
<td>1.09±0.63</td>
<td>1.05±0.32</td>
<td>1.03±0.36</td>
<td>0.96±0.31</td>
</tr>
<tr>
<td>Heart</td>
<td>7.58±2.23</td>
<td>4.73±0.61</td>
<td>6.48±0.79</td>
<td>3.16±0.24</td>
</tr>
<tr>
<td>Lungs</td>
<td>6.69±0.78</td>
<td>5.09±0.65</td>
<td>6.40±0.24</td>
<td>3.36±0.68</td>
</tr>
<tr>
<td>Sm. Intestine</td>
<td>3.14±0.06</td>
<td>2.56±0.64</td>
<td>3.99±0.84</td>
<td>2.94±0.57</td>
</tr>
<tr>
<td>Lg. Intestine</td>
<td>0.82±0.07</td>
<td>1.05±0.58</td>
<td>0.96±0.08</td>
<td>5.12±0.56</td>
</tr>
<tr>
<td>Femur</td>
<td>7.21±0.64</td>
<td>2.16±0.27</td>
<td>1.27±0.31</td>
<td>4.78±0.32</td>
</tr>
<tr>
<td>Muscle</td>
<td>1.86±0.61</td>
<td>0.83±0.04</td>
<td>1.27±0.31</td>
<td>0.82±0.12</td>
</tr>
<tr>
<td>Ovary</td>
<td>4.96±0.57</td>
<td>6.11±1.72</td>
<td>5.83±0.38</td>
<td>2.47±1.50</td>
</tr>
<tr>
<td>Thyroid*</td>
<td>0.23±0.02</td>
<td>0.24±0.05</td>
<td>0.25±0.04</td>
<td>0.13±0.04</td>
</tr>
</tbody>
</table>

* % injected dose in whole organ

Table 4 Radiation absorbed dose, rads

The estimated absorbed radiation doses to an average patient (70 kg) from an intravenous injection of 10 and 20mCi of $^{99m}$Tc anti-hCG-beta-F(ab')$_2$ is given below.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Rads/mCi</th>
<th>Rads/mCi</th>
<th>Rads/mCi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidneys</td>
<td>0.000712</td>
<td>7.12</td>
<td>14.24</td>
</tr>
<tr>
<td>Liver</td>
<td>0.000136</td>
<td>1.36</td>
<td>2.72</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.000062</td>
<td>0.62</td>
<td>1.24</td>
</tr>
<tr>
<td>Testis</td>
<td>0.000025</td>
<td>0.25</td>
<td>0.50</td>
</tr>
<tr>
<td>Ovaries</td>
<td>0.000057</td>
<td>0.57</td>
<td>1.14</td>
</tr>
<tr>
<td>Bladder wall</td>
<td>0.000132</td>
<td>1.32</td>
<td>2.64</td>
</tr>
<tr>
<td>Whole body</td>
<td>0.000013</td>
<td>0.13</td>
<td>0.26</td>
</tr>
</tbody>
</table>

studies were made during the injection. Next an early, i.e. 5 minute, image of the blood pool in the chest or other region of interest was obtained. Static images were obtained 3~6 hours post injection. At times a delayed, i.e. 16~24 hour image, was obtained or a background subtracted image was obtained after the 3~6 hour image. To make the background subtracted image, $^{113m}$InCl$_3$ was injected to label the circulating transferin. After normalization of the two images, the $^{113m}$In image, i.e. the blood pool image, was computer subtracted from the $^{99m}$Tc image. At times the subtracted image would reveal a lesion not seen in the unsubtracted image.

5.2 Localization of tumors in patients

Morrison, et al.\textsuperscript{20} were able to visualize choriocarcinomas, and adenocarcinoma and squamous cell tumors in the lungs. Also an occasional metastasis to bone was visualized. The results with murine monoclonal antibody fragments were essentially the same as those previously obtained with sheep polyclonal antibody fragments. Primary lesions were detected with a higher frequency than metastasis with the highest detection rate being for the choriocarcinomas. Sixty nine percent of lung tumors were detected. The overall lesion detection rate was 49% when the results of all known lesions both primary and secondary were evaluated.

6. Summary

6.1 Conclusions

The used of $^{99m}$Tc labelled F(ab)$_2$ fragments for tumor imaging has been demonstrated clinically in man. The imaging can be done within the usable life of $^{99m}$Tc. The procedure
has been shown to be safe for human use. The detection rates are not as high as desired; this may be due in part to lack of expression of the antigenic determinant by the tumor.

6.2 Prospects for the future

Recent innovations in the use of radio-labelled monoclonals for tumor imaging give great promise to this approach to tumor diagnosis. For example, Berche, et al. have shown that by the use of tomography the detection rate of tumors is greatly improved. Ehrlich and Moyle have shown that mixtures of monoclonal antibodies can be used to enhance the sensitivity of antigen detection. Also we can expect that improved antibodies will be available in the future, including human monoclonal antibodies. Finally, there is now developing an expectation that tumor detection rates may be a function of the actual amount of antibody injected. Thus current studies may not have been done with optimized dosages. Hence, as these various new parameters are more fully evaluated and put into clinical utilization we can anticipate decided improvements in the diagnostic efficacy of radioimmunoimaging.

The true future of this diagnostic methodology is probably its use in selecting patients for antibody directed tumor therapy. Recent reviews of the development of this approach to tumor therapy indicated that extensive work is being completed which is leading to the clinical application of cancer immunotherapy in the near future.

References
