Uptake and Metabolism of $^{111}$In-labeled Monoclonal Antibody B6.2 by the Rat Liver

Peter L. Jones, Beverly A. Brown, and Howard Sands

Abstract

When $^{111}$In-labeled murine monoclonal antibodies are used in radioimmunodiagnostic procedures, a large fraction of the injected radionuclide is sequestered by the liver. Neither the cells responsible for the uptake nor the mechanism of uptake are known. Little is known about either the site within the liver of antibody metabolism or the form of the products of metabolism. In these studies, the uptake and metabolism of a monoclonal antibody, B6.2 radiolabeled with $^{111}$In or $^{125}$I (either intact B6.2 or F(ab')2) were determined in rats. One h after injection of either $^{111}$In- or $^{125}$I- $^{111}$In-diethylenetriaminepentaacetic acid ($^{111}$In-DTPA)-labeled B6.2, the predominant liver cell in which the radionuclide was found was the parenchymal cell. At this time, the absolute uptake of $^{111}$In in the liver was 0.23 ± 0.06% (SD) of the injected dose compared to 0.61 ± 0.06% when the radionuclide was $^{125}$I. Removal of the Fc portion of the antibody reduced the absolute liver uptake of $^{125}$I to 0.10 ± 0.01 and the absolute uptake of $^{111}$In to 0.16 ± 0.06. Both radionuclides were still associated predominantly with the parenchymal cell. Using size exclusion high performance liquid chromatography analysis of liver supernatants the metabolism of radiolabeled B6.2 was followed for 24 h. Of the radioactivity recovered, 47.9% of the $^{125}$I was precipitable by centrifugation (and presumed bound to cell membranes) while 15.4% was attached to B6.2 found in the cytosol. In contrast, when $^{111}$In-DTPA-B6.2 was administered, 16.0% of $^{111}$In recovered from the liver was precipitable by centrifugation, and 6.5% was attached to B6.2 found in the cytosol. Sixty % of the $^{111}$In was recovered as a low molecular weight (less than 1000) component in the cytosol. This metabolite was not immunoreactive, nor did it comigrate with ferritin, and was resolved into four components by ion exchange high performance liquid chromatography. Of these, only a minor component cochromatographed with an $^{111}$In-DTPA standard. These data suggest that the large accretion of radionuclide by the liver is due to uptake of monoclonal antibodies by an Fc receptor-mediated mechanism and the subsequent accumulation of low molecular weight metabolites, presumably $^{111}$In-DTPA, attached to one or more amino acids. The reasons for the entrapment of metabolites in the liver are under investigation.

Introduction

$^{111}$In-labeled monoclonal antibodies have been used widely both in animal studies (1–5) and in clinical studies (6–10) to detect solid tumors. The advantages of $^{111}$In as a diagnostic radionuclide are its relatively short half-life of 2.8 days and its γ emission energies of 173 and 274 keV which are suitable for scintillation imaging. Moreover, $^{111}$In can be conveniently attached to tumor-specific monoclonal antibodies via bifunctional chelators such as DTPA2 (11). In most clinical and preclinical studies the accretion of $^{111}$In-labeled antibodies by tumors has been greater than that of radioiodinated antibodies (6–9, 12). Unfortunately, as both animal and clinical studies have shown, the accumulation of $^{111}$In in the liver is very high relative to radioiodinated monoclonal antibodies. For example, in tumor-bearing mice the liver uptake of $^{111}$In is typically within the range of 15–20% of the injected dose/g (9, 11, 13). The consequences of liver accumulation are: (a) less $^{111}$In-labeled antibody is available for binding to tumors; (b) imaging of metastases in liver and surrounding areas is difficult, if not impossible; and (c) with therapeutic isotopes, the radiation dose to the liver may be significant. The high liver accretion of $^{111}$In when attached to a monoclonal antibody has, therefore, limited its practical utility as a diagnostic radionuclide. As a step in an attempt to develop a rational approach to addressing the problem of non-antigen-specific $^{111}$In uptake by the liver, this study had two objectives. The first was to identify the liver cell types in the rat which are responsible for the uptake of $^{111}$In following administration of intact immunoglobulin or F(ab')2 labeled with $^{111}$In-DTPA. The second objective was to compare the labeled metabolites found in the liver following injection of $^{111}$In-DTPA labeled monoclonal antibody with those found after injection of $^{125}$I-labeled monoclonal antibody.

Materials and Methods

Antibody Purification. The hybridoma line for B6.2, a murine IgG1, was generated by immunizing BALB/c mice with a membrane-enriched fraction of a human breast carcinoma metastasis to the liver (14). The antibody which was then obtained from the ascites fluid of BALB/c mice was purified by ammonium sulfate precipitation followed by ion exchange chromatography (14). Protein concentrations were determined spectrophotometrically by measurement of absorbances at 280 nm using an extinction coefficient of 1.88. F(ab')2 fragments of B6.2 were produced by pepsin digestion. In this procedure, B6.2 (0.5–2.0 mg/ml) was dialyzed into 0.1 mM sodium acetate buffer, pH 4.0. Pepsin (Fisher Scientific, Pittsburgh, PA) was diluted to 2 mg/ml in acetate buffer, added to the B6.2 solution to a final B6.2:pepsin ratio of 2% (w/w), and incubated for 15 h at 37°C. The mixture was neutralized by the addition of 0.2-mI volumes of Tris-Cl at pH 8.8 and dialyzed exhaustively in 0.05 M sodium phosphate buffer, pH 6.5, using dialysis tubing with a molecular weight cutoff of 50,000. The purity of the final product was confirmed to be greater than 95% by sodium dodecyl sulfate-polyacrylamide gel electrophoresis performed under reducing and non-reducing conditions.

Radiolabeling. Radiiodination was carried out by the Iodo-Gen method as has been described previously (15). B6.2 (20 μg) was reacted with 1 mCi Na125I for 5 min in a reaction tube which had been coated with 20 μg Iodo-Gen. $^{125}$I-B6.2 was subsequently separated from unreacted Na125I using an AG 1-X8 column (Bio-Rad, Richmond, CA). B6.2 was modified with the bis(cyclic anhydride) of DTPA by the method described by Hnatowich et al. (11) as modified by Brown et al. (4) and radiolabeled with $^{111}$In. Labeled antibodies were stored at 4°C and used within 24 h of labeling. $^{99m}$Tc-Microlite (DuPont-NEN) and $^{99m}$Tc-Hepatolite (DuPont-NEN) were prepared according to the manufacturer's instructions and used within 1 h of preparation. $^{99m}$Fe-DTPA, $^{111}$In-DTPA, and $^{111}$In-ferritin were prepared using 59FeSO4 or $^{111}$InCl3 (DuPont, Boston, MA) and 10−3 M DTPA or ferritin as appropriate by the same method as has been described earlier for labeling B6.2.

Animals. Female CD rats weighing 250–300 g were supplied by Charles River Laboratories. Prior to surgery, rats were anesthetized by ether inhalation.

Liver Cell Separation. Either $^{111}$In- or $^{125}$I-labeled antibodies (50 μCi) or $^{99m}$Tc-labeled cell markers (100 μCi) were injected i.v. via the carotid vein which was exposed by a ventral incision at the base of the neck. In all experiments, collagenase perfusion was started 1 h after antibody administration. The procedure adopted for perfusing the rat liver has...
was then perfused through the liver at a flow rate of 40 ml/min as a 1-
perfused with oxygenated DPBS without calcium and magnesium for
been described in detail elsewhere (16). Briefly, the liver was first
gently agitating the liver. A single cell suspension was produced by
2 minutes with DPBS and a crude cell suspension was obtained by
filtering the crude cell suspension through a series of nylon filters.
Parenchymal and nonparenchymal cells were purified by sedimentation
in Percoll (17). Briefly, the crude cell suspension obtained following collagense perfusion was centrifuged for 2 min at 50 x g in a
Beckman T3-6 centrifuge at 4°C. The cell pellet which contained both
viable and nonviable parenchymal cells was resuspended in 10 ml DPBS
and mixed with an equal volume of Percoll (Pharmacia, Uppsala,
Sweden) which had been diluted 1:10 in 10 times concentrated DPBS.
The cell suspension was centrifuged at 1500 x g for 15 min. Cells in
the resulting pellet were resuspended in DPBS and counted using a
hemacytometer. The radioactivity in the cell pellet was measured using
an LKB Gamma Counter. Nonparenchymal cells were purified from the
initial 50 x g supernatant which was layered on a two-step Percoll
gradient, consisting of 2.5% Percoll in DPBS layered on 5% Percoll in
DPBS. The tubes were subsequently centrifuged at 800 x g for 15 min
at 4°C. The lower Percoll zone and the interface of the two Percoll
layers, which were enriched in nonparenchymal cells, were combined,
mixed with an equal volume of DPBS, and centrifuged at 1400 x g for
10 min at 4°C. The resulting nonparenchymal cells were resuspended
in DPBS and counted using a hemacytometer, and the radioactivity in
the cells was determined by gamma scintillation counting.
Metabolism Studies. In the liver fractionation and metabolite analysis
experiments, rats were given injections of 25-250 µCi each of either
"Tc-Microlite or "In-labeled B6.2 or 250 µCi of "Fe-DTPA or "In-DTPA. At
selected times after antibody injection, the liver of each rat was washed
in situ with DPBS for 2 min at 4°C via a catheter inserted in the hepatic
vein. The rat was then sacrificed by ether inhalation, liver samples were
weighed, and the radioactivity was measured by gamma scintillation
counting. To determine the distribution of radioactivity relative to the
membrane fraction and the cytosol, several liver samples were freeze-
thawed three times and sonicated using a Branson Sonifier (Model 185)
for 2 min at 4°C. The sonicated liver samples were centrifuged in a
Sorvall RT6000 at 400 x g for 10 min at 4°C to remove cell debris.
The resulting supernatants were centrifuged in a Beckman Airfuge at
95,000 x g for 30 min at room temperature and the radioactivity in the
resulting supernatants was determined. The pellets were washed twice
by resuspension in DPBS followed by recentrifugation. The radioactiv-
ty in washed pellets and supernatants was then determined by scintil-
lation counting.
Estimation of the molecular weight of the radioactive products in the
supernatants obtained by ultracentrifugation was initially determined by
SEC-HPLC using a Gilson HPLC system fitted with a DuPont GF250XL column. The mobile phase, which was used at a flow rate of 2 ml/min, was 300 mM sodium phosphate, pH 7.0.
Radioactivity was detected with a Berthold radioactivity monitor
(Model LB 506) interfaced with an IBM-AT computer. Fractions (0.3
ml) were collected and peak radioactivity fractions were stored at -70°Cfor
further analysis. These fractions were subsequently analyzed by sepa-
ration on a Ultrahydrogel column (Waters, Milford, MA) using 40
mM Tris, pH 8.5, as the aqueous phase at a flow rate of 0.5 ml/min. Fractions were collected and counted. Using polyethylene glycol standards,
the linear molecular weight separation range of the column was shown to be 600 to 4250.
Table 1 Recovery of parenchymal and nonparenchymal cells from the rat liver
One h after injection of radiolabeled cell markers or radiolabeled antibodies, liver cells were separated on Percoll gradients as described in "Materials and Methods." Each data point is expressed as the product of the counts recovered per cell and the percentage of each cell type recovered in the crude cell supernatant (see "Materials and Methods" for experimental details).

<table>
<thead>
<tr>
<th>Cell markers</th>
<th>Parenchymal</th>
<th>Nonparenchymal</th>
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<tbody>
<tr>
<td>&quot;Tc-Hepatolite</td>
<td>89.7 ± 8.4</td>
<td>10.3 ± 8.4</td>
</tr>
<tr>
<td>&quot;Tc-Microlite</td>
<td>11.8 ± 9.7</td>
<td>88.4 ± 9.8</td>
</tr>
<tr>
<td>&quot;In-DTPA-B6.2</td>
<td>68.8 ± 11.5</td>
<td>31.24</td>
</tr>
<tr>
<td>&quot;In-DTPA-B6.2</td>
<td>65.4 ± 13.9</td>
<td>34.58</td>
</tr>
<tr>
<td>&quot;In-DTPA-B6.2 (F(ab')2)</td>
<td>81.5 ± 12.0</td>
<td></td>
</tr>
<tr>
<td>&quot;In-DTPA-B6.2 F(ab')3</td>
<td>77.3 ± 22.9</td>
<td></td>
</tr>
<tr>
<td>&quot;Tc-Microlite</td>
<td>68.8 ± 11.5</td>
<td>31.24</td>
</tr>
</tbody>
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Each data point is the mean of three determinations, except for those marked with Footnote b, where the values represent the mean of two determinations.
were both immunoreactive (Table 2). These data suggest that when assayed for their ability to bind antigen, Peaks 2 and 3 correspond to the molecular weight range of a proteolytic fragment of an IgG, M, 15,000-50,000, increases with time from 10% of the supernatant at 1 min after antibody injection to almost 75% of the supernatant radioactivity at 24 h. Unlike the time course seen for 125I-labeled antibody, the 111In-labeled B6.2 fragment (Peak 3) showed only a transient increase, reaching approximately 30% of the supernatant radioactivity at 1 h and then decreasing to less than 10% 24 h postinjection. As seen with 125I-B6.2, only Peaks 2 and 3 (Table 2) retained the ability to bind antigen.

Fig. 3 shows the recovery of 125I and 111In at each step of the liver fractionation protocol using livers obtained from rats 24 h after injection of radiolabeled antibody. When the data shown in Figs. 1 and 2 were back-calculated to determine the percentage of each peak that was found in the intact liver (Fig. 3), approximately one-half of the radioactivity following 125I-B6.2 injection was found associated with the cell membrane fraction. Of the remainder which was recovered in the cytosol, approximately one-half comprised labeled proteolytic fragments. In contrast, 80% of the 111In was cytosolic. The low molecular weight Peak 4 accounted for almost 60% of the total 111In found in the liver.

Characterization of the Low Molecular Weight Metabolite of 111In-DTPA-B6.2. To determine whether 111In is processed in vivo in a manner similar to that for iron, liver supernatants taken from rats given injections of 111In-DTPA were compared with those from rats given injections of 59Fe-DTPA (Fig. 4). In supernatants from rats given injections of 59Fe-DTPA, 59Fe was recovered principally as a single peak with a molecular weight of approximately 40,000-50,000 (Fig. 4C). As expected, this coeluted with one of the two peaks seen on HPLC analysis of [59Fe]ferritin (Fig. 4D). 111In, however, was recovered as a low molecular weight species of less than 10,000 (Fig. 4B) which did not coelute with either of the two major peaks of [59Fe]ferritin but which did coelute with the low molecular weight metabolite (Peak 4) of 111In-DTPA-B6.2 (Figs. 1D and 4A). In order to compare the behavior of the low molecular weight metabolite of 111In-DTPA-labeled monoclonal antibody with

125I is found in Peak 2, corresponding to a M, 150,000 IgG. The contribution of this peak to the total radioactivity in the supernatant decreases with time to 30% at 24 h. Peak 3, corresponding to the molecular weight range of a proteolytic fragment of an IgG, M, 15,000-50,000, increases with time from 10% of the supernatant at 1 min after antibody injection to 50% of the total supernatant radioactivity recovered at 24 h. When assayed for their ability to bind antigen, Peaks 2 and 3 were both immunoreactive (Table 2). These data suggest that Peak 2 represents unmetabolized, cytosolic 125I-labeled antibody, while Peak 3 represents proteolytic fragments containing the intact antigen-binding site.

In contrast, parallel studies of the metabolic products generated after the injection of 111In DTPA-B6.2 showed a different metabolic time course (Fig. 2). Metabolic analysis at early time points indicated that, similar to 125I-B6.2, 60% of the radioactivity in the supernatant corresponded to intact IgG. This peak rapidly decreased until, at 24 h, it represented only 8% of the supernatant radioactivity. Concomitant with the observed decrease in intact B6.2 (Peak 2) was a rapid increase in the low molecular weight metabolite (Peak 4), which increased from less than 8% of the radioactivity in the supernatant at 1 min after antibody injection to almost 75% of the supernatant radioactivity at 24 h. The integrals of the major radioactivity peaks were obtained after separation by SEC-HPLC. A, B, 125I at 1 and 24 h, respectively; C, D, 111In at 1 and 24 h, respectively. Major peaks are labeled 1 through 4.
that of $^{111}$In-DTPA standard on ion exchange chromatography, samples of the metabolite and $^{111}$In-DTPA were reanalyzed on SEC-HPLC in a buffer containing 40 mM Tris, pH 8.5 (Fig. 5A). In this buffer, although $^{111}$In-DTPA migrated as a single species, the metabolite peak chromatographed not as a single entity, but as a triple peak (Peaks A, B, and C). Peaks A, B, and C were then further analyzed by ion exchange chromatography. Only 4% of the original triple peak which had been recovered from SEC-HPLC in a buffer containing 40 mM Tris, pH 8.5 (Fig. 5A) had a retention time similar to that of $^{111}$In-DTPA (Table 3). When Peaks A, B, and C were combined, the pooled peak was shown by size exclusion chromatography using an UltraHydrogel column to have a molecular weight of approximately 1000 (Fig. 5B).

Discussion

Numerous studies have described antibody binding sites on both parenchymal and nonparenchymal cells of the liver (18–20). Carbohydrate receptors (18, 19) and Fc receptors (20) which bind immunoglobulin occur on both parenchymal and nonparenchymal cells. Consequently, both may contribute to the hepatic uptake of a radioisotope which is attached to a monoclonal antibody. The data showed that on a per cell basis the parenchymal cells and nonparenchymal cells equally sequestered $^{125}$I and $^{111}$In, although the absolute uptake of $^{111}$In was higher than that of $^{125}$I for both the intact and the F(ab')2 forms of the antibody. Since the parenchymal cells account for approximately 80% of total liver cells (17) the majority of the radioactivity found in the liver was found in these cells. This was the case whether the radiolabel was attached to either intact B6.2 or F(ab')2.

The mechanism by which $^{111}$In-labeled antibodies are metabolized by the liver is poorly understood. Binding of $^{111}$In to transferrin in the serum (6, 21, 22) or ferritin in liver cells (23, 24) has been proposed as a possible mechanism for sequestration of $^{111}$In by the liver. In addition, if antigen is present in the circulation, the formation and uptake of immune complex may also play a role in elevating the levels of $^{111}$In (8, 25) in the liver. Since these studies were performed in non-tumor-bearing animals, the uptake of radiolabeled immune complex by the liver was not a factor. In order to investigate the mechanism of liver metabolism, the nature of the metabolic products of $^{111}$In and $^{125}$I-labeled B6.2 was evaluated. $^{111}$In-labeled metabolites which were recovered from the liver differed from the $^{125}$I-labeled metabolites. At early times after injection, $^{111}$In which was recovered in the cytosol fraction was attached to a $M_\text{r}$ 40,000–50,000 metabolite which retained the ability to bind to LS174T tumor membranes. This metabolite was subsequently further metabolized to a species with a molecular weight of <1000. This finding is in agreement with that of Shochat et al. (24) who found a low molecular weight metabolite of $^{111}$In-labeled antibody in the liver. In contrast are the data found after $^{125}$I-B6.2 injection. At 24 h after injection, approximately 50% of $^{125}$I in the cytosol was attached to B6.2. There were no $^{125}$I-labeled low molecular weight species detected in the cytosol. Comparison of the time course for the various radioactive species in the liver supernatants suggests that the amount of intact radiolabeled B6.2 taken up by the liver may be the same regardless of the isotope. However, the low molecular weight $^{125}$I diffuses out of the liver, while the $^{111}$In-DTPA is “trapped” and remains.

The precise identity of the primary metabolite of $^{111}$In-DTPA-B6.2 is uncertain. The possibility exists that $^{111}$In is cleaved from the antibody and then bound to ferritin in the liver. However, when $^{111}$In-DTPA was injected into rats, $^{111}$In was not transferred to ferritin but remained as $^{111}$In-DTPA. The low molecular weight metabolite found in the liver after injection of $^{111}$In-DTPA-B6.2 did not cochromatograph with $^{111}$In-DTPA, suggesting that it was not simply $^{111}$In-DTPA. Thus, it appears possible that the DTPA-amino acid linkage remains intact.

These data suggest that the sequestration of $^{111}$In after the injection of $^{111}$In-DTPA-labeled antibody is the result of a normal hepatic cellular mechanism which is largely dependent on the presence of the Fc portion of the molecule. Further metabolism of the antibody results in the production of a low molecular weight (<1000) metabolite which is trapped within the cell, possibly as a result of ionic charge. This metabolite is most probably $^{111}$In-DTPA attached to one or more amino
acids. The results reported here indicate that reduction of liver uptake of $^{111}$In is not simply a case of utilizing a chelator with better affinity for $^{111}$In, since $^{111}$In apparently does not dissociate from the chelator. Liver accretion may be reduced by the use of chelators which result in the formation of a $^{111}$In metabolite which is actively or passively extruded from the liver cells.

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References


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