Uptake of Indium-111 in the Liver of Mice Following Administration of Indium-111-DTPA-Labeled Monoclonal Antibodies: Influence of Labeling Parameters, Physiologic Parameters, and Antibody Dose

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Liver uptake of indium-111 (^{111}In) in mice was investigated following administration of ^{111}In-DTPA murine monoclonal antibodies (^{111}In-DTPA-MAbs) labeled by the cyclic anhydride method. Biodistribution of HPLC-purified ^{111}In-DTPA-MAb preparations was checked with a low (0.2 μg) and a high (8.0 μg) MAb dose. Using Bio Gel P-30 for desalting the MAb-conjugates, ^{111}In uptake in the liver amounted to 8%–9% of the injected dose (ID) and was independent from the MAb dose, the DTPA-to-MAb molar ratio, tumor growth and biologic variability (different MAbs and different strains of mice). Using Sephadex G-25 for desalting, 0.2 μg doses from 7 out of 26 preparations showed increased liver accumulation of ^{111}In in non-tumor mice ranging from 15%–25% of ID. Corresponding high doses led to a "normal" value of 8%–9%. Increased liver uptake of the low dose could not be reduced by coadministration of the unconjugated MAb, but was normal after reinjection of "in vivo filtered" material. An inverse intracellular distribution of ^{111}In activity between sediment and supernatant of liver homogenates, following the administration of the low and the high MAb dose, indicated an artifact of the labeling procedure rather than an inherent biological property of labeled MAbs.

J Nucl Med 1990; 31:1084–1093

High uptake of Indium-111 (^{111}In) by the reticuloendothelial system (RES), especially by the liver, of patients after administration of ^{111}In-diethylenetriaminepentaacetic acid (DTPA) labeled monoclonal antitumor antibodies (MAbs) severely limits immunoscintigraphy in the abdominal region (*1–6*). Administration of higher doses of MAbs, e.g., by addition of 20 mg of unlabeled MAbs to the usual dose of 1–2 mg of labeled MAbs, generally resulted in higher blood levels and lower spleen activity, while liver uptake was significantly reduced in some investigations (*1,3,5*) but remained unaffected in others (*3,4,7*). Regardless of the MAb dose administered, the liver was the organ of predominant radioactivity accumulation in all scintigraphic images.

There might be several causes for the observed high ^{111}In-liver uptake in these studies.

1. Physiologic phenomena might influence biodistribution of labeled MAbs. Since all the MAbs investigated by the above authors were of murine origin, the dose dependence of biodistribution in patients may have resulted from saturation of binding sites for murine antitumor MAbs present in human non-tumor tissues (*8*). Additionally, tumor antigen, reactive with MAb, might be present in blood and give rise to the formation of antigen-MAb complexes, which are mainly cleared by the liver (*9*).
2. High ^{111}In-liver uptake might be an inherent consequence of the labeling method. All MAbs used were conjugated according to either the cyclic anhydride (CA-DTPA) method of Hnatowich (*10*) or the mixed anhydride (MA-DTPA) method of Krejcarek (*11*). These bifunctional chelating agents are suspected to cause a special type of intramolecular crosslinking of MAbs (*12*), which cannot be detected by polyacrylamide electrophoresis (SDS-PAGE). Additionally, a kinetic and thermodynamic instability in vivo of the resulting protein-diethylenetriaminetetraacetic acid (DTTA) complex with ^{111}In might entail a contin-

Received Jun. 14, 1989; revision accepted Mar. 6, 1990
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uous leakage of indium, which might result in a transferrin-mediated uptake of indium by the liver (*13*).

3. Modifications of the conjugation procedure as introduced by some authors might have influenced ^{111}In-liver accumulation.

The most clear-cut situtation with respect to the biodistribution of ^{111}In-DTPA labeled murine MAbs is encountered in normal mice. Since in this system the injected MAb is syngeneic with the recipient, no cross expression of tumor-associated antigens in the liver is expected and ^{111}In liver uptake should be MAb dose-independent. Inherent consequences of the DTPA labeling method should result in a constant, reproducible ^{111}In-liver uptake and only modifications of the labeling procedure might result in variable liver uptake. The same should be valid for tumor-bearing mice, provided that no tumor antigen is shed into the circulation.

Current literature on the biodistribution of murine CA-DTPA or MA-DTPA conjugated MAbs of the IgG-type in mice reports markedly diverging results. While four investigations resulted in an ^{111}In liver uptake ranging from 15% to 20% of injected dose (ID) per gram (*13–16*), other authors describe a 7%–11% uptake (*17–21*). Additionally, a MAb dose-dependent liver uptake in non-tumor-bearing mice was reported (*22*). The contradictory interpretations of these results led us to reinvestigate several details of ^{111}In-DTPA labeling of MAbs and their relation to the ^{111}In liver uptake. This was thought to be essential for further work on developing new bifunctional agents with low ^{111}In liver uptake. From the presently available DTPA labeling methods, we have chosen the CA-DTPA method, because it is frequently used, is simple to carry out, it is not time-consuming and CA-DTPA is readily available from commercial sources.

MATERIALS AND METHODS

Iodine-131 Labeling of MAbs

Twenty micrograms of protein in 100 μl of 0.5 *M* phosphate buffer pH 7.4 was placed in a glass tube coated with 10 μg of IODO-GEN (Pierce, Rockford, IL). After addition of ^{131}I (740 MBq/μg; Amersham Buchler, Braunschweig, FRG) the mixture was allowed to stand for 8–10 min. Unreacted ^{131}I was separated by a centrifugation through a Sephadex G-25 desalting column (see below). Labeled MAbs were subsequently purified by size exclusion high performance liquid chromatography (HPLC) (Superose HR12, 10 × 300 mm, MW range 1–300kD; Pharmacia, Freiburg, FRG). The iodination yield was 70% and specific activity was adjusted to 0.1 MBq/μg of MAb.

Indium-111 Labeling of MAbs

Bicyclic anhydride of DTPA (Aldrich, Steinheim, FRG) was dissolved in anhydrous dimethylsulfoxide (11.6 mg CA-DTPA per ml) which had been stored over a 3-Å molecular sieve (Merck, Darmstadt, FRG). For most preparations, 300 μl of MAb solution, containing 5 mg of MAb per ml of 0.01 *M* phosphate-buffered saline (PBS) pH 7.1, was mixed with either 10.5 μl or 3.5 μl of the CA-DTPA solution, resulting in a molar ratio of chelate-to-MAb of 34:1 and 11.3:1, respectively. The mixture was allowed to react for 1 hr at room temperature. To remove unconjugated DTPA and to transfer DTPA-coupled MAbs in a buffer suitable for ^{111}In labeling, we used a centrifuged column procedure (*23,24*). Small columns fitted with a 0.2-μm membrane filter (Schleicher & Schaüll, Dassel, FRG) were filled with 1.5 ml of either Bio Gel P-30 (Bio-Rad München, FRG) or Sephadex G-25 (Pharmacia) and equilibrated with 100 μl of a solution of 100 mg/ml human serum albumin (HSA) in PBS. Columns were converted into the citrate form by 5 cycles of centrifugation (1,000 rpm, 5 min), loading 0.8 ml of 0.1 *M* citrate buffer of pH 6 on the columns. After separation of uncoupled DTPA and buffer exchange, the MAb concentration in the 0.1 *M* citrate effluent was determined at 280 nm.

Indium-111 labeling (^{111}In, no-carrier added, 3.7 GBq/ml; NEN-Du Pont, Dreieich, FRG) was carried out by addition of an amount of radioactivity sufficient for obtaining specific activities of 0.15–0.2 MBq/μg of DTPA-coupled MAb. In some experiments, commercially supplied ^{111}In was subjected to a HPLC ion exchange purification (Nucleosil SA, 5 μ, 4 × 250 mm, Latek, Heidelberg, FRG). One hundred microliters of ^{111}In (370 MBq) were injected onto a column which was operated with 0.05 *M* HCl containing 0.5% NaCl, and adjusted to pH 2.0 at a flow rate of 1.0 ml/min. Ultraviolet (UV) absorption and radioactivity were monitored. By collecting the peak eluting at a retention time of 5.5 min, a separation of ^{111}In from divalent metal ions and unidentified UV absorbing impurities was achieved. Indium-111 was allowed to react with DTPA-coupled MAbs for 1 hr at room temperature. Indium-111 not incorporated into the DTPA-MAb conjugate was chelated by adding 10 μl of a 0.01 *M* EDTA solution. A small aliquot was taken and subjected to paper chromatography for determination of the labeling efficiency (Whatmann Nr.1; methanol:water 55:45). The ^{111}In-DTPA-MAb preparations were freed from oligomers by size-exclusion HPLC on a Superose HR 12 column operated with PBS at a flow rate of 0.4 ml/min. Monomeric MAbs eluted in fractions 29–33 (11.6–13.2 ml), but only peak fractions 30–32 containing 70% of the injected protein were used for experiments. Radioactivity and MAb concentration in each fraction were measured by gamma counting and UV absorbance at 280 nm.

For determination of the molar ratio of DTPA coupled to MAbs, the same procedure was used as described above with the exception that carrier-free ^{111}In was replaced by low specific activity indium prepared by addition of 55 MBq of ^{111}In to 15 μg of high purity $InCl_3$ (Fluka, Buchs, Switzerland) dissolved in 0.1 *M* citrate buffer, pH 6.0. The amount of low specific activity indium added was adjusted such as to exceed the expected incorporation by a factor of 10. In one experiment, a DTPA-MAb conjugate saturated with indium was checked for its biodistribution.

Determination of Intra-Hepatic Blood Volume

The blood volume remaining in the liver after partial exsanguination was measured by means of mouse erythrocytes labeled with ^{111}In-acetylacetone (*25*). One hour after the ad-

ministration of labeled erythrocytes, ~0.4 ml of blood was collected from the retroorbital plexus of six NMRI mice. Animals were killed by extensive ether anesthesia and livers were removed. Indium-111 activity in the blood and in the whole liver was determined by gamma counting.

SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

To determine intra-molecular, inter-chain crosslinking of MAbs due to CA-DTPA coupling, the monomeric fractions of the HPLC effluent were checked on a 10% slab gel in the presence of beta-mercaptoethanol according to Laemmli (*26*). Gels were fixed and stained with Coomassie Brilliant Blue 250R.

Immunoreactivity

For determining the immunoreactive fraction as well as for estimating the binding affinity of the ^{111}In-DTPA labeled MAb A2.6, the Lineweaver-Burk approach was used as described previously (*27*). One hundred microliters of a suspension of 14 ASML-1 target cells were stepwise diluted by a factor of two, starting with a concentration of $1.25 \cdot 10^7$ cells per ml, and placed into U-shaped Microtest II plates (Greiner, Nürtingen, FRG). After pelleting (900 rpm), supernatants were removed by decantation of the whole plates and antibody solution (HEPES-buffered culture media containing 5 ng of labeled MAbs per 0.1 ml) was added. Plates were agitated on a gyratory shaker for 2 hr at 37°C, 5% CO_2 in air. Thereafter, cells were washed twice with media containing 2% calf serum and counted in a gamma counter. Nonspecific adsorption of labeled MAbs was determined by incubation with ASML-cells that had been preloaded with an excess of unlabeled A2.6.

Monoclonal Antibodies

Most experiments were carried out with the murine MAb A2.6 (IgG1) directed against a rat sarcoma antigen (*28*). In one experiment, murine anti-melanoma MAb M.2.9.4 (IgG2A) (*29*) and murine anti-CEA MAb (BW431/26 (IgG1) (*30*) were additionally used. The latter was obtained as a DTPA-conjugate in lyophilized form (Behringwerke, Marburg, FRG). A2.6 and M.2.9.4 were purified from ascitic fluid by chromatography on protein A and MonoQ ion exchange columns (Pharmacia) (*31*). Purity was checked by SDS-PAGE. MAbs were concentrated to 5 mg per ml, dialyzed into PBS and stored at −20°C.

Biodistribution of Labeled MAbs in Normal Mice

In biodistribution experiments, we used either NMRI or C3H/Hc mice (28–35 g body weight, Zentralinstitut (für Versuchstiere, Hannover, FRG) or athymic mice (CD-1 nu/nu; Charles River-WIGA, Sulzfeld, FRG), all being reared under SPF conditions. Animals were injected into the tail vein with a solution of labeled MAbs together with 20 μg of HSA in 0.2 ml PBS. For each In-DTPA-MAb preparation dose, dependence of liver uptake was investigated with a 0.2 μg or 8.0 μg dose. At the time points indicated, animals were anesthetized with ether, bled from the retroorbital plexus (0.3–0.5 ml), and killed by cervical dislocation. Liver, spleen, and kidneys were removed, weighed, and counted for radioactivity. Total blood volume was assumed to be 6% of body weight.

In two experiments, 0.2 ml of pooled blood from donor mice, which had been treated with 8 μg of labeled MAbs 24 hr before exsanguination, were re-injected into new recipients. MAb dose was calculated from the activity present in blood of donor mice, assuming that all radioactivity was bound to MAbs.

Biodistribution of Labeled MAbs in Tumor-Bearing Mice

To investigate the influence of tumor growth on ^{111}In liver uptake, 12 athymic mice (CD-1 nu/nu) were inoculated subcutaneously with 5×10^6 14-ASML-1 cells derived from a rat sarcoma (BSp 73). Three weeks after inoculation tumors had reached an average weight of 300 ± 150 mg. One μg of In-DTPA A2.6 (molar ratio DTPA:MAb 1:1, desalted with BioGel P-30) was injected via tail vein and biodistribution was measured at 48 hr and 120 hr as described for normal mice.

Distribution of ^{111}In Activity in Liver Homogenates

The amount of ^{111}In in the sediment and the supernatant of liver homogenates was checked with two different ^{111}In-DTPA-MAb preparations administered at a dose of 0.2 μg or 8 μg per mouse. Liver samples were disintegrated with a Potter homogenizer in a threefold volume of saline. An aliquot of the homogenate was centrifuged for 1 hr at 100,000 g. The supernatant was divided in two equal parts, one containing all membranes, the other being essentially free from membranes. Both fractions, as well as the sediment containing nuclei, microsomes, and mitochondria, were counted for radioactivity.

RESULTS

Biodistribution of ^{131}I-labeled MAbs

Data representative of a short-term biodistribution obtained with ^{131}I-labeled A2.6 are shown in Table 1. The 50% loss of ^{131}I-MAbs from blood, 1 hr postinjection and the observed ^{131}I activity level of 9%–10% of ID in the liver are in good agreement with data reported by Holton et al. (*32*) for an irrelevant murine MAb (MOPC-21, IgG_1). No dose dependence of liver uptake was detected. Reinjection of blood from donor mice previously injected with ^{131}I-labeled MAbs led to a biodistribution which was virtually identical with that of the original MAb, indicating no labeling damage. Addition of 0.2 ml of blood from untreated mice had no influence on MAb biodistribution. Immunoreactive fraction of ^{131}I-A2.6 was fairly high (Fig. 1). In SDS-PAGE solely, bands at 25 and 50 kD were observed as expected.

Intrahepatic Blood Volume

To gain an estimate of the contribution of intravascular radioactivity to the total activity found in the liver, liver blood volume after partial exsanguination by eye bleeding was determined using ^{111}In-labeled erythrocytes. Labeling did not affect viability of the erythrocytes, which was indicated by a 96.5% ± 6.5% recovery of ^{111}In activity in whole blood, assuming that total blood amounts to 6% of body weight, and an ^{111}In level in the spleen below 1%. From the ^{111}In activity

TABLE 1
Biodistribution of ^{131}I-Labeled A2.6* in Normal NMRI Mice†

	Time (hr)	%ID per organ			
		blood	liver	spleen	kidneys
0.2 μg MAb in saline	1	51.2 ± 3.9‡	9.05 ± 0.73	0.78 ± 0.15	2.13 ± 0.23
0.2 μg MAb mixed with 0.2 ml of blood from untreated mice	1	49.4 ± 5.2	9.49 ± 0.29	0.74 ± 0.13	2.45 ± 0.15
0.2 ml of blood from donor mice previously injected with 8 μg MAb§	1	54.6 ± 5.8	8.92 ± 0.79	0.86 ± 0.15	2.14 ± 0.34
8 μg MAb in saline	1	52.9 ± 3.5	9.84 ± 0.54	0.68 ± 0.08	2.04 ± 0.25
8 μg MAb in saline	24	29.4 ± 3.0	4.68 ± 0.33	0.29 ± 0.04	1.19 ± 0.05

* Specific activity 3 μCi ^{131}I/μg MAb.
† Body weight of mice 34.0 ± 1.4 g; liver = 1.49 ± 0.11 g; spleen = 0.17 ± 0.03 g; and kidneys = 0.39 ± 0.05 g.
‡ Mean ± s.d. from six mice. Data from organs represent total ^{131}In activity.
§ From activity in the pooled blood of donor mice, an amount of 0.26 μg of MAb per 0.2 ml was calculated.

present in the liver, a blood volume of 4.65% ± 0.70% of total blood was calculated. Data of liver blood volume were obtained with animals of 28.5 ± 1.7 g body weight and a liver weight of 1.32 ± 0.11 g. Using the biodistribution data of ^{131}I-labeled MAbs (see Table 1), 2.4%–2.5% of ID found in the liver 1 hr postinjection were due to intravascular radioactivity.

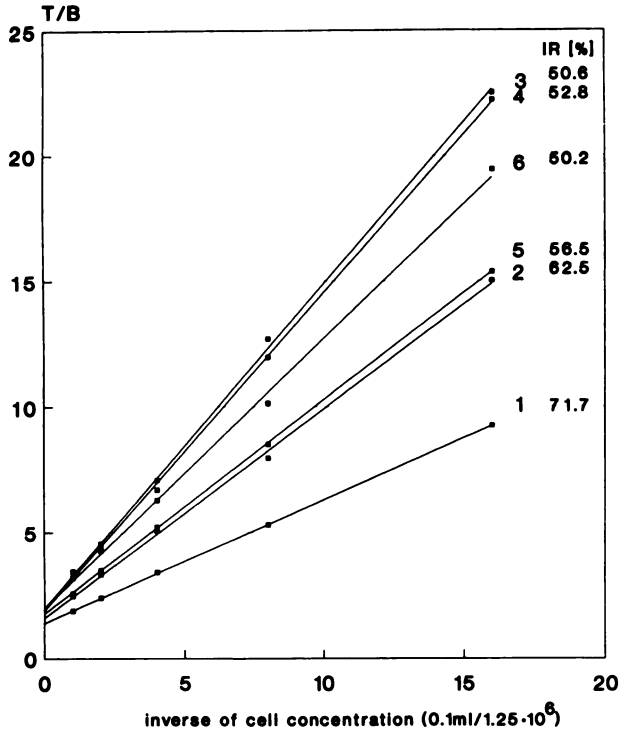

FIGURE 1
Immunoreactive fraction (IR) of labeled A2.6: Lineweaver-Burk plot. Living 14ASML-1 cells were incubated with ^{131}I-labeled MAb (*1*) and different ^{111}In-DTPA MAb preparations (*2,6*). T = total activity; B = activity bound to cells.

Nr.	DTPA:MAb	Desalting	HPLC purification of	
			^{111}In	^{111}In-DTPA-MAb
2.	1,14:1	Bio Gel	+	+
3.	2,44:1	Bio Gel	–	+
4.	2,70:1	Sephadex	–	+
5.	1,14:1	Bio Gel	–	+
6.	2,44:1	Bio Gel	–	–

Immunoreactivity

Immunoreactivity data of labeled MAbs are presented according to the Lineweaver-Burk approach as a double inverse plot of MAb-binding to the target cells over the cell concentration (Fig. 1). The immunoreactive fraction (IR) at infinite antigen excess was obtained by calculating the reciprocal of the intercept on the T/B axis. IR of In-DTPA-MAbs varied only slightly with the DTPA-to-MAb molar ratio, being 56.5%–62.5% for a 1.1:1 and 50.2%–52.8% for a 2.5:1 substitution. Affinity constants of labeled MAbs, which are inversely correlated with the slopes of the T/B data, showed a stronger dependence on the DTPA-to-MAb molar ratio, probably due to an increase in DTPA conjugation near the binding sites of MAbs. The highest IR and the highest affinity constant were obtained for ^{131}I-labeled MAbs. However, labeling of MAbs at a specific activity of 0.1 MBq ^{131}I per μg results in a comparably low molar ratio of ^{131}I to MAbs of ~0.15:1.

Indium-111-DTPA Labeling of MAbs.

Conjugation of MAbs was carried out using CA-DTPA dissolved in dimethylsulfoxide. Coupling yields as calculated from HPLC-purified MAbs ranged from 7%–9% of CA-DTPA added and were independent from the initial molar ratio of CA-DTPA to MAbs used in these experiments (11.3:1 and 34:1). The molar ratio of DTPA-to-MAbs and the labeling efficiency with ^{111}In was determined for all preparations. Additionally, each preparation was tested for a dose dependent liver uptake of ^{111}In with a small number (*4–6*) of animals.

For separation of unbound DTPA from DTPA coupled MAbs and for buffer exchange prior to ^{111}In labeling, two types of column material were used, Sephadex G-25 and Bio Gel 30. Out of 26 ^{111}In-MAb preparations produced using Sephadex G-25, seven showed an in-

creased ^{111}In liver uptake ranging from 15% to 26% of ID 1 hr after administration of the low MAb dose (i.e., 0.2 μg per mouse). The high doses (i.e., 8 μg per mouse) of the same preparations always led to "normal" ^{111}In levels in the liver in the range of 8%–9% of ID. In the remaining 19 preparations, liver uptake of ^{111}In ranged from 8%–9% of ID regardless of the injected MAb dose. Common to all 26 preparations was comparably low labeling efficiency amounting to 72% ± 12% (range: 55%–95%) of added ^{111}In for an 1-hr incubation. One of the ^{111}In DTPA-A2.6 preparations exhibiting a pronounced dose dependence was investigated in more detail (Table 2). It must be stressed that no impurities were detected in this preparation upon HPLC chromatography (Fig. 2) or SDS-PAGE. The immunoreactive fraction was similar to all other ^{111}In-DTPA-MAb preparations and was only moderately reduced compared to that of the iodinated MAb (Fig. 1).

Increased liver uptake following administration of the 0.2 μg MAb dose was accompanied by a significantly lower blood level. Liver activity decreased from 26% to 18% of ID during 24 hr, but then remained twice as high as the one resulting from the 8-μg MAb dose. Reinjection of blood from donor mice, containing an amount of MAb corresponding to the low dose, showed a "normal" ^{111}In liver uptake of 8% of ID. The addition of 8 μg unlabeled MAb to the low dose of ^{111}In-DTPA-MAb did not decrease ^{111}In uptake in the liver. This indicated the absence of a physiologic, saturable process of murine MAb binding by mouse liver tissue.

When analyzing subcellular ^{111}In distribution in the liver, increased uptake in the liver observed 1 hr after injection of the low dose was reflected by an increased radioactivity level in the sediment of liver homogenates but not in the supernatants containing membranes, cytoplasm and extracellular fluid (Table 3). Since this effect was never observed when Bio Gel P-30 was used instead of Sephadex G-25 during the conjugation and labeling procedure, we tried to identify impurities in the Sephadex G-25 effluent endowed with the capacity of adsorbing ^{111}In. Obvious candidates to be trapped in the liver were low molecular weight dextrans, since Sephadex is produced from natural dextrans by cross-linking with epichlorohydrine. This, however, could not be substantiated experimentally.

When Sephadex G-25 was replaced by Bio Gel P-30, a fully synthetic material manufactured by copolymerization of acrylamide and N,N'methylene-bis-acrylamide, none of ten consecutive ^{111}In-DTPA-MAb preparations tested so far showed a dose-dependent liver uptake of ^{111}In. In addition, incorporation of ^{111}In into these DTPA-MAb conjugates was found to be increased to 93% ± 3%. Results of biodistribution of several batches of ^{111}In-DTPA-MAb prepared by the use of Bio Gel P-30 are presented in Table 4.

It is conceivable that ^{111}In-DTPA-MAb preparations may have contained varying amounts of DTPA conjugates which had either no metal incorporated or which were saturated with inactive metal impurities contained in commercial ^{111}In preparations, e.g., Fe and Cd. Thus, we compared the biodistribution of DTPA-MAb conjugates labeled with HPLC-purified, no-carrier-added ^{111}In to the biodistribution of DTPA-MAb conjugates which were fully saturated by an excess of low-specific activity (carrier-added) ^{111}In. No differences were found (Table 4, compare lines b and c). In addition, biodistri-

TABLE 2
Biodistribution of an ^{111}In-DTPA A2.6 Preparation* Showing a Dose-Dependent ^{111}In Liver Uptake in Normal NMRI Mice†

Experiment		Time (hr)	% ID per organ			
			blood	liver	spleen	kidneys
a	0.2 μg labeled MAb in saline	1	40.3 ± 2.4‡	26.03 ± 3.48	0.88 ± 0.17	2.31 ± 0.14
b	8.0 μg labeled MAb in saline	1	54.5 ± 2.9	8.29 ± 0.68	0.66 ± 0.11	2.39 ± 0.26
c	0.2 μg labeled MAb in saline	24	20.5 ± 1.0	17.80 ± 1.69	0.72 ± 0.05	2.79 ± 0.20
d	8.0 μg labeled MAb in saline	24	31.8 ± 2.1	8.10 ± 0.93	0.74 ± 0.07	3.46 ± 0.20
e	0.2 ml of blood from donor mice, previously injected with 8 μg of labeled MAbs§	1	53.9 ± 4.0	8.02 ± 0.39	0.71 ± 0.08	2.30 ± 0.23
f	0.2 μg of labeled MAbs mixed with 8 μg of unconjugated, unlabeled A2.6 MAb	1	41.8 ± 3.0	24.00 ± 1.24	0.92 ± 0.08	2.36 ± 0.29

* Before ^{111}In labeling, DTPA coupled A2.6 was separated from unconjugated DTPA by a centrifuged column procedure using Sephadex G-25. Molar ratio DTPA: MAb was 2.7:1; specific activity was 4.1 μCi ^{111}In per 1 μg MAb.

† Body weight = 25.8 ± 1.3 g; liver = 1.25 ± 0.17 g; spleen = 0.11 ± 0.02 g; and kidneys = 0.34 ± 0.04 g.

‡ Mean ± s.d. from six mice, data from organs represent total ^{111}In activity.

§ From ^{111}In activity in the pooled blood of donor mice, an amount of 0.32 μg MAb per 0.2 ml was calculated.

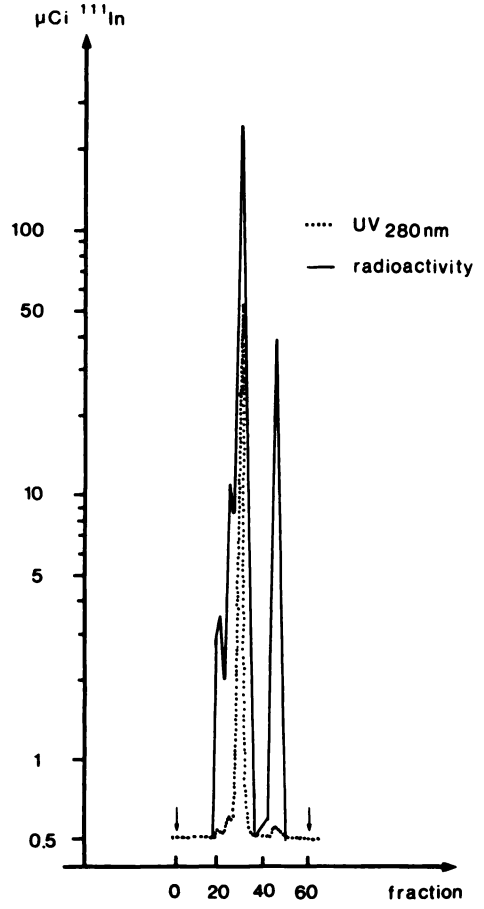

FIGURE 2
Size exclusion HPLC. Elution profile (UV and ^{111}In activity) of an ^{111}In-DTPA-A2.6 preparation showing a dose-dependent ^{111}In liver uptake. Radioactivity was measured per fraction and amounted: 0,9% (fr. 20–22) oligomeres, 4,3% (fr. 23–27) dimeres, 80,9% (fr. 28–34) monomeres, 12,7% (fr. 43–50) low molecular weight species including ^{111}In-EDTA. No difference could be detected between this elution profile and those obtained from ^{111}In-DPTA-A2.6 preparations showing no dose-dependent ^{111}In liver uptake.

bution was not affected by a difference in the DTPA-to-MAb molar ratio (Table 4, compare lines a and d).

The clearance of ^{111}In from the blood indicated a rapid equilibration of labeled MAbs into the extravascular space identical to the one observed with ^{131}I-labeled MAbs. This process was followed by a much slower component, presumably representing catabolic elimination of labeled MAbs (Table 4, d–f). Total ^{111}In activity in the liver decreased by 2%–3% of ID during a five-day observation period.

No differences in biodistribution and liver uptake were observed when mice of different strains (NMR I, CH_3/Hc, and CD-1 nu/nu) were injected with ^{111}In-DTPA or ^{131}I-labeled A2.6 (data not shown). Using ^{111}In-DTPA labeled MAbs A2.6 and M.2.9.4, which were not purified by size exclusion HPLC after labeling, considerable variation in their liver uptake was observed, indicating differential sensitivity of MAbs to interchain crosslinking by CA-DTPA and/or a different degree of aggregation of MAbs prior to labeling (Table 5). This component of liver uptake, however, did not depend on the injected dose. A commercial preparation of ^{111}In-labeled MAb BW431/26 (supplied as a lyophilized DTPA-MAb conjugate) showed low liver uptake irrespective of HPLC purification, provided that correction was made for the amount of ^{111}In-EDTA present in the non-HPLC treated solution. With this DTPA-MAb conjugate, binding of ^{111}In was, however, slower and less complete, i.e., 30% and 85% after 1 hr and 2.5 hr, respectively, compared to 93% after 1 hr obtained with our own DTPA-MAb preparations using Bio Gel P-30.

Indium-111 liver uptake in tumor-bearing mice 48 hr and 120 hr postinjection amounted to 6.12 ± 1.11% and 5.79 ± 1.38% of ID per liver (n = 6 ± 1 s.d.) and was identical to data obtained with normal mice (table 4 lines e, f). Indium-111 activity in blood, spleen, and kidneys were also very similar. Tumor uptake of ^{111}In-DTPA labeled A2.6 was 28.3 ± 10.4 and 31.6 ± 9.1% ID per g at 48 hr and 120 hr, respectively.

DISCUSSION

Using a labeling technique with CA-DTPA dissolved in DMSO, Bio Gel P-30 for desalting and size exclusion HPLC for purification, the main result of our investigations on the biodistribution of ^{111}In-DTPA labeled MAbs in normal mice was that liver uptake of ^{111}In was highly reproducible with many consecutive DTPA-MAb preparations. Liver uptake was independent from the molar ratio of DTPA to MAb in the range of 1:1 up to 2.7:1 and, consequently, from a noticeable decrease in binding affinity of the more heavily DTPA substituted MAbs, from the administered MAb dose in the range of 0.2–8.0 μg per animal and from the amount and purity of ^{111}In used for labeling. No influence on ^{111}In liver uptake was observed using different MAbs or different strains of mice. Total ^{111}In activity in the liver amounted to 7%–9% of ID 1 hr postinjection and slowly decreased to 5%–6% of ID during a five-day observation period. This decrease could be fully attributed to the decline of the ^{111}In activity in blood, as was calculated from the data of intrahepatic blood volume and ^{111}In blood activity. Thus, ~5–6% of ID was taken up by the liver tissue as early as 1 hr postinjection and remained unchanged over a long time period. This leaves little room for a leakage of ^{111}In from the protein-DTTA complex and transchelation of ^{111}In by transferrin, not regarding the fact that in our hands a deliberate injection of ^{111}In-transferrin resulted in a radioactivity accumulation in kidneys and bone rather than in the liver (data not shown). Whether or not the observed ^{111}In activity in the liver following administration of ^{111}In-DTPA labeled MAbs represents the physiologic level of endogeneous IgG cannot unequivocally be

TABLE 3
Distribution of ^{111}In Activity Between Sediment and Supernatant of Liver Homogenates of NMRI Mice after Administration of Two ^{111}In-DTPA A2.6 Preparations* Showing a Dose-Dependent and a Dose-Independent Liver Uptake

	Dose-dependent		Dose-independent	
	0.2 μg MAb	8.0 μg MAb	0.2 μg MAb	8.0 μg MAb
Liver weight [g]	1.37	1.24	1.85	1.77
^{111}In liver uptake [% ID per organ]	25.16†	10.37†	9.42†	10.13†
½ supernatant + membranes [%]‡	7.7	34.6	42.1	35.6
½ supernatant, membrane free [%]‡	14.4	37.3	41.9	43.9
Sediment [%]‡	77.9	28.1	16.0	20.5

* The two preparations of ^{111}In-DTPA-A2.6 differed only in the use of Sephadex G-25 (dose-dependent) and Bio Gel P-30 (dose-independent) for separation of unconjugated DTPA prior to ^{111}In labeling. Preparations are those described in Tables 2 and 4.
† Animals were killed 1 h after MAb administration without exsanguination.
‡ % of activity present in the liver.

stated, because labeled MAbs might have been affected by chemical alterations due to the isolation procedure from ascites fluid or from hybridoma cell cultures or by the labeling procedure. However, comparing ^{111}In-DTPA-MAbs with ^{131}I-MAbs, the early biodistribution (1 hr postinjection) was virtually identical and no influence of MAb dose or in vivo filtration was observed. Iodine-131-labeled MAbs were prepared with a specific activity of <150 kBq/using IODO-GEN as an oxidant. This method has been reported to produce only a small number of twice-substituted molecules and to have only a slight influence on immunoreactivity (*33,34*). Moreover, MAbs, labeled endogenously by the addition of ^{75}Se-methionine to hybridoma cell cultures, showed a very similar liver uptake of 7%–10% of ID/g 4–6 hr postinjection (*15,35*). This parallelism in the early total radioactivity uptake in the liver observed with differently labeled MAbs suggests that the physiologic level of antibodies should also be in the range of 7%–10% of total IgG.

In contrast to data obtained with Bio Gel P-30, the use of Sephadex G-25 for desalting MAb-conjugates drastically increased ^{111}In liver uptake after administration of a 0.2-μg ^{111}In-DTPA MAb dose, while being "normal" with an 8-μg MAb dose and after reinjection of in vivo filtered material. Although we failed to localize an ^{111}In impurity in the Sephadex effluent with the commonly-used analytical methods (electrophoresis

TABLE 4
Biodistribution of ^{111}In-DTPA-A2.6 Preparations* Showing a Dose-Independent ^{111}In Liver Uptake in Normal NMRI Mice†

Experiment	MAb dose (μg)	Molar ratio DTPA:MAb	^{111}In	Time p.i. (hr)	% ID per organ			
					blood	liver	spleen	kidneys
a	0.2	1.15:1	Unprocessed‡	1	57.7 ± 5.6§	8.10 ± 0.45	0.65 ± 0.12	2.07 ± 0.19
	8.0	1.15:1	Unprocessed	1	55.0 ± 3.6	7.88 ± 0.31	0.81 ± 0.19	2.06 ± 0.29
b	0.2	1.15:1	HPLC	1	54.9 ± 3.2	8.71 ± 0.95	0.72 ± 0.10	2.28 ± 0.26
	8.0	1.15:1	HPLC	1	55.7 ± 3.9	8.30 ± 0.47	0.73 ± 0.14	2.22 ± 0.17
c	0.2	1.15:1	Low specific	1	53.8 ± 6.5	7.52 ± 0.35	0.61 ± 0.05	2.36 ± 0.38
	8.0	1.15:1	activity¶	1	53.4 ± 3.6	7.89 ± 0.70	0.67 ± 0.11	2.91 ± 0.19
d	0.2	2.44:1	Unprocessed	1	53.3 ± 3.7	7.93 ± 0.57	0.69 ± 0.16	1.86 ± 0.14
	8.0	2.44:1	Unprocessed	1	57.9 ± 4.6	8.36 ± 0.84	0.76 ± 0.14	2.35 ± 0.14
e	0.2	2.44:1	Unprocessed	48	19.6 ± 1.5	6.34 ± 0.40	0.59 ± 0.06	2.83 ± 0.19
	8.0	2.44:1	Unprocessed	48	21.4 ± 1.8	6.29 ± 0.71	0.64 ± 0.07	3.18 ± 0.47
f	0.2	2.44:1	Unprocessed	120	9.17 ± 0.91	5.83 ± 0.41	0.50 ± 0.09	3.17 ± 0.36
	8.0	2.44:1	Unprocessed	120	8.12 ± 0.65	5.21 ± 0.29	0.42 ± 0.06	2.70 ± 0.23

* Before ^{111}In labeling, DTPA coupled A2.6 was separated from unconjugated DTPA by a centrifuged column procedure using Bio Gel P-30.
† Body weight = 30.4 ± 1.2 g; liver = 1.31 ± 0.07 g; spleen = 0.14 ± 0.03 g; and kidneys = 0.33 ± 0.04 g.
‡ Specific activities of ^{111}In-DTPA-A2.6: Experiment a = 3.9, b = 4.4, c = 0.1, d–f = 4.5 μCi ^{111}In per μg MAb.
§ Mean ± s.d. from six mice, data from organs represent total ^{111}In activity.
¶ DTPA coupled MAbs completely saturated with ionic indium.

TABLE 5
Indium-111 Liver Uptake of Different ^{111}In-DTPA Labeled MAbs With and Without HPLC Purification of the Labeled MAbs, 1 Hour Postinjection in Normal NMRI Mice*

	MAb dose (μg)	Liver (% ID per organ)		MAb dose (μg)	Liver (% ID per organ)
A2.6† – HPLC‡	0.2	10.53 ± 1.52¶	M294 + HPLC	0.2	10.51 ± 0.98
	8.0	10.06 ± 0.78		8.0	8.92 ± 0.72
A2.6 + HPLC	0.2	7.93 ± 0.57	BW431/26§	0.2	7.53 ± 0.40 (8.86 ± 0.47)
	8.0	8.36 ± 0.84		8.0	7.18 ± 0.57 (8.45 ± 0.67)
M294† – HPLC	0.2	21.5 ± 2.4	BW431/26 + HPLC	0.2	8.84 ± 0.55
	8.0	20.4 ± 3.8		8.0	8.64 ± 0.55

* Body weight = 30.6 ± 1.3 g; liver = 1.29 ± 0.08 g.

† Prior to ^{111}In labeling, DTPA coupled A2.6 and M294 were separated from unconjugated DTPA by a centrifuged column procedure using Bio Gel P-30. Molar ratios of DTPA-to-MAb were 2.4:1 and 2.5:1, specific activities were 4.5 and 5.0 μCi ^{111}In per μg of A2.6 and M294, respectively.

‡ HPLC means that HPLC purification of ^{111}In-DTPA-MAb preparations was replaced by a centrifuged column procedure using Bio Gel P-30 equilibrated with PBS.

§ BW431/26 was prepared following the instructions of the manufacturer: incubation with ^{111}In, chelation of nonantibody bound ^{111}In by 0.1 ml of 0.01 *M* EDTA, no further treatment. Because ^{111}In incorporation into the DTPA-MAb conjugate was only 85%, the remaining ^{111}In activity was present as ^{111}In-EDTA. Indium-111 liver uptake, corrected for the presence of ^{111}In-EDTA, is indicated in brackets. Molar ratio of DTPA-to-BW431/26 was 0.82:1, specific activity 4.0 μCi ^{111}In per μg MAb.

¶ Mean ± s.d. from six mice, data from organs represent total ^{111}In activity.

and HPLC), the markedly different subcellular distribution of ^{111}In activity in liver homogenates indirectly suggested that the injected solution contained a species of ^{111}In activity different from native ^{111}In-DTPA MAb, which was processed by liver cells and ^{111}In being stored in sedimenting organelles. These adverse effects of Sephadex on ^{111}In-DTPA labeling of MAbs might be taken into account to explain data reported by Adams et al. and Halpern and co-workers. Compared with our results, Adams found a strikingly similar dose dependence of ^{111}In liver uptake in non-tumor mice using a 0.2-μg and a 20-μg dose of ^{111}In-labeled, p-isothiocyanatobenzyl-EDTA conjugated MAb, but dose dependence was interpreted by a hypothetical isotype specificity of hepatic immunoglobulin Fc receptors (*22*). Halpern noted a 50% reduced ^{111}In liver uptake in non-tumor mice following the administration of an in vivo filtered ^{111}In-DTPA labeled human IgM-preparation as compared to the original material. No explanation for this effect was given (*36*). Both authors, however, indicate the use of Sephadex for desalting.

Increased liver uptake found by Esteban et al. (*13*) and Brown et al. (*16*) cannot be attributed to the use of Sephadex because these authors desalted DTPA-MAb conjugates by extensive dialysis. According to our experience with ^{111}In-DTPA A2.6, preparations dialysis per se did not increase ^{111}In liver uptake (data not shown). It is obvious, however, that both authors and, additionally, Sands et al. (*14*) used the same model system, the murine MAb B 72.3 in mice bearing the LS174T tumor, which is derived from a human colon carcinoma. Thus, it might be argued that special features of this MAb-tumor model might have influenced ^{111}In in liver uptake. Recently, Beatty et al. (*337*) have shown that ^{111}In liver uptake in LS174T tumor-bearing mice following administration of different ^{111}In-DTPA labeled anti-CEA MAbs depend on tumor size and pretreatment with corresponding unlabeled MAbs. In this study, increased liver uptake was attributed to CEA-MAb complexes isolated from plasma and liver. Accordingly, circulating TAG72 antigen, which is reactive with the B72.3, might explain the different ^{111}In liver uptake of the ^{111}In-DTPA labeled B72.3 reported by Brown (*16*). While LS174T tumor-bearing mice accumulated 20% of ID in the liver, this was only 9% in animals bearing the antigen-negative tumor HCT-T5. However, immune complex formation between TAG72 antigen and B72.3-MAb does not appear to be an inherent consequence in LS174T tumor-bearing mice. This might be due to a modulation of antigen secretion of different LS174T cell lines used for tumor transplantation. A low secretion of antigen may explain the low (9%–10% of ID) ^{111}In liver uptake for the CA-DTPA and isothiocyanatobenzyl-DTPA (SCN-Bz-DTPA) conjugated B72.3 found by Roselli et al. (*38*) and Esteban et al. (*13*), respectively. More difficult to explain is a comparison of different chelating agents made by Esteban et al. (*13*), who described a decrease of ^{111}In liver uptake in LS174T tumor-bearing mice from 17% to 9% of ID when replacing the CA-DTPA conjugation of B72.3 by the SCN-Bz-DTPA coupling. The liver uptake of CA-DTPA conjugated B72.3 found in this study appears to be rather high, however, when compared with data of Roselli (*38*). Additionally, the uptake of CA-DTPA and SCN-Bz-DTPA coupled B72.3 in LS147T tumors seemed to be inversely correlated with the liver uptake, while in Roselli's study tumor levels were similar for the differently labeled B72.3. These

contradictory results suggest other sources for increased ^{111}In liver uptake of the CA-DTPA labeling technique than those described in this paper. These sources, however, could not be elucidated from the descriptions in the material and methods sections presented in both studies.

Data of several biodistribution studies with tumor-bearing mice (*17–18,20–21*), including our own, demonstrated that the presence per se of a tumor did not necessarily increase ^{111}In liver uptake. This situation may change when necrosis, antigen shedding or metastases accompany tumor growth. HPLC purification of labeled DTPA-MAb preparations proved to be necessary for separation of aggregated or CA-DTPA cross-linked MAbs and thus for obtaining a reproducible low liver uptake. This is in agreement with results of other investigations (*13,39*).

CONCLUSION

Indium-111-labeled, CA-DTPA conjugated murine MAbs prepared under strictly defined conditions are of good in vivo stability and show an ^{111}In activity in the livers of mice which appear to be closely related to the level of endogeneous IgG. From observed total ^{111}In activity in the liver 1 hr postinjection, ~2%–3% resulted from intravascular activity. Five to six percent of ID were taken up by the extravascular liver tissue, and this level did not decrease with time. Thus, the generally higher ^{111}In liver uptake seen in patients following administration of ^{111}In-DTPA labeled murine MAbs is not likely to be an inherent property of the ^{111}In-DTPA chelate but should be due to an increased initial uptake of labeled MAbs caused by physiologic phenomena as outlined in the introduction. To reduce activity levels in the liver, two strategies are conceivable. The elimination of physiologic phenonema compromising a low liver uptake by using bispecific MAbs as proposed and outlined by Goodwin et al. (*40*) or the development of metal chelates which remain intact during the catabolism of MAb-conjugates and thus ensure the excretion of the labeled species. However, to improve scintigraphic contrast, excretion should proceed at a slower rate in the tumor than in normal tissues.

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