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Prevention of Radiolysis of Monoclonal Antibody during Labeling

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Monoclonal antibody may undergo loss of immunoreactivity due to radiation damage when labeled with large amounts of ¹³¹I or ⁹⁰Y for therapy. Our aim was to develop a method to protect an antibody during the labeling procedure. **Methods:** As a model we used T101, a murine monoclonal antibody directed against CD5 antigen. Iodine-125-T101 (100 µg, 1 ml) was mixed with ⁹⁰Y-DTPA (0.64 MBq to 165.9 MBq) for 24 hr in order to deliver doses of 5 Gy to 1280 Gy to the solution. In separate experiments, ¹²⁵I-T101 solutions were irradiated with ⁶⁰Co external beam delivering radiation doses of 40 Gy to 1280 Gy. The effect of radiation on T101 immunoreactivity was tested by using the CCRF-CEM cell line, and the bound T101 radioactivity was determined. In a final experiment, we directly labeled a DTPA conjugated T101 using 561 MBq of ⁹⁰Y under conditions delivering ~640 Gy to the solution. Previously used radioprotectants including human serum albumin, cysteamine and glycerol were evaluated. We focused on ascorbic acid because it is an FDA approved drug that does not interfere with the radiolabeling process. **Results:** The immunoreactivity of ¹²⁵I-T101 was ~83%, but at 640 Gy the immunoreactivity decrease to 7%. In contrast, in the presence of radioprotectants this decrease could be abrogated. External irradiation also showed a dose dependent decrease in

immunoreactivity to as low as 0.3% at 1280 Gy. Adding ascorbic acid (5.5 mg/ml) to the solutions prior to the irradiation largely abrogated this decrease. The immunoreactivity of T101 labeled with ⁹⁰Y without protectant showed 46% immunoreactivity whereas, in presence of ascorbic acid (11 mg/ml) full retention of immunoreactivity was observed. **Conclusion:** Various radioprotectants can successfully prevent the loss of immunoreactivity or breakdown as a result of radiolysis. Ascorbic acid is an effective radioprotectant that can be used to prevent loss of antibody immunoreactivity during the labeling process.

Key Words: monoclonal antibody; labeling damage; immunoreactivity; yttrium-90; radioimmunotherapy; radiolysis

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The use of radiolabeled antibodies as tumor targeting reagents for radioimmunotherapy is under evaluation (1). Several reports treating hematological malignancies with ¹³¹I or ⁹⁰Y radiolabeled antibodies have shown promising results (2-4). Several radionuclides have been proposed for radioimmunotherapy including ¹³¹I, ⁹⁰Y and ¹⁷⁷Lu (5). These radionuclides deliver a large amount of radiation to the antibody solution during the labeling process as well as during the storage prior to injection. Several reports have confirmed significant damage to antibodies as a result of the labeling and storage (6,7,9,10). The

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damaged antibody cannot preferentially bind to the tumor and, thus, the radioactivity can contribute to toxicity with little targeted delivery to tumor sites. The mechanism for radiation damage is thought to be mediated by the generation of free radicals (8). Free radical scavengers such as human serum albumin (HSA) and cysteamine have been shown to considerably decrease or completely abrogate radiation induced damage to antibody (7,9). In addition, freezing the antibody after radiolabeling up to the time of administration can considerably improve the immunoreactivity by decreasing the diffusion and interaction of free radicals with antibody (10). Our purpose was to develop a method that would protect an antibody from damage during the radiolabeling process. In this study, we evaluate the effect of ionizing radiation from ^{90}Y or external beam on the stability and immunoreactivity of radiolabeled T101 murine monoclonal antibody and the ability of HSA, cysteamine, glycerol and ascorbic acid to act as radioprotectants. We focused on ascorbic acid since it is an FDA-approved pharmaceutical that could be used during the labeling process without interference and without potential toxicity to the patients.

MATERIAL AND METHODS

Antibody System

T101 murine monoclonal antibody was used as a test system. T101 is an IgG2a that recognizes CD5, a pan T-cell antigen (11). The monoclonal antibody was purified from hybridoma ascites of BALB/c mice by precipitation with 18% ammonium sulfate. The T101 antibody was provided in normal HSA (1:1 ratio) and was purified by isoelectric focusing to remove the albumin. Size-exclusion HPLC showed that the unlabeled T101 consisted of a single IgG peak. T101 was labeled with ^{125}I using the chloramine-T method (12) and T101-bound iodine was purified from free iodine by gel filtration chromatography using a PD10 column. The specific activities ranged from 296–370 MBq/mg. Quality control of these preparations typically showed $94.6\% \pm 2.7\%$ (mean \pm s.d) T101-bound ^{125}I and had an immunoreactivity of $83\% \pm 2.4\%$ ($n = 11$).

The immunoreactivity of T101 was determined by a cell binding assay using CCRF-CEM cells (13,14). CCRF-CEM is a CD5 positive human T-cell line that grows in cell suspension. In brief, serial dilutions (0.5×10^6 to 16×10^6) CCRF-CEM cell were incubated with 5 ng/75 μl of radiolabeled T101 at 4°C for 2 hr. The cells were then separated by centrifugation in PBS/1% bovine serum albumin. The cell pellets were counted in a gamma counter and the percent of the total radioactivity that bound to cells was determined from the total added. Once the conditions of antigen excess were determined, subsequent immunoreactivity measurements were performed as single point assays at antigen excess (8×10^6 cells).

Trichloroacetic acid precipitation and size exclusion HPLC were used to determine the T101 bound radioactivity. Aliquots of the ^{125}I T101 MAb in PBS/1% BSA (100 μl) were mixed with 0.5 ml of 25% trichloroacetic acid precipitation and immediately centrifuged at 1500 g for 5 min. The supernatant was decanted and the pellet was counted in a gamma counter. The counts in the pellet were then expressed as a percentage of the total counts added. In addition, size-exclusion HPLC was performed using a TSK G-2000 SW \times L column that was eluted with 0.067 M PBS with 100 mM KCl, pH 6.8, at a flow rate of 0.5 ml/min. The HPLC had an on-line NaI gamma detector which could detect both ^{125}I and ^{90}Y and correct for spillover during simultaneous counting.

When counting samples with both ^{125}I and ^{90}Y in the gamma counter, corrections for spillover of radioactivity were performed.

Subsequent recounting of specimens after ^{90}Y had decayed yielded similar results.

Radiolysis Experiments

Various radioprotectants were evaluated under different irradiation conditions. These included ascorbic acid USP (500 mg/ml, CEVALIN[®]) which contained ascorbic acid, sodium carbonate granular, monothioglycerol and water for injection; Na ascorbate in concentrations ranging from 0.0042 mg/ml to 15 mg/ml; 5 mM cysteamine hydrochloride; 5% glycerol anhydrous; diethylenetriaminepentaacetic acid (DTPA) 0.001 M, pH 6.0; and 2% human serum albumin. These solutions were prepared freshly in sterile PBS at pH 7.4. Three methods of irradiating the antibody were used: (a) "internal irradiation", where ^{125}I -T101 was used as a tracer and was irradiated by ^{90}Y -DTPA added to the solution; (b) "external irradiation" where ^{125}I -T101 was used as a tracer and was irradiated by external beam and (c) "direct irradiation" where T101 was labeled directly with ^{90}Y with irradiation occurring during the labeling process.

Internal irradiation was delivered to aliquots of ^{125}I -T101 (~ 8.88 MBq/100 $\mu\text{g/ml}$) placed in 3.6-ml snap top round bottom polypropylene tubes. The final mass of T101 was adjusted to 100 μg by adding the necessary amounts of unlabeled T101 (~ 70 – 75 μg). Yttrium-90-DTPA was prepared (15) by adding 2 M Na-acetate, pH 4.2 (100 μl) to 0.001 M DTPA solution (1 ml), pH 6.2, followed by adding 740 MBq ^{90}Y , then allowed to stand 15 min at room temperature. Iodine-125-T101 solutions were incubated with various amounts of ^{90}Y -DTPA (0.629 MBq, 1.258 MBq, 2.553 MBq, 5.106 MBq, 10.18 MBq, 20.35 MBq, 41.07 MBq, 82.14 MBq, 164.28 MBq) in a final volume of 1 ml. The solutions were incubated at 4°C for 24 hr to deliver radiation doses ranging from 5 Gy to 1280 Gy. The radiation doses were calculated using the MIRD techniques (16) and assuming 69% absorbed fraction under these geometric conditions, which is in the range calculated by Dr. John C. Roeske using a point kernel method (17) (*personal communication*, 1995) and is also in the range calculated for 1-g spheres (18). In brief, the radiation dose was calculated by multiplying the mean energy emitted for unit cumulative activity times the absorbed dose fraction for the geometry of interest (0.69×1.99 g \cdot rad/ $\mu\text{Ci}\cdot$ hr). As a control, ^{125}I -T101 was incubated in PBS or PBS and DTPA (without ^{90}Y). At the end of the incubation the protein bound fraction and the immunoreactivity were determined.

External irradiation was delivered to aliquots of ^{125}I -T101 (~ 8.88 MBq/100 $\mu\text{g/l}$ ml) prepared in the same containers and geometry as the above experiments. The aliquots that had been placed on ice at $\sim 4^\circ\text{C}$ were removed and immediately irradiated (at room temperature) using a ^{60}Co Gamma cell 220 irradiator (Atomic Energy of Canada Ltd., Ottawa, Canada). The preparations received graded doses of 40 Gy, 80 Gy, 160 Gy, 320 Gy, 640 Gy, 1280 Gy at a dose rate of 108 Gy/min (22 to 711 sec). Although temperature changes were not monitored only a minor increase in the temperature of the solutions was expected. The preparations were then examined for protein bound fraction and immunoreactivity.

For direct irradiation T101 that had been conjugated with 1B4M-DTPA (2-p-isothiocyanatobenzyl-6-methyl-DTPA) chelate (19) was radiolabeled. Direct labeling of the 1B4M-T101 was performed under three different conditions. As a control the conjugated T101 was labeled with 37 MBq of In-111 (100 $\mu\text{g/l}$ ml) in 3.6 ml sterile round bottom polypropylene tube. The ^{111}In -labeled T101 represented the near maximum binding of this 1B4M-T101 preparation. Labeling with ^{90}Y was performed under the same geometry as the ^{111}In , in the presence or absence of 11 mg/ml of ascorbic acid (Na ascorbate). In short, 0.5 mg of

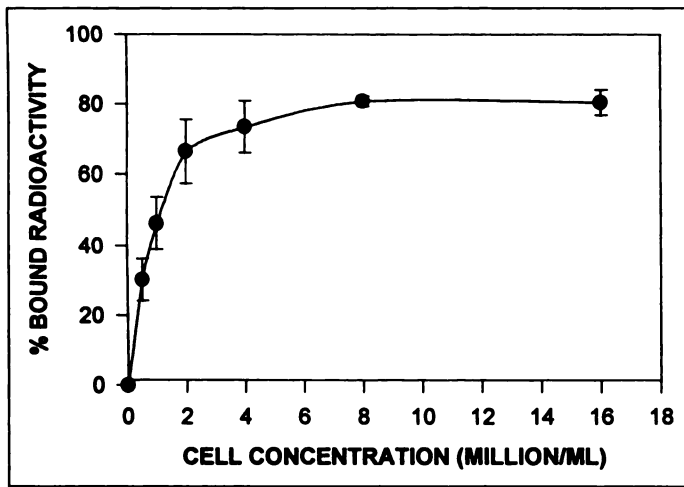


FIGURE 1. Saturation binding curve of ¹²⁵I-T101 (●). The maximum binding of ¹²⁵I-T101 (5 ng) by different concentration of CCRF-CEM cell was determined.

1B4M-T101 were reacted with 555 MBq of ⁹⁰Y for 2.75 hr in a 1-ml volume, pH 4.2 at room temperature. The reaction was then stopped by adding 0.001 M DTPA (50 μl) at pH 6.0 and allowed to stand for an additional 0.5 hr. This was estimated to deliver approximately 640 Gy in 3.25 hr. Labeling yields were 90%–100% for both cases as determined by ITLC in the presence of 10% ammonium acetate in water: methanol (1:1). The nonantibody bound ⁹⁰Y was then separated from the ⁹⁰Y-1B4M-T101 by passing the mixture through anion-exchange column in a volume of 3 ml. The eluted ⁹⁰Y-T101 was further diluted (2 ml) into solutions with 2% HSA in PBS for subsequent immunoreactivity study. The specific activity of these diluted samples were 999 to 1110 MBq/mg and specific concentration was 0.091 MBq/ml.

RESULTS

A full cell binding assay of the ¹²⁵I-T101 (n = 3) showed a plateau in the maximum binding with approximately 8 million cells indicating antigen excess (Fig. 1). Therefore, most assays were subsequently performed at a single point with 8 million cells. A maximum binding of ~83% ± 2% (mean ± s.d., n = 11) was seen.

The immunoreactivity of preparations internally radiated with graded doses of 5-640 Gy decreased drastically as a function

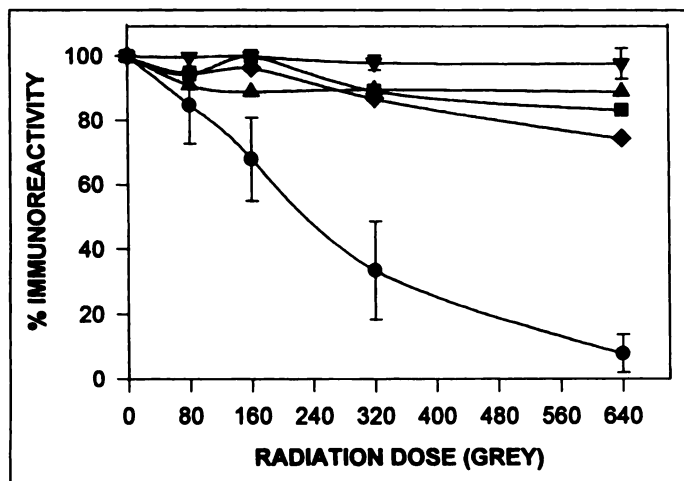


FIGURE 2. Immunoreactivity of ¹²⁵I-T101 was evaluated after delivering 0 and 640 Gy by mixing with ⁹⁰Y-DTPA ("internal irradiation"). The immunoreactivity was determined in the presence and absence of various radioprotectants: ● control (n = 6); ■ hsa (n = 2), ▲ cysteamine (n = 2), ▼ ascorbic acid (n = 7), ◆ glycerol (n = 2). Data are expressed as mean ± s.d.

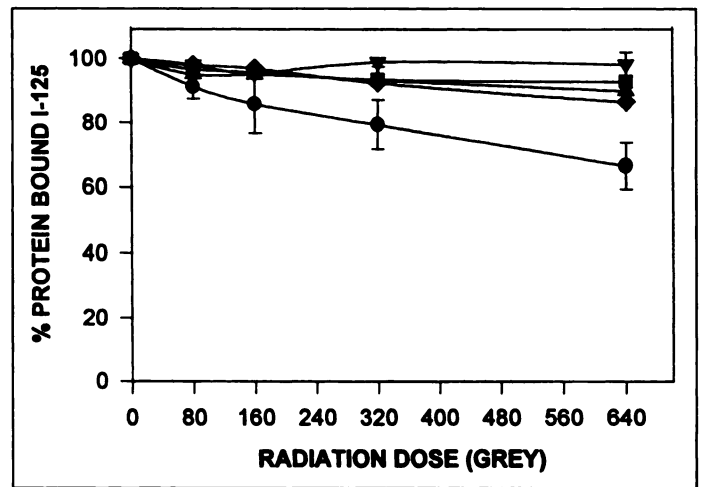


FIGURE 3. Percent protein bound ¹²⁵I-T101 exposed to doses ranging from 0 to 640 Gy delivered by mixing with ⁹⁰Y-DTPA ("internal irradiation") was determined. Effect of various radioprotectants: ■ hsa (n = 2), ▲ cysteamine (n = 2), ▼ ascorbic acid (n = 7), ◆ glycerol (n = 2) on the prevention of this breakdown was evaluated and compared to no protectant: ● control (n = 6). Data point represents the mean ± s.d.

of dose (Fig. 2) a limited number of studies performed at 1280 Gy also showed this large decrease (data not shown). The immunoreactivity of the various preparations was expressed as a fraction of the immunoreactivity of the ¹²⁵I-T101 control that was not exposed to ⁹⁰Y radiation. Doses of 640 to 1280 Gy resulted in a 7.9% and 4.1% retention of the original immunoreactivity, respectively. In contrast, the radioprotectants prevented the loss of immunoreactivity (Fig. 2). In addition to preventing a drop in immunoreactivity the radioprotectants also decreased the breakdown of ¹²⁵I from the T101 (Fig. 3). Whereas, ¹²⁵I-T101 without protectant (n = 2) showed non-TCA precipitable fractions that were 66% of the control (n = 6), the use of radioprotectants (n = 2) prevented breakdown, with more than 90% protein bound ¹²⁵I. These findings were confirmed by HPLC which showed similar results as the TCA assays (data not shown). These results of ascorbic acid (11 mg/ml) were very reproducible from one experiment to another (n = 7).

Graded doses of external irradiation resulted in a dose

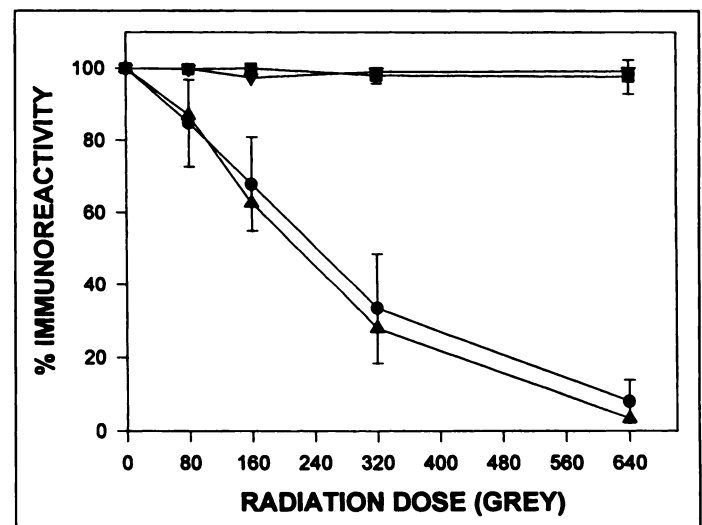


FIGURE 4. Immunoreactivity of ¹²⁵I-T101 exposed to radiation by external beam with ⁶⁰Co or "internal radiation" with ⁹⁰Y-DTPA in absence or presence of ascorbic acid was determined. External irradiation without protectant ▲ (n = 2), external irradiation with ascorbic acid ▼ (n = 2); internal irradiation without protectant ● (n = 6); internal irradiation with ascorbic acid ■ (n = 6). All data are reported as mean ± s.d.

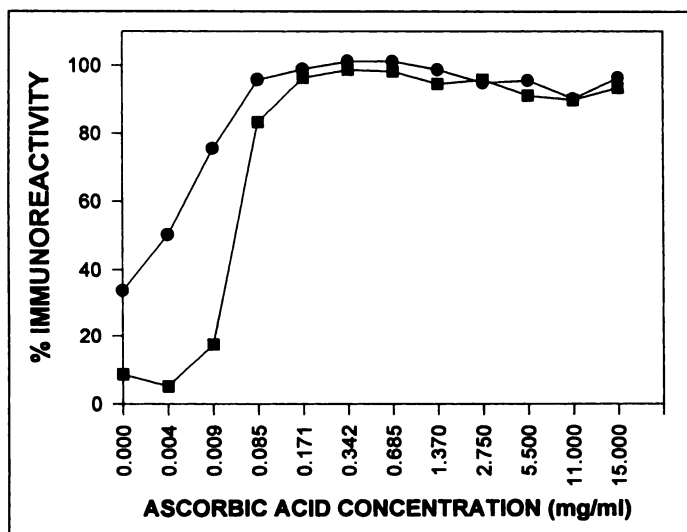


FIGURE 5. Immunoreactivity of ^{125}I -T101 exposed to 320 Gy (●) and 640 Gy (■) using ^{90}Y -DTPA ("internal" irradiation) was determined in the presence of graded doses of ascorbic acid.

dependent decrease in immunoreactivity which was abrogated by the use of ascorbic acid (Fig. 4). These findings were similar to the results seen with internal irradiation. While most of our internal irradiations using ascorbic acid as the protectant used a commercially available preparation, similar results were also obtained with sodium ascorbate. Graded doses of external irradiation also resulted in similar increases in non-protein bound ^{125}I as seen with internal irradiation and the breakdown was also prevented by addition of ascorbic acid or Na-ascorbate.

The radioprotectant effect of various concentrations (0.0042–15 mg/ml) of ascorbic acid was evaluated using ^{125}I -T101 internally irradiated with 320 or 640 Gy and compared to control preparations receiving no irradiation (Fig. 5). Concentrations greater than 0.085 mg/ml were all effective in protecting ^{125}I -T101 from damage as measured by retention of immunoreactivity (Fig. 5) and a decrease in ^{125}I breakdown (data not shown). Addition of ascorbic acid did not affect the immunoreactivity of ^{125}I -T101 adversely.

Indium-111 labeling of T101 showed a maximal binding of $74\% \pm 11.7\%$ ($n = 4$). Direct labeling of ^{90}Y to T101 under conditions delivering doses of 640 Gy to the solution resulted in an immunoreactive fraction of $46.3\% \pm 7.0$, whereas in the presence of ascorbic acid the immunoreactivity was $75.7\% \pm 5.3$ (Table 1). The incorporation of ^{90}Y into the chelated T101 antibody was not adversely affected by the ascorbic acid.

TABLE 1
Effect of Ascorbic Acid on Direct Labeling of T101 with Yttrium-90*

Ascorbic Acid		No Ascorbic Acid	
% Immunoreactivity	Specific activity	% Immunoreactivity	Specific activity
69	1110MBq/mg	40	1110MBq/mg
78	999MBq/mg	51	1110MBq/mg
81	1258MBq/mg	56	1184MBq/mg
79	1036MBq/mg	39	962MBq/mg

*Labeling conditions resulted in 640 Gy to each antibody solution (see Material and Methods).

DISCUSSION

The loss of immunoreactivity of ^{125}I -T101 was similar whether the radiation was delivered "internally" or "externally", and in both cases ascorbic acid prevented loss of immunoreactivity (Fig. 4). This study demonstrated that graded doses of radiation resulted both in a decrease in immunoreactive fraction of T101 as well as antibody breakdown with drop in protein bound radioactivity. Although the external irradiation was given over a few seconds and the internal irradiation was delivered over 24 hr, when the data were plotted in terms of rads, the drop in immunoreactivity was similar for both methods. The drop in immunoreactive fraction (Fig. 2) could not be completely accounted for by the breakdown products (Fig. 3) and is likely due to damage by free radicals interacting with binding site. This dose-dependent radiation-damage is similar to the radiation induced damage reported by others (7,9). We also confirmed the radioprotectant effect of HSA (7,9) and cysteamine (9). In addition, both glycerol and ascorbic acid were shown to be effective radioprotectants.

We focused on ascorbic acid because it is a USP approved formulation. Initially, we evaluated an FDA-approved formulation which, in addition, to ascorbic acid contained 10 mg/ml monothiolglycerol which was expected to have an additional radioprotective effect. Because this product was removed from the market and was not available for our clinical trials, we evaluated Na ascorbate. Na ascorbate was very effective at doses of 0.085 to 15 mg/ml. The radiation doses that we evaluated are in the range of doses that could be delivered to the antibody solution during the process of radiolabeling with ^{90}Y , in particular, when large dose of ^{90}Y radiolabeled antibody are required for therapy. A benefit to the use of ascorbic acid is that it does not interfere with complexation of radiometals isotopes into DTPA derivatives and possibly may be of benefit by binding any iron in the solution. Similar doses of Na ascorbate have been shown to have a negligible role in protein reduction (20). Additionally, because ascorbic acid is an FDA-approved drug with little toxicity, incorporation into a labeling protocol would be feasible. We did not use HSA during the labeling process in order to minimize potential problems with nonspecific binding of isotope to HSA and difficulties in separating free from protein bound product.

As shown in this study under typical conditions of use (direct labeling), we were able to prevent significant drop in immunoreactivity. We have shown similar protection with two other monoclonal antibodies undergoing clinical use (data not shown).

CONCLUSION

With the importance of immunoreactivity in vivo tumor targeting (21), efforts to minimize loss of immunoreactivity are important. Because Na ascorbate is an effective radioprotectant and has the advantage that it is an USP-approved drug that is easily available, does not interfere with the labeling procedure and can be easily separated during the purification step, we now use sodium ascorbate in our standard procedure for radiolabeling MAb.

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Inhibition of Renal Uptake of Indium-111-DTPA-Octreotide In Vivo

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Indium-111-DTPA-octreotide has been successfully used for imaging of somatostatin receptor-positive lesions. However, significant renal uptake of ¹¹¹In-DTPA-octreotide exists, reducing the scintigraphic sensitivity for detection of small tumors in the perirenal region and the possibilities for radiotherapy. The aim of the present study was to determine whether renal uptake of ¹¹¹In-DTPA-octreotide could be reduced in vivo in rats. **Methods:** Male Wistar rats (200–250 g) were placed in metabolic cages and injected with ¹¹¹In-DTPA-octreotide (0.2 MBq and 0.5 μg octreotide), in the presence or absence of re-uptake blockers. At time t = 20 hr after injection, rats were sacrificed and organs were isolated and counted for radioactivity. **Results:** Adding NH₄Cl or NaHCO₃ to the food, resulting in the production of more acid or alkaline urine respectively, resulted in less radioactivity in the kidneys 20 hr after injection compared to controls. Lysine in a single dose of 400 mg/kg resulted in an inhibition of kidney uptake of 40%. When lysine was injected 30 min before ¹¹¹In-DTPA-octreotide, the inhibition was 25%. Arginine had less effect on tubular uptake of ¹¹¹In-DTPA-octreotide than lysine (20% inhibition). Sodium maleate inhibited kidney uptake of ¹¹¹In-DTPA-octreotide most successfully. Acetazolamide (100 mg/kg), succinylacetone (100 mg/kg), cystine dimethylester (340 mg/kg) and increase in urinary flow did not influence ¹¹¹In-DTPA-octreotide retention in the kidneys. **Conclusion:** It appeared possible to reduce re-uptake of ¹¹¹In-DTPA-octreotide in the rat kidney in vivo. The most pronounced effects were seen after administration of sodium maleate or lysine but, because of the described toxic effects of maleate, we will study further only the effects of lysine in a clinical setting.

Key Words: indium-111-DTPA-octreotide; renal tubular re-uptake; sodium maleate; lysine; urine pH

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Indium-111-DTPA-octreotide is a radiopharmaceutical that binds to somatostatin receptors (subtypes 2, 3 and 5) present in certain tissues. It is being used for scintigraphic imaging of somatostatin receptor-positive lesions, such as gastrointestinal pancreatic tumors, neuroblastoma, pheochromocytoma, breast cancer, Hodgkin's lymphoma and small-cell lung cancer (1,2). This peptide is cleared from the body mostly by the kidneys, 50% within the first four hours after injection. However, a significant amount of the dose accumulates in the renal parenchyma (in humans about 7% dose, 4 hr after injection), reducing the scintigraphic sensitivity for detection of small tumors in the perirenal region in the abdomen (3).

In this study we investigated if this renal accumulation could be reduced in vivo in rats. The infusion of certain amino acids, particularly lysine and arginine, has been shown to block renal tubular peptide re-absorption (4). An infusion of synthetic amino acids, containing lysine and arginine among other amino acids, significantly reduced parenchymal uptake (up to 50%) of ¹¹¹In-DTPA-octreotide 4 hr after injection in eight patients, without an effect on glomerular filtration rate (5). Also in mice, reduction of renal tubular reabsorption of ¹¹¹In-labeled Fab fragment was affected by systemic administration of lysine (6). We have, therefore, tested the influence of a single dose of lysine or arginine on renal tubular uptake of ¹¹¹In-DTPA-octreotide in vivo in the rat. We also investigated the influences of: increased urine production; changes in the pH of the urine, the latter by addition of NaCl, NaHCO₃ or NH₄Cl to the food; and sodium maleate on the re-uptake process. Maleate is known to produce a generalized defect of renal tubular transport, causing an immediate and transient diuresis, natriuresis, glucosuria and proteinuria, probably by inhibition of renal cortical Na-K-ATPase and ATP production (7–9). In addition, we tested the compounds succinylacetone, acetazolamide and cys-

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