Residualizing Iodine Markedly Improved Tumor Targeting Using Bispecific Antibody-Based Pretargeting

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Previous studies have shown that pretargeting allows rapid visualization of renal cell carcinomas (RCC) with an ^{111}In-labeled bivalent peptide. For radioimmunotherapy, a β-emitting radionuclide labeled to a bivalent peptide is required. Therapeutic efficacy of these radionuclides depends on the E_{max}, physical half-life, and residence time of the radiolabel in the tumor. The ^{131}I radiolabel generally clears rapidly from the tumor after internalization and subsequent degradation of the bivalent L-amino acid peptide (L-a.a. peptide) in the tumor cells. To improve the residence time of the iodine label in the tumor, a new bivalent peptide was synthesized that is peptidase resistant and consists of 4 D-amino acids (D-a.a. peptide). Here we investigated the characteristics of the residualizing iodine label in SK-RC-52 RCC tumors. **Methods:** The D-a.a. peptide was manually synthesized according to standard solid-phase Fmoc/HBTU (2-[1H-benzotriazole-1-yl]-1,1,3,3-tetramethyluronium hexafluorophosphate) chemistry. The uptake and retention in the tumor of ^{111}In-/^{125}I-labeled bivalent peptides (L-a.a. peptide and D-a.a. peptide) were studied in female BALB/c athymic mice with subcutaneous SK-RC-52 RCC tumors. Tumors were pretargeted with the bispecific monoclonal antibody (bs-mAb) G250xDTIn-1 and, 72 h later, mice were injected intravenously with one of both radiolabeled peptides. The effect of bs-mAb–diDTPA–bs-mAb (DTPA is diethylenetriaminepentaacetic acid) bridging at the tumor cell surface on the internalization of the bs-mAb–diDTPA complex was investigated in SK-RC-52 tumor-bearing mice. **Results:** The maximum uptake and retention of ^{125}I-labeled L-a.a. peptide in the tumor were significantly lower compared with that of the ^{111}In-labeled L-a.a. peptide. In contrast, the tumor uptake and retention of the ^{125}I-labeled D-a.a. peptide) were similar to that of the ^{111}In-labeled L-a.a. peptide but were superior at later time points. The biodistribution of the radioiodinated D-a.a. peptide was highly similar to that of the ^{111}In-labeled D-a.a. peptide, and both radiolabeled peptides were retained significantly better in the tumor than the ^{111}In-labeled L-a.a. peptide. bs-mAb–diDTPA–bs-mAb bridge formation did not affect internalization of the bs-mAb–diDTPA complex. **Conclusion:** Uptake and retention in the tumor of the iodinated peptide after pretargeting with a bs-mAb can be significantly improved using D-a.a. peptides. Accordingly, the radiation dose to the tumor, correlating with the therapeutic efficacy of pretargeted RCC, can be enhanced substantially.

Key Words: pretargeting strategy; residualizing iodine; internalization; bs-mAb G250xDTIn-1

J Nucl Med 2005; 46:1016–1022

Monoclonal antibodies (mAbs) directed against tumor-associated antigens have excellent characteristics to target tumor cells selectively (*1–3*). So far, radioimmunotherapy (RIT) has only induced significant therapeutic effect in patients with hematologic tumors, presumably due to their relatively high radiosensitivity (*4,5*). The combination of the long circulating half-life of the radiolabeled mAb and the radiosensitivity of the bone marrow limits the radioactive doses of labeled antibodies that can be administered safely (*6,7*). The separation of the mAb (targeting agent) and the radionuclide (effector agent) as proposed by Goodwin et al. (*8,9*) could improve the efficacy of RIT. In this approach a nonradiolabeled bispecific monoclonal antibody (bs-mAb) with antitumor-associated antigen (TAA) and antihapten specificity is injected in the first phase. In the second phase a radiolabeled hapten is administered, which is trapped in the tumor by the bs-mAb.

We have developed an effective pretargeting system based on a biologically produced bs-mAb (antirenal cell carcinoma [RCC] x anti-DTPA(In) bs-mAb: G250xDTIn-1) and an ^{111}In-labeled bivalent L-amino acid peptide (L-a.a. peptide) (Ac-Phe-Lys(DTPA)-Tyr-Lys(DTPA)-NH_2) (DTPA is diethylenetriaminepentaacetic acid) (*10*). In previous studies we have shown that with this strategy relatively high uptake of the ^{111}In label in various RCC mouse models can be achieved

Received Dec. 1, 2004; revision accepted Jan. 14, 2005.

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(maximum uptake from 54.1 to 287 %ID/g) (%ID/g is percentage injected dose per gram) (*11*). The β-emitting radionuclide ^{131}I is a commonly used radionuclide for RIT. Unfortunately, after internalization and degradation of the ^{131}I-labeled peptide in the lysosomes, the metabolite ^{131}I-Tyr is rapidly cleared from tumor cells (*12–15*). In contrast, peptides labeled with a radiometal via a substituted aminopolycarboxylate (such as DTPA or 1,4,7,10-tetraazacyclododecane-*N,N′,N″,N‴*-tetraacetic acid [DOTA]) showed enhanced retention in tumor cells after internalization (*16*). The metabolite Lys-chelate-(metal) is trapped in the lysosomes because it cannot pass the lysosomal membrane (*17,18*). Stein et al. (*19,20*) and Govindan et al. (*21*) postulated that radioiodine can also be trapped in the lysosomes when the tyrosine residue is conjugated to the DTPA-substituted lysine residue via a peptidase-resistant peptide bond by using the D-analogs of both amino acid residues involved. In the present study, these findings were exploited to enhance the tumor retention of ^{131}I-labeled peptide in the pretargeting approach. A new bivalent tetrapeptide was synthesized, consisting of 4 D-amino acid (D-a.a.) residues and both lysine residues were conjugated with a DTPA moiety (D-a.a. peptide). These D-a.a. peptide bonds are resistant to endo- and exopeptidases (*22*). Because such a peptide cannot be degraded in the lysosomes, the ^{131}I label will be retained in the tumor. In these studies the iodinated D-a.a. bivalent peptide was studied in the pretargeting strategy of SK-RC-52 tumors.

Various studies have demonstrated markedly improved tumor targeting with a bivalent peptide instead of a monovalent hapten in the pretargeting strategy (*23–27*). Internalization of the bs-mAb–peptide complex might also play a role in the tumor retention of the radiolabel after tumor targeting.

Cross-linking of 2 cell-surface antigens via a mAb can induce acceleration of internalization of the antigen–mAb complex (*28*). In the pretargeting system, cross-linking of tumor antigens can occur via the bivalent peptide. Here we examined the influence of G250 antigen cross-linking on the internalization rate of the peptide.

MATERIALS AND METHODS

bs-mAb G250xDTIn-1

The characteristics of the mAb anti-G250 (IgG1), directed against the RCC-associated antigen G250, identified as carbonic anhydrase isoform IX (MN/CA IX), have been described by Oosterwijk et al. (*29*). The MN/CA IX antigen is expressed on the cell surface of virtually all clear-cell RCCs (*30*).

The characteristics of anti-DTPA-In mAb (DTIn-1, IgG2a), the isolation of bispecific antibody–producing quadroma cells, and the characterization and purification of the bs-mAb G250xDTIn-1 have been described in detail previously (*31*).

^{111}In-G250xDTIn-1. The bs-mAb G250xDTIn-1 was conjugated with ITC-DTPA (ITC is *p*-isothiocyanatobenzyl) according to Ruegg et al. (*32*). Briefly, to 100 μL bs-mAb (5 mg/mL 50 mmol/L Na_2CO_3, pH 9.5), 0.117 mg ITC-DTPA was added and, after 1-h incubation at room temperature, the reaction mixture was dialyzed against 50 mmol/L NaAc, pH 5.5, in a Slide-A-Lyzer (10-kDa cutoff; Pierce Chemical Co.) to eliminate the excess ITC-DTPA. bs-mAb–ITC-DTPA was labeled with ^{111}In: To 155 μg bs-mAb–ITC-DTPA, 11.1 MBq (300 μCi) $^{111}InCl_3$ (Tyco Health Care) were added and the reaction mixture was incubated for 1 h at room temperature. The radiochemical purity (RCP) was determined by instant thin-layer chromatography on silica gel strips using 0.15 mol/L citrate buffer (pH 6.0) as the mobile phase (RCP, >98%).

^{125}I-G250xDTIn-1. The bs-mAb was radioiodinated with ^{125}I using the IODO-GEN-method (Pierce Chemical Co.) (*33*). To a 100-μg IODO-GEN vial, 140 μL bs-mAb (1.475 mg/mL), 20.4 MBq (550 μCi) $Na^{125}I$, and 50 μL phosphate buffer, pH 7.4, were added. After incubating the reaction mixture at room temperature for 10 min, the iodinated bs-mAb was purified on a PD-10 column, eluted with 0.5% bovine serum albumin and phosphate-buffered saline.

Bivalent Peptide

L-a.a. Peptide. The synthesis of the bivalent L-a.a. peptide (Ac-Phe-Lys(DTPA)-Tyr-Lys(DTPA)-NH_2) was described previously (*10,34*).

D-a.a. Peptide. The bivalent D-a.a. peptide Ac-D-Phe-D-Lys-(DTPA)-D-Tyr-D-Lys(DTPA)-NH_2 was manually synthesized by using standard solid-phase Fmoc/HBTU (2-[1H-benzotriazole-1-yl]-1,1,3,3-tetramethyluronium hexafluorophosphate) chemistry on a rink amide resin. The lysine residue was coupled with its C-terminus to an Argogel rink resin (Argonaut). Subsequently, D-tyrosine, D-lysine, and D-phenylalanine were conjugated. After deprotection of the N-terminal amino acid residue from the resin, the peptide was acetylated at the N-terminus by treatment with acetic acid anhydride/HOBt/DIPEA solution (HOBt is 1-hydroxybenzotriazole hydrate; DIPEA is diisopropylethylamine). The peptide was deprotected and cleaved from the resin using a trifluoroacetic acid (TFA)/H_2O mixture. Next, monoactivated DTPA chelates (DTPA-(t-Bu)$_4$) (*35*) were conjugated to the ϵ-amino group of both lysine residues, using benzotriazole-1-yl-oxy-tris-(dimethylamino)phosphonium hexafluorophosphate (BOP) as a coupling reagent. The peptide was purified by preparative reverse-phase high-performance liquid chromatography (RP-HPLC) on a C-8 column (Adsorbosphere XL, Allech; 250 × 22 mm) using a H_2O/MeCN/TFA buffer system. Fractions were collected and lyophilized. The product was characterized using matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) mass spectrometry and electrospray ionization (ESI) mass spectroscopy (MH^+ = 1,376.96 g/mol) and analyzed by RP-HPLC.

^{111}In Labeling of L-a.a. Peptide and D-a.a. Peptide. To 15 μL of the peptide solution (11 ng/μL), 15 μL 50 mmol/L NH_4Ac (pH 5.4) and 7.4 MBq (0.2 mCi) $^{111}InCl_3$ (Tyco Health Care) were added. The reaction mixture was incubated for 60 min at room temperature. The RCP was determined by RP-HPLC on an Agilent 1100 series liquid chromatography system (Agilent Technologies) and a Zorbax Rx-C18 column (5 μm, 4.6 × 250 mm). The column was eluted with a flow rate of 1 mL/min with a gradient from 10 mmol/L NH_4Ac, pH 7.4, to 100% acetonitrile within 15 min. Radioactivity was detected with an in-line Radiomatic A-500 series flow detector (Canberra-Packard). A 3-fold molar excess $InCl_3$ was added when the RCP was >95%, to saturate the remaining DTPA chelates with stable In^{3+}.

^{125}I Labeling of L-a.a. Peptide and D-a.a. Peptide. The peptides were iodinated with ^{125}I according to the IODO-GEN method (*33*). To a vial coated with 100 μg IODO-GEN, 5 μL (10 μg/mL) peptide, 15 μL phosphate buffer, pH 7.2, and 11.1 MBq (300 μCi) ^{125}I were added. After a 10-min incubation, the reaction mixture

was transferred to a reaction vial containing 1/10 volume 1 mol/L NH_4Ac, pH 5.4. Subsequently, a 3-fold molar excess of stable $InCl_3$ was added and incubated for 30 min to saturate the DTPA chelates. The peptide was purified on an activated C-18 SepPak cartridge (Waters), washed with 0.1 mol/L NH_4Ac, pH 7.4, and eluted with 100% MeOH. After evaporation of the MeOH, the peptide was dissolved in 50 mmol/L NH_4Ac, pH 5.4, and the RCP of the sample was determined by RP-HPLC as described (*34*).

In Vivo Experiments

Female BALB/c *nu/nu* mice were injected subcutaneously with 200 μL SK-RC-52 cell suspension (2×10^6 cells per 200 μL). After 2–3 wk, when tumor sizes were 50–300 mm^3, the biodistribution experiments were initiated. SK-RC-52 RCC was derived from a clear-cell RCC metastatic lesion in the mediastinum (*36*) and was obtained from the Memorial Sloan-Kettering Cancer Center.

Mice with subcutaneous SK-RC-52 tumors were pretargeted with 15 μg bs-mAb G250xDTIn-1. After 72 h, mice were injected intravenously with 6 ng ^{125}I-/^{111}In-labeled peptide (L-a.a. peptide or D-a.a. peptide). All reagents were injected intravenously via the tail vein (200 μL).

To investigate the effect of cross-linking MN/CA IX antigens by bs-mAb–diDTPA bridge formation on the internalization of the Ag–bs-mAb complex, tumors were pretargeted with 15 μg of directly labeled bs-mAb (bs-mAb–^{111}In/bs-mAb–^{125}I). At 24 and 96 h after injection, 2 groups of mice were killed by CO_2 asphyxiation without administrating the bivalent L-a.a. peptide (Table 1). Seventy-two hours after injection of the radiolabeled bs-mAb, 2 groups of mice were injected intravenously with an In^{3+}-labeled bivalent L-a.a. peptide; 24 and 72 h after injection of the unlabeled bivalent peptide, mice were killed. Blood was obtained by cardiac puncture. Tissues (tumor, muscle, lung, spleen, kidney, liver, and small intestine) were dissected and weighed and the radioactivity was measured in a γ-counter (Wallac Wizard 3″ 1480 automatic γ-counter). To allow calculation of the radioactive uptake in each organ as a fraction of the injected dose, an aliquot of the injection dose was counted simultaneously. Results were expressed as % ID/g. All groups consisted of 4 or 5 mice.

Statistical Analysis

All mean values are given as mean ± SD. Statistical analysis was performed using the unpaired *t* test when 2 groups were analyzed, and the 1-way ANOVA was used when >2 groups were analyzed. The level of significance was set at $P < 0.05$.

RESULTS

Biodistribution Results of Radiolabeled Bivalent Peptides

The uptake and retention of the ^{125}I-/^{111}In-labeled D-a.a. peptide and L-a.a. peptide in SK-RC-52 tumors are depicted in Figure 1. The tumor uptake of the ^{111}In-labeled L-a.a. peptide was relatively high after pretargeting of SK-RC-52 tumors with bs-mAb G250xDTIn-1 (Fig. 1B; maximum uptake, 22.0 ± 6.9 %ID/g at 4 h after injection). Although the ^{111}In label is considered as a residualizing radionuclide, the radiolabel cleared from the tumor in time. The maximum uptake in the tumor of the ^{125}I-iodinated L-a.a. bivalent peptide was significantly lower compared with that of the ^{111}In-labeled L-a.a. peptide. In addition, the ^{125}I label cleared from the tumor much more rapidly (Fig. 1A; 5.1 ± 0.8 %ID/g at 4 h after injection to 0.05 ± 0.01 %ID/g at 48 h after injection). In contrast, using the newly synthesized D-a.a. peptide, the uptake of the ^{125}I radiolabel in the tumor was significantly higher and the radiolabel was retained in the tumor much better (Fig. 1A; 22.3 ± 5.3 %ID/g at 24 h after injection to 14.8 ± 5.0 %ID/g at 7 d after injection) than that of the iodinated L-a.a. peptide. The biodistribution of ^{111}In and ^{125}I, linked to the D-a.a. peptides, was highly similar. Moreover, the residence time of ^{125}I-labeled D-a.a. peptide in the tumor was remarkably higher than that of the ^{111}In-labeled L-a.a. peptide.

The biodistribution data of the ^{111}In-/^{125}I-labeled D-a.a. peptide and L-a.a. peptides in pretargeted SK-RC-52 tumor-bearing mice are shown in Figure 2. The radioactivity was cleared relatively fast from the normal organs as well as from the circulation, regardless of the radiolabeled peptide used. This indicates no specific uptake of the radiolabeled peptides in the normal tissues (the hepatobiliary tract or renal excretion).

Effect of bs-mAb–diDTPA–bs-mAb Cross-Linking on Internalization

mAbs can induce or accelerate internalization of the antigen–mAb complex by cross-linking of the antigens. With our approach, using monovalently bound antigen–bs-mAb, dimerization of the antigens can be accomplished by bridge formation via a bivalent peptide. The influence of

TABLE 1
Design of Experiment To Determine Effect of Cross-Linking of MN/CA IX Antigens

Group	Day 0	Day 1	Day 3	Day 4	Day 6
1	G250xDTIn-1	Dissection			
2	G250xDTIn-1	—	—	Dissection	
3	G250xDTIn-1	—	diDTPA	Dissection	
4	G250xDTIn-1	—	diDTPA	—	Dissection

SK-RC-52 RCC tumor-bearing mice were pretargeted with 15 μg ^{111}In-/^{125}I-dual-labeled bs-mAb G250xDTIn-1. Mice were killed at various time points after injection of radiolabeled bs-mAb and biodistribution of radiolabels was determined with (groups 3 and 4) or without (groups 1 and 2) administration of bivalent peptide (diDTPA).

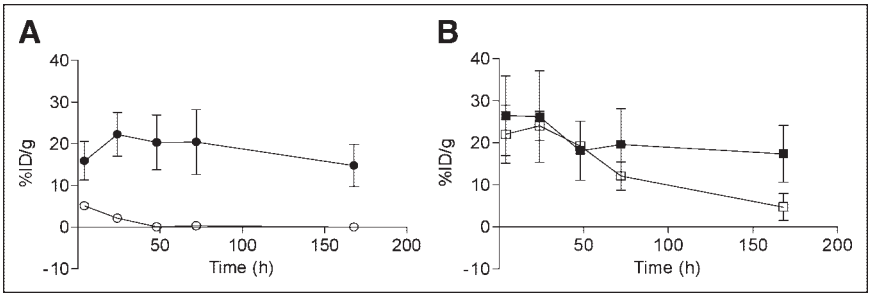

FIGURE 1. Uptake and retention of dual-labeled bivalent peptides in SK-RC-52 tumors. Tumor-bearing mice received 6 ng radiolabeled peptides, 72 h after pretargeting tumors with 15 μg bs-mAb G250xDTIn-1. (A) ^{125}I-L-a.a. peptide (○) and ^{125}I-D-a.a. peptide (●). (B) ^{111}In-L-a.a. peptide (□) and ^{111}In-D-a.a. peptide (■). Biodistribution was determined 4, 24, 48, and 72 h after injection and 7 d after injection Uptake is expressed as %ID/g (mean ± SD).

bridging 2 adjacent MN/CA IX antigens via a bivalent peptide on internalization of the Ag–bs-mAb complex was studied in vivo with ^{111}In-/^{125}I-labeled bs-mAb G250xDTIn-1 (Tables 1 and 2; Fig. 3). At 24 h after injection of the radiolabeled bs-mAb (no bivalent peptide administered), the ^{111}In label showed a significantly higher uptake in the tumor than that of the ^{125}I label (25.7 ± 9.4 %ID/g and 14.3 ± 2.1 %ID/g, respectively; $P < 0.05$). From 24 to 96 h after injection, the uptake of the ^{111}In label in the tumor increased from 25.7 ± 9.4 %ID/g at 24 h after injection to 34.0 ± 10.3 %ID/g at 96 h after injection The uptake of the ^{125}I label in the tumor decreased from 14.3 ± 2.1 %ID/g at 24 h after injection to 9.9 ± 2.2 %ID/g at 96 h after injection Administration of the unlabeled bivalent peptide (96 h after injection of the radiolabeled bs-mAb) did not lead to significant alterations in tumor targeting within 24 h (^{111}In: 32.5 ± 10.8 %ID/g and 34.0 ± 10.3 %ID/g, respectively; ^{125}I: 8.7 ± 2.3 %ID/g and 9.9 ± 2.2 %ID/g, respectively).

DISCUSSION

Pretargeting RCC tumors with a bs-mAb directed against MN/CA IX antigen, in combination with ^{111}In-labeled bivalent peptide, leads to excellent tumor targeting together with a very rapid and complete background clearance. For RIT, the ^{131}I radiolabel is a commonly used radionuclide with suboptimal physiologic characteristics for the treatment of small tumor lesions. Unfortunately, after internalization and degradation of the radiolabeled peptide, the metabolite ^{131}I-Tyr is rapidly excreted from the tumor cells. Enhanced tumor cell retention of the ^{131}I label is required to achieve therapeutic efficacy. Therefore, a new bivalent peptide, more resistant to proteolytic degradation, was synthesized, consisting of 4 D-a.a. and substituted with 2 DTPA chelates.

In the SK-RC-52 RCC tumor model, the pretargeting strategy was tested by comparing the use of the iodinated bivalent D-a.a. peptide and the iodinated L-a.a. peptide.

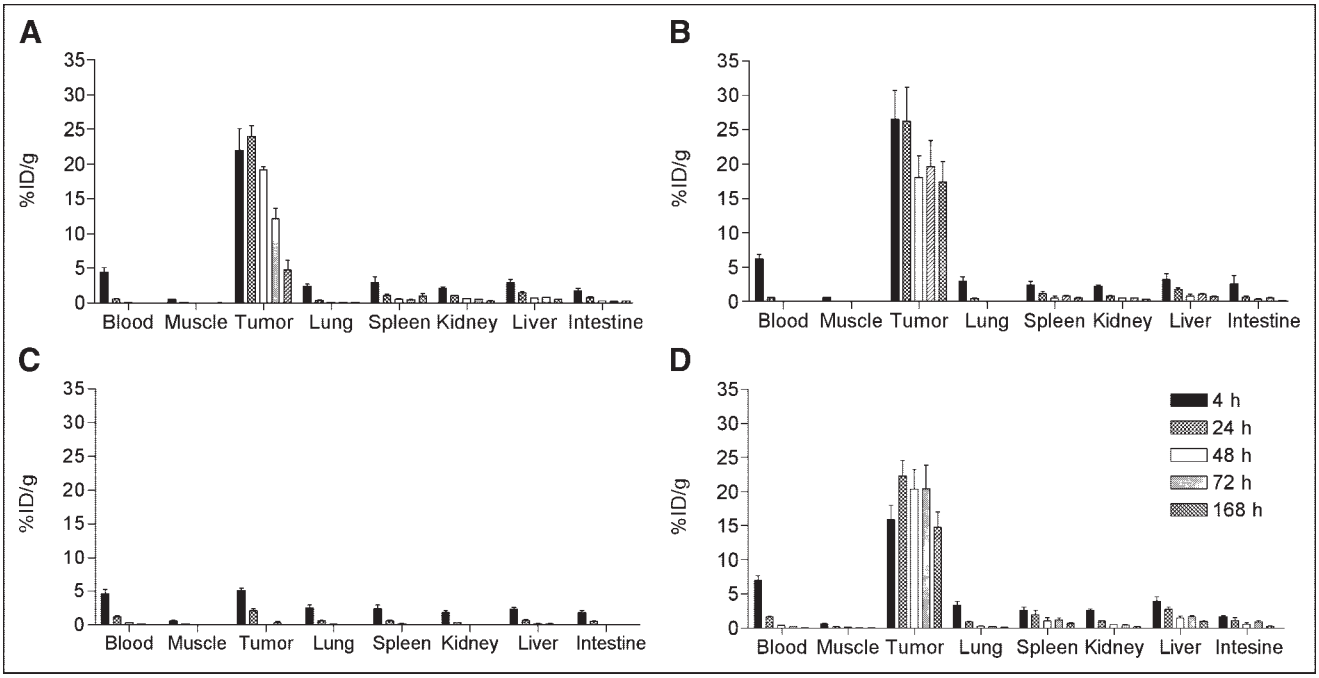

FIGURE 2. Biodistribution of radiolabeled bivalent peptides: ^{111}In-L-a.a. peptide (A), ^{111}In-D-a.a. peptide (B), ^{125}I-L-a.a. peptide (C), and ^{125}I-D-a.a. peptide (D). After pretargeting of SK-RC-52 tumor-bearing mice with 15 μg bs-mAb G250xDTIn-1, 72 h later mice were injected intravenously with 6 ng peptide. Mice were killed at various time points after injection of radiolabel and biodistribution was determined. Uptake is expressed as %ID/g (mean ± SD).

TABLE 2
Biodistribution Results of Pretargeting of SK-RC-52 RCC with ^{111}In-/^{125}I-Labeled bs-mAb G250xDTIn-1

Biodistribution	Group 1	Group 2	Group 3	Group 4
		^{111}In–bs-mAb		
Blood	13.9 ± 1.1	12.1 ± 1.3	11.6 ± 0.6	8.1 ± 1.9
Muscle	1.4 ± 0.2	1.3 ± 0.1	1.0 ± 0.1	0.9 ± 0.1
Tumor	25.7 ± 9.4	34.0 ± 10.3	32.5 ± 10.8	30.0 ± 3.6
Lung	8.5 ± 0.7	6.6 ± 0.5	6.6 ± 0.8	3.4 ± 0.9
Spleen	16.9 ± 7.8	9.6 ± 4.2	6.8 ± 1.7	8.0 ± 3.9
Kidney	5.0 ± 0.5	4.2 ± 0.7	3.9 ± 0.5	3.2 ± 0.5
Liver	8.4 ± 1.2	5.9 ± 0.5	7.1 ± 0.9	6.0 ± 1.8
Intestine	5.7 ± 1.5	4.5 ± 1.7	3.4 ± 0.6	3.4 ± 0.7
T/B	1.9 ± 0.6	2.8 ± 0.8	2.8 ± 1.0	3.8 ± 1.1
		^{125}I–bs-mAb		
Blood	13.5 ± 2.1	12.3 ± 1.4	11.8 ± 1.0	9.0 ± 1.9
Muscle	1.6 ± 0.1	1.5 ± 0.1	1.2 ± 0.1	1.0 ± 0.1
Tumor	14.3 ± 2.1	9.9 ± 2.2	8.7 ± 2.3	7.5 ± 0.7
Lung	9.2 ± 0.5	8.0 ± 2.5	6.7 ± 0.4	5.2 ± 1.5
Spleen	6.6 ± 1.7	4.3 ± 2.0	2.8 ± 0.7	2.5 ± 1.0
Kidney	4.6 ± 0.3	4.0 ± 0.5	3.4 ± 0.2	2.7 ± 0.6
Liver	3.9 ± 2.2	3.6 ± 0.4	3.2 ± 0.5	2.4 ± 0.6
Intestine	4.3 ± 0.7	3.9 ± 1.0	3.0 ± 0.2	2.5 ± 0.7
T/B	1.1 ± 0.04	0.8 ± 0.2	0.8 ± 0.2	0.9 ± 0.3

T/B = tumor-to-blood ratio.
Groups 3 and 4 were injected after 72 h with 6 ng bivalent L-a.a. peptide, loaded with nonlabeled indium.

Peptide-containing endosomes, appearing after internalization of the bound antigen, fuse with lysosomes. Peptides are degraded in the lysosomes by peptidases to single amino acids, and the majority of the amino acid residues are subsequently excreted from the cell. However, when the lysine residue is conjugated with ^{111}In-labeled DTPA, the Lys-DTPA(^{111}In) metabolite is trapped in the lysosomes. Foulon et al. ascribed the capturing of the Lys-DTPA(^{111}In) in the lysosomes to the positively charged lysine-DTPA(In) complex at lysosomal pH (*37*), whereas Stein et al. (*19,20*) and Govindan et al. (*21*) reported that the enhanced hydrophilicity, caused by the present DTPA chelate, was responsible for trapping of the ^{111}In-labeled lysine.

The newly synthesized D-a.a. peptide as well as the L-a.a. peptide were blocked on both termini to reduce the susceptibility to proteolytic degradation in the circulation, but lysosomal endopeptidases could still metabolize the L-a.a. peptide. When iodinated, the metabolite ^{125}I-L-Tyr will rap-

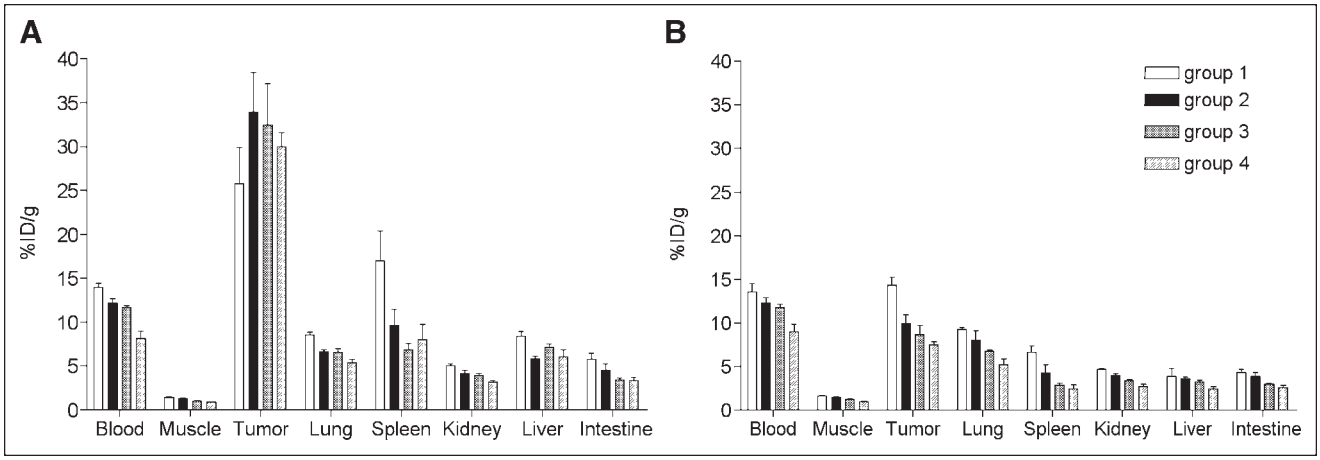

FIGURE 3. Biodistribution of ^{111}In label (A) and ^{125}I label (B) in tumor-bearing mice, pretargeted with 15 μg ^{111}In-/^{125}I-labeled bs-mAb G250xDTIn-1. Seventy-two hours later, 2 groups of mice were injected intravenously with 6 ng bivalent peptide (groups 3 and 4, DTPA chelates loaded with unlabeled indium). Mice were killed at various time points after injection of radiolabel (Table 1) and biodistribution was determined. Uptake is expressed as %ID/g (mean ± SD).

idly be excreted from the cell. In contrast, the residence time of the radiolabel was significantly improved when the D-a.a. peptide was used. Presumably, in contrast to the L-a.a. peptides, the D-a.a. peptides remained intact in the lysosomes because the D-a.a. peptide bonds are more resistant to peptidase degradation. The iodinated tyrosine residue remained bound to both adjoining DTPA-substituted lysine residues, which cannot cross the lysosomal membrane. Accordingly, the nonresidualizing property of iodine was converted into a residualizing character.

The uptake of the residualizing radiolabeled peptide in the tumor increased or was stabilized from 4 to 24 h after injection (Fig. 1). The uptake of the residualizing ^{111}In label in the tumor was significantly higher at 4 h after injection than the uptake of the nonresidualizing ^{125}I label when labeled to the L-a.a. peptide. These results indicated a rapid internalization rate of the radiolabeled bivalent peptide. In addition, previously published data showed an increased uptake and an enhanced retention of the radiolabel in the tumor when the pretargeting strategy was performed with a bivalent ^{111}In-labeled peptide instead of a monovalent hapten (*23–25*). It has been shown that cross-linking of the cell-surface-antigen by antibodies could induce accelerated internalization (*28*). Therefore, we hypothesized that bridging 2 MN/CA IX antigens via a bivalent peptide might be necessary to influence internalization. The improved tumor targeting using a bivalent peptide instead of a monovalent hapten could be due to bridge formation of the MN/CA XI antigen by a bivalent peptide to induce internalization, which is not feasible with a monovalent hapten. This cross-bridge formation was previously designated the "affinity enhancement system," as described by Devys et al. (*26*) and Barbet et al. (*27*).

The effect of cross-linking 2 MN/CA IX antigens was examined with pretargeting of directly labeled bs-mAb (^{111}In–bs-mAb and ^{125}I–bs-mAb) exploiting the residualizing character differences between both radiolabels. The pretargeted tumors were targeted with or without a bivalent peptide loaded with unlabeled indium. By 24 h after injection of the labeled bs-mAb, a significant difference in uptake of both radiolabels was observed, suggesting that internalization occurred with monovalently bound bs-mAb. Second, no significant difference in uptake of the individual radiolabels in the tumor was observed between groups 2 and 3, indicating that cross-bridge formation did not affect the internalization rate. In addition, even up to 72 h after the injection of the bivalent peptide (group 4), the uptake of the radiolabel in the tumor did not change significantly. Cross-bridge formation does not affect the internalization rate in this tumor model, which displays the significance of an accurate determination of the optimal protein and peptide dose and the optimal interval between both administrations.

In summary, a significant improvement in the uptake and retention of radioiodine in the tumor can be achieved in the pretargeting strategy with iodinated D-a.a. peptides instead of L-a.a. peptides. Internalization of the antigen–bs-mAb complex was not induced or enhanced by bridge formation of the MN/CA IX antigens.

CONCLUSION

With the pretargeting strategy, the uptake and retention of the radiolabel in tumors are improved when radiolabeled D-a.a. peptides were used for both indium and iodine.

ACKNOWLEDGMENTS

This work was supported by a research grant from the Dutch Cancer Society (KUN 2000-2306). We thank Gerry Grutters, Hennie Eijkholt, and Bianca de Weem (University of Nijmegen, Central Animal Laboratory) for technical assistance in the animal experiments.

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