Methods to Avoid Adverse Effect of Circulating Antigen on Biodistribution of ^{125}I-labeled AntiTac dsFv: Preinjection of Intact Antibody Versus Clearance of Antigen with Avidin-Biotin System

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The presence of circulating antigen may adversely affect the biodistribution of a radiolabeled antibody. The α subunit of the interleukin-2 receptor (IL-2Rα) is a cell-surface receptor that is overexpressed in various hematologic malignancies and in benign disorders. This receptor is cleaved from the cell surface and can be found in high concentrations in serum. Radiolabeled antiTac antibodies are being evaluated to target this receptor. Previous studies have shown that circulating soluble IL-2Rα (sIL-2Rα) adversely affected the biodistribution of radiolabeled antiTac disulfide-stabilized (ds)Fv. In this study, we compared blocking and clearing sIL-2Rα to see which better minimized its interference with the biodistribution of radiolabeled antiTac dsFv. **Methods:** Two models of sIL-2Rα were used: one consisted of mice given intravenous sIL-2Rα and the other consisted of mice bearing SP2/Tac tumor xenografts (IL-2Rα positive), which shed sIL-2Rα. We biotinylated humanized antiTac monoclonal antibody (bt-HuTac) and radiolabeled it with ^{125}I. We then compared its biodistribution with that of humanized antiTac monoclonal antibody IgG (HuTac). We examined the biodistribution of an injected dose of ^{125}I-labeled antiTac dsFv after a preinjection of HuTac to block the sIL-2Rα epitope and after a preinjection of bt-HuTac, followed by an avidin chase. **Result:** The ^{125}I-labeled bt-HuTac cleared from the serum at a rate similar to that of HuTac. The avidin chase effectively cleared >92% of circulating ^{125}I-labeled bt-HuTac within 20 min and was also effective in clearing sIL-2Rα. In comparison, HuTac prolonged the retention of ^{125}I-labeled sIL-2Rα in the circulation, and the avidin chase decreased ^{125}I-labeled sIL-2Rα to <18% of control. Although the two-step antigen-clearing system effectively cleared the antigen from the circulation and improved the biodistribution of ^{125}I-labeled dsFv, the HuTac preinjection method had a similar but longer lasting beneficial effect on ^{125}I-labeled dsFv biodistribution. **Conclusion:** Preinjection of either HuTac or bt-HuTac with avidin chase improved the biodistribution of subsequently administered ^{125}I-labeled antiTac dsFv by preventing the dsFv from binding to the sIL-2Rα, but the HuTac blocking method is simpler and longer lasting.

Key Words: Fv fragment; radioimmunodetection; monoclonal antibody; interleukin-2; avidin

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The effectiveness of radiolabeled antibodies for radioimmunodetection and radioimmunotherapy of tumors has been studied extensively (*1,2*). Most of the antigens targeted are not shed from the cell surface. However, the several antigens that are released from the cell surface are present at significant levels in the circulation (*3–5*). In theory, these circulating antigens interfere with tumor targeting by immediately neutralizing the antigen-binding site of the injected antibody (*6*). The resulting complexes may lead to faster clearance of the antibody (*7*). In some cases in which an intact antibody is used, circulating antigen is not associated with poor tumor localization or altered biodistribution (*8*), whereas in others, a detrimental effect in the biodistribution is observed (*9*).

With the advent of low-molecular-weight, genetically engineered antibody reagents (*10*), the effect of circulating antigen on biodistribution must be revisited. The use of antibody fragments such as the 25-kD antiTac disulfide-stabilized Fv fragment (dsFv) requires a reagent that rapidly clears the blood and targets the tumor, thus resulting in high tumor-to-nontumor ratios (*11*). We have recently shown that in targeting the α subunit of the interleukin-2 receptor (IL-2Rα) with a radiolabeled dsFv fragment, antigen-antibody complexes were formed in vivo in the circulation. These complexes adversely affected the biodistribution by prolonging the retention of the fragment in the circulation (*12*). These findings are analogous to the prolonged circula-

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tion of insulin (a low-molecular-weight protein) that is seen in patients who have anti-insulin antibodies (*13*).

IL-2Rα is a cell-surface receptor that is overexpressed in various hematologic T-cell malignancies (*14*) and in autoimmune diseases (*15,16*). In contrast, it is not expressed or is expressed at very low levels in normal lymphocytes. IL-2Rα has been used as a target for immunotherapy and radioimmunotherapy (*8,14*). AntiTac is an IgG2a murine monoclonal antibody that recognizes IL-2Rα (*17*). This antibody has shown some effectiveness in the treatment of adult T-cell leukemia (*8*) and in the prevention of allograft rejection in both a monkey heart transplant model as well as in randomized, placebo-controlled, renal transplant clinical trials (*18,19*). However, trials targeting IL-2Rα with radiolabeled antiTac have shown that soluble IL-2Rα (sIL-2Rα) is often present in the circulation and results in the formation of complexes that block the antibody-binding site (*8*).

We previously proposed a method to overcome the problem of prolonged blood clearance and low tumor accumulation of radiolabeled dsFv in the presence of sIL-2Rα: blocking sIL-2Rα with humanized antiTac IgG (HuTac). In this study, we evaluated an alternate method based on avidin-biotin binding. The avidin-biotin system has been studied extensively as a way to optimize radioimmunodetection and radioimmunotherapy (*20–22*). A two-step method has been used to clear circulating antibody: A streptavidin-conjugated antibody is injected and then followed by a chase of a biotinylated compound that will result in antibody clearance by the reticuloendothelial system (*23*). Conversely, avidin itself works as a strong "chase" reagent for a biotinylated antibody (*24,25*). In this study, we modified this concept to clear sIL-2Rα antigen from the circulation by administering biotinylated HuTac (bt-HuTac), followed by an avidin chase before injection of radiolabeled dsFv.

MATERIALS AND METHODS

Biologic Reagents

We used a genetically engineered, dsFv fragment that binds to the IL-2Rα (*26*). This dsFv was derived from antiTac IgG2a, murine monoclonal antibody (*17*). Briefly, the V_H and V_L domains were expressed in separate *Escherichia coli* cultures and recovered as cytosolic inclusion bodies and refolded. The refolded antiTac dsFv was purified by ion-exchange and size-exclusion chromatography. The dsFvs were >98% pure as determined by size-exclusion high-performance liquid chromatography (HPLC) with an ultraviolet detector.

We also used HuTac monoclonal antibody that was constructed, as previously described, by combining the complementarity-determining regions of the murine antiTac antibody with the human IgG1κ framework and constant regions (*27*). HuTac was produced in a continuous-perfusion bioreactor from SP2/0 cells that had been transfected with the genes encoding the heavy and light chains of the hyperchimeric antibody and purified on an IL-2R affinity column. The eluted antibodies were further purified to contain >99% IgG.

sIL-2Rα (R&D Systems Inc., Minneapolis, MN) was used to mimic shedding of the receptor into the serum.

Biotinylation of Monoclonal Antibodies

The monoclonal antibodies were biotinylated as reported previously (*24*). Briefly, 5 mg/mL HuTac were mixed with sulfosuccinimidyl-6-(biotinamido) hexanoate (NHS-LC-biotin) (Pierce Chemical Co., Rockford, IL) in 0.05 mol/L phosphate-buffered saline (PBS) (pH 7.4) for 30 min at room temperature. The molar ratio of NHS-LC-biotin to antibody was 20:1. The biotinylated antibody was then separated from the unconjugated NHS-LC-biotin by PD-10 gel chromatography (Pharmacia, Uppsala, Sweden). To determine the number of biotin molecules per HuTac, we used the 2-(4′-hydroxyazobenzene)benzoic acid (HABA) method (*28*). The HABA reagent (Pierce Chemical Co.) was prepared according to the manufacturer's instructions by adding 10 mg avidin and 600 μL of 10 mmol/L HABA to 19.4 mL PBS. We added 100 μL bt-HuTac to 900 μL of the avidin-HABA solution and measured the absorbance at 500 nm. We determined that there were approximately 3.7–4.2 NHS-LC-biotin complexes conjugated to each antibody.

Avidin-Biotin Binding Assay of Radiolabeled Biotinylated Antibodies

To test the ability of biotinylated antibody to bind to avidin, a 100-μL aliquot of each batch of the radiolabeled biotinylated antibody (100,000 cpm/ 7–10 ng) was incubated with approximately 0.5 mL of avidin-Sepharose gel (Pierce Chemical Co.) for 10 min at room temperature. The gel was then washed with 0.05 mol/L PBS. We counted the radioactivity in the gel and in the supernatant and calculated the fraction of the radioactive biotinylated antibody bound to avidin (*29*). More than 88% of the antibodies had avidin-binding activity.

Radiolabeling

AntiTac dsFv and sIL-2Rα were labeled with ^{125}I using the Iodo-Gen method (*30*). Briefly, approximately 37–74 MBq $Na^{125}I$ were added to 100 μg dsFv or sIL-2Rα in 80 μL PBS, pH 7.2, in a conical polypropylene vial coated with 10 μg Iodo-Gen. After incubation for 10 min at room temperature, the radiolabeled product was purified using a PD-10 column (Pharmacia). The specific activities of the ^{125}I-labeled antiTac dsFv and ^{125}I-labeled sIL-2Rα were 370 and 444 MBq/mg, respectively. The radiochemical purity of ^{125}I-labeled antiTac dsFv was >98%, as confirmed by instant thin-layer chromatography (ITLC) and size-exclusion HPLC.

HuTac and bt-HuTac were labeled with ^{125}I using the chloramine-T method, as previously described (*24*). Briefly, bt-HuTac or HuTac (100 μg) in 0.05 mol/L phosphate buffer (PB) (pH 7.5) and 14.8 MBq ^{125}I were mixed with 12 μg chloramine-T, which had been dissolved in 0.05 mol/L PB. After reacting for 5 min, the radiolabeled products were purified using a PD-10 column. The specific activities of both ^{125}I-labeled HuTac preparations were approximately 129.5–159.1 MBq/mg, and their purity was >98%, as confirmed by ITLC and size-exclusion HPLC.

Binding Ability of ^{125}I-labeled sIL-2Rα to HuTac

The ability of ^{125}I-labeled sIL-2Rα to bind to HuTac was examined with size-exclusion HPLC. Briefly, a 100-fold molar excess of HuTac was mixed with ^{125}I-labeled sIL-2Rα and analyzed using a size-exclusion HPLC with a TSK G2000SW column (TosoHaas, Philadelphia, PA; 0.067 mol/L PBS with 0.1 mol/L KCl; pH 6.8; 0.5 mL/min) equipped with an online NaI gamma detector (γ RAM; IN/US Systems, Inc., Tampa, FL).

Greater than 90% of radioactivity in the ^{125}I-labeled sIL-2Rα fraction shifted into a higher-molecular-weight fraction (complexes).

Cell Lines and Animal Models

Tumor xenografts were generated with an IL-2Rα antigen-positive (SP2/Tac) cell line genetically developed (*31*). This line was generated by transfecting the gene that encodes for the IL-2Rα into the antigen-negative SP2/0 cells. The SP2/0 cells are of a nonimmunoglobulin-secreting murine myeloma line that does not express IL-2Rα (CRL 1581; American Type Culture Collection, Rockville, MD). The cells were provided by Dr. Thomas Waldmann of the Metabolism Branch of the National Cancer Institute. All cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (GIBCO Laboratories, Grand Island, NY) and 0.03% L-glutamine at 37°C in 5% CO_2.

Animal studies were performed under a protocol approved by the Institutional Animal Care and Use Committee. Female athymic nude mice (nu/nu) (5–7 wk old and 15–20 g), were obtained from Harlan Sprague-Dawley (Frederick, MD). Tumor xenografts were established in the mice by subcutaneous inoculation of 4×10^6 SP2/Tac. Experiments on tumor-bearing mice were performed 11–15 d after implantation, when the tumors weighed a mean of 1219 mg (512–2213 mg). Such tumors shed sIL-2Rα into the circulation in predictable amounts (*12,31*).

All mice were killed by CO_2 inhalation and exsanguinated by cardiac puncture before dissection. The organs were harvested, blot dried and weighed on an analytical balance, and the radioactivity was then counted in a gamma counter.

Immunoreactivity

Immunoreactivity was determined by use of a modification of the cell-binding assay of Lindmo et al. (*32*). Aliquots of the ^{125}I-labeled dsFv (1.0 ng), HuTac (5 ng) and bt-HuTac (4 ng) in 100 μL PBS were incubated for 2 h at 4°C with 2×10^6 to $\times 10^7$ of SP2/Tac and SP2/0 cells. The cell-bound radioactivity was separated by centrifugation and counted in a gamma counter. Nonspecific binding to the cells was examined under conditions of antibody excess (25 μg nonradiolabeled antiTac antibody). Immunoreactivity of ^{125}I-labeled antiTac dsFv, HuTac and bt-HuTac was >80%. Binding to the antigen-negative SP2/0 was <5%.

Amino Acid Preparations

To block renal accumulation of the radiolabeled dsFv, we coinfused L-lysine (*33,34*). A stock solution of lysine (300 mg/mL) was prepared in 0.1 mol/L PB, pH 7.5, using L-lysine monohydrochloride (Pierce Chemical Co.). ^{125}I-labeled antiTac dsFv was coinjected with 50 mg lysine at a final concentration of 250 mg/mL. Coinjection of lysine was done to prevent renal uptake and catabolism of the radiolabeled dsFv, which would have resulted in elevated low-molecular-weight catabolites of ^{125}I being reabsorbed into the blood, thus complicating our pharmacokinetic assessment (*33*).

Biodistribution of ^{125}I-labeled bt-HuTac and ^{125}I-labeled HuTac

We determined whether the addition of biotin to HuTac affected its biodistribution. Four groups of normal, athymic mice (n = 3 per group) and mice containing SP2/Tac xenografts (n = 3 per group; tumor weight 0.6–1.1 g) received intravenous injections of 111 kBq/5 μg either ^{125}I-labeled HuTac or ^{125}I-labeled bt-HuTac in 0.05 mol/L PBS/bovine serum albumin (BSA) in a final volume of 200 μL. Normal mice were killed 30 min after injection; tumor-bearing mice were killed 1 d after injection. All organs were removed and weighed, and the radioactivity was counted in a gamma counter. The data were expressed as percentage of injected dose per gram (% ID/g) of tissue. At kill, an aliquot of each mouse's serum was analyzed using the size-exclusion HPLC system and the avidin-Sepharose affinity gel, as mentioned above.

Timing of Avidin Injection as a Chase of ^{125}I-labeled bt-HuTac

We determined the importance of timing of avidin chase on the biodistribution of ^{125}I-labeled bt-HuTac. Three groups of normal, athymic mice (n = 3 per group) received intravenous injections of 111 kBq/5 μg ^{125}I-labeled bt-HuTac in 0.05 mol/L PBS/BSA in a final volume of 200 μL. All mice were killed at 30 min postinjection. Two of these groups were injected intravenously with 20 μg avidin, either at 20 min before kill (−20 min) or 5 min before kill (−5 min). The third group served as a control and did not receive avidin. Two additional groups of mice (n = 3 per group) received 111 kBq/5 μg ^{125}I-labeled HuTac. One group received ^{125}I-labeled HuTac followed by avidin 5 min before kill and the other was a control group (no avidin) that received ^{125}I-labeled HuTac alone. The data were expressed as %ID/g. Serum samples were obtained from these mice at the time of kill and analyzed by the following methods: (a) size-exclusion HPLC, (b) avidin-Sepharose affinity gel and (c) analysis of antibody-bound activity using 10% trichloroacetic acid (TCA) precipitation, in which 200 μL 10% TCA was added to 30 μL serum and centrifuged at 1000*g* for 5 min, with subsequent gamma counting of the pellet and supernatant (*29*).

Blood Clearance and Biodistribution of ^{125}I-labeled sIL-2Rα with HuTac or bt-HuTac Followed by Avidin Chase

We quantitated the effect of HuTac or bt-HuTac on ^{125}I-labeled sIL-2Rα in circulation and in tissues. Two groups of normal athymic mice (n = 4 per group) were injected with 500 ng (11 pmol)/148 kBq ^{125}I-labeled sIL-2Rα intravenously. After 10 min, one of the groups was injected with 20 μg (133 pmol) HuTac; the other group received 20 μg (133 pmol) bt-HuTac, followed by 60 μg (920 pmol) avidin intravenously 25 min after the injection of ^{125}I-labeled sIL-2Rα. Serial blood sampling from the tail vein was performed at 10, 25, 30, 45, 90 and 180 min after administration of ^{125}I-labeled sIL-2Rα using calibrated 5-μL micropipets. The data were expressed as %ID/g blood.

Two groups of four normal athymic mice were injected with 250 ng (6 pmol)/74 kBq ^{125}I-labeled sIL-2Rα intravenously and then injected with either HuTac or bt-HuTac plus avidin, as above. These mice were killed 45 min after administration of ^{125}I-labeled sIL-2Rα and biodistribution studies were performed. The data were expressed as %ID/g. In addition, serum samples at the time of kill were analyzed with size-exclusion HPLC.

Biodistribution of ^{125}I-labeled AntiTac dsFv with Preinjection of HuTac or bt-HuTac

One group of four mice bearing SP2/Tac tumor xenografts that weighed approximately 1 g received intravenous preinjections of 20 μg (133 pmol) HuTac, followed by 185 kBq/300 ng ^{125}I-labeled antiTac dsFv with 50 mg L-lysine at 15, 30, 60 or 180 min after administration of HuTac. The mice had approximately 90 ng (2 pmol) /mL sIL-2Rα in their serum (*12*). Another group of four mice received intravenous preinjection of 20 μg (133 pmol) bt-HuTac followed by 60 μg (920 pmol) intravenous avidin 10 min later. These mice then received 185 kBq/300 ng ^{125}I-labeled antiTac dsFv

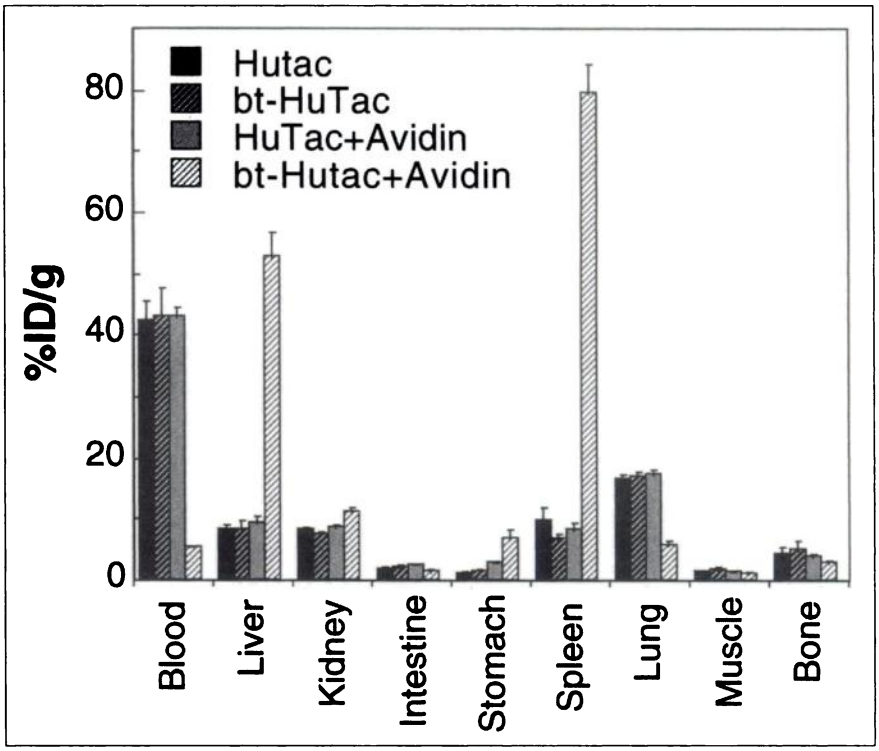

FIGURE 1. Biodistribution of HuTac or bt-HuTac in normal mice: effect of avidin chase. Biodistribution of 5 μg/111 kBq ^{125}I-labeled HuTac alone, ^{125}I-labeled bt-HuTac alone, ^{125}I-labeled HuTac or ^{125}I-labeled bt-HuTac followed by 20-μg avidin chase 10 min after antibody injection (20 min before kill) was plotted. Three normal nude mice per group were killed at 30 min postinjection of antibody. Values are mean ± SD.

with 50 mg L-lysine at 15, 30, 60 or 180 min after administration of bt-HuTac. All mice were killed 15 min after injection of ^{125}I-labeled antiTac dsFv, and biodistribution studies were performed. Data were expressed both as %ID/g and as tumor-to-normal tissue ratios. In addition, serum samples were collected and analyzed with size-exclusion HPLC. We then calculated the percentage of the injected dose present in the high-molecular-weight fraction (complex), the dsFv fraction and the low-molecular (catabolite) fraction.

RESULTS

Biodistribution of ^{125}I-labeled bt-HuTac and HuTac

Biotinylation did not adversely affect the biodistribution of HuTac. There were no significant differences in the biodistribution of ^{125}I-labeled HuTac and ^{125}I-labeled bt-HuTac in normal athymic mice (Fig. 1) or in SP2/Tac tumor-bearing mice (Fig. 2). An avidin chase was effective

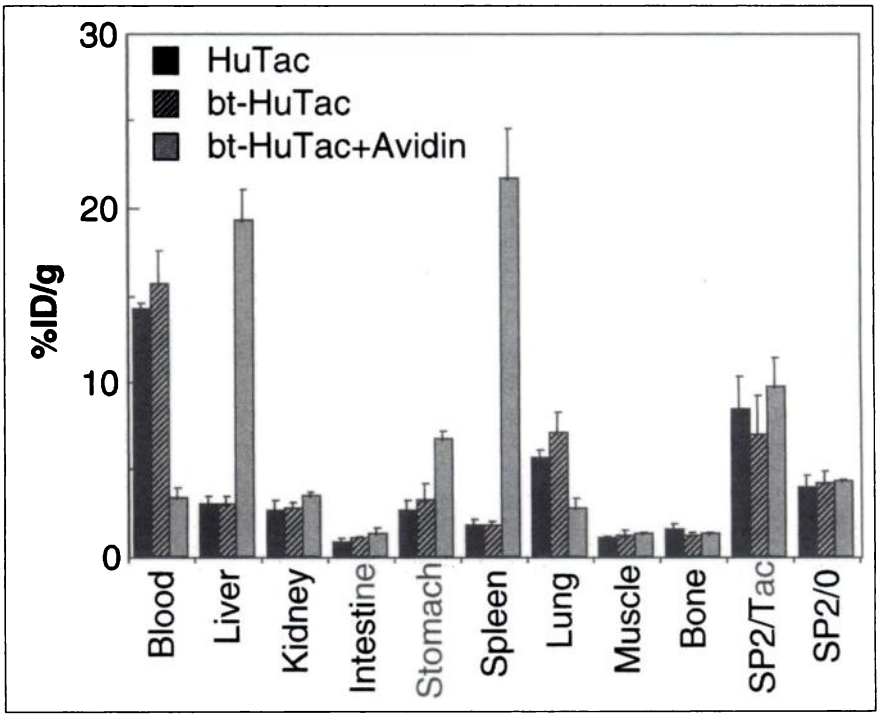

FIGURE 2. Biodistribution of HuTac or bt-HuTac in tumor-bearing mice: effect of avidin chase. Biodistribution of 5 μg/111 kBq ^{125}I-labeled HuTac alone, ^{125}I-labeled bt-HuTac alone and ^{125}I-labeled bt-HuTac followed by 20 μg avidin chase 23 h 40 min after antibody injection (−20 min) was plotted. Three mice bearing SP2/Tac and SP2/0 tumors were killed per group at 24 h postinjection of radiolabeled antibody. Values are mean ± SD.

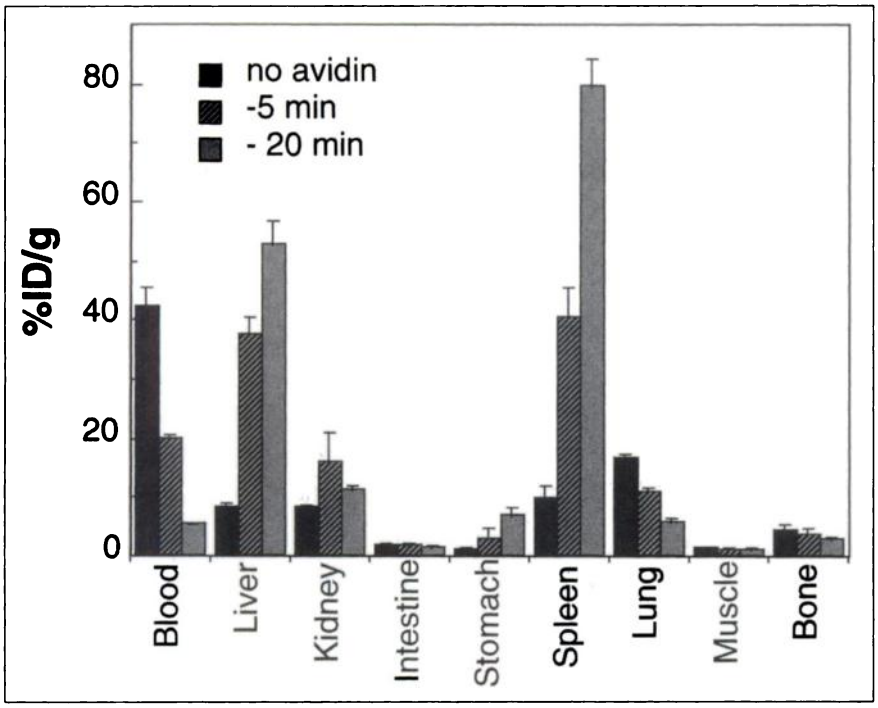

FIGURE 3. Plot of biodistribution of 5 μg/111 kBq ^{125}I-labeled bt-HuTac alone or ^{125}I-labeled bt-HuTac followed by 20-μg avidin injection at 5 min or 20 min before kill. Three normal nude mice per group were killed 30 min after injection of ^{125}I-labeled bt-HuTac. Values are mean ± SD.

in clearing the ^{125}I-labeled bt-HuTac from the circulation, but it did not affect the ^{125}I-labeled HuTac (Fig. 1). The serum of normal mice analyzed with size-exclusion HPLC showed that most of the radioactivity was in the intact IgG fraction in mice receiving the HuTac or bt-HuTac (data not shown).

Timing of Avidin Injection

The clearance from the blood of ^{125}I-labeled bt-HuTac was greater when more time elapsed between avidin administration and kill. When the avidin chase was injected 5 min before kill, the ^{125}I-labeled bt-HuTac level in the blood dropped to 47% that of the control group, compared with 12% of the control when the avidin chase was given 20 min before kill (Fig. 3).

When HuTac or bt-HuTac was administered without an avidin chase, most of the injected radioactivity was in the circulation and was protein bound (precipitable) 30 min after injection (Table 1). As expected, ^{125}I-labeled HuTac did not have significant binding to avidin, because only a mean of 3.8% of the circulating activity was bound to avidin beads (probably represented nonspecific binding) compared with a mean of 3.6% when avidin was infused 5 min before kill (Table 2). When avidin was administered 5 min before kill in animals receiving ^{125}I-labeled bt-HuTac, the amount of ^{125}I remaining in the blood at the time of kill was 17 %ID/g, which represented 41 %ID/g of the controls (Table 1). In contrast, when more than 20 min elapsed between avidin chase and kill, the amount of bt-HuTac (precipitable ^{125}I) remaining in the serum was much lower than that of the −5 min chase. Values were 3.1 versus 17 %ID/g, respectively. Compared with the no-avidin control, in which 95% of the ^{125}I was precipitable, in the mice receiving the avidin chase at −5 min only 83% of the circulating ^{125}I was precipitable, and 69.3% was bound to avidin beads.

Blood Clearance and Biodistribution of ^{125}I-labeled sIL-2Rα with HuTac or bt-HuTac Followed by Avidin Chase

As previously described (*35,36*), ^{125}I-labeled sIL-2Rα survived longer in the circulation in the presence of HuTac than in the no-HuTac control mice. The amount of ^{125}I-

TABLE 1
Amount of Precipitable ^{125}I Present in Serum After Administration of ^{125}I-labeled HuTac or Biotinylated HuTac

	HuTac	bt-HuTac	bt-HuTac	HuTac	bt-HuTac
Time of avidin injection*	—	—	−5 min	−5 min	−20 min
Percent precipitable ^{125}I (mean ± SD)	96.5 ± 1.0	95.2 ± 0.9	82.5 ± 1.5	95.5 ± 1.2	60.5 ± 5.4
%ID/g of precipitable ^{125}I in whole blood	41.4 ± 4.8	40.2 ± 3.4	16.5 ± 0.6	41.0 ± 1.2	3.1 ± 0.3

*Time of avidin injection represents time before mice were killed.
HuTac = humanized antiTac; bt = biotinylated; %ID/g = percentage of injected dose per gram.
All mice were killed 30 min after antibody injection.

TABLE 2
Amount of ^{125}I in Serum Capable of Binding to Avidin Gel After Administration of ^{125}I-labeled HuTac or Biotinylated HuTac

	HuTac	bt-HuTac	bt-HuTac	HuTac	bt-HuTac
Time of avidin injection*	—	—	−5 min	−5 min	−20 min
Percent circulating ^{125}I bound to avidin-gel (mean ± SD)	3.8 ± 1.1	83.0 ± 1.0	69.3 ± 2.1	3.6 ± 1.1	30.3 ± 2.2
%ID/g whole blood of ^{125}I bound to avidin gel	1.6 ± 0.5	35.0 ± 2.8	13.9 ± 0.6	1.6 ± 0.5	1.6 ± 0.1

*Time of avidin injection represents time before mice were killed.
HuTac = humanized antiTac; bt = biotinylated; %ID/g = percentage of injected dose per gram.
All mice were killed 30 min after antibody injection.

labeled sIL-2Rα circulating in the blood at 180 min after injection was 32 %ID/g in the group receiving HuTac versus 16 %ID/g in the control group (Fig. 4). The group receiving bt-HuTac followed by the avidin chase 15 min afterward showed very rapid clearance of ^{125}I-labeled sIL-2Rα from the blood (5.0 %ID/g) (Fig. 4). The activity that cleared the blood accumulated predominantly in the liver and the spleen (Fig. 5).

HPLC analysis of the serum obtained from animals receiving HuTac after injection of ^{125}I-labeled sIL-2Rα showed 27 %ID/g of ^{125}I to be present in complexes; this represented 73% of the circulating activity. In the animals receiving the bt-HuTac and avidin chase, there was 3.6 %ID/g of the ^{125}I present in complexes and this represented 35% of the remaining activity in serum. In mice receiving ^{125}I-labeled sIL-2Rα alone, 7.3 %ID/g was present in complexes, and this represented 31% of the remaining activity in serum. The amount of ^{125}I present as intact IL-2Rα was least in the mice receiving the avidin chase and greatest in those receiving sIL-2Rα alone (Fig. 6).

Biodistribution of ^{125}I-labeled AntiTac dsFv with Preinjection of HuTac or bt-HuTac Followed by Avidin Chase

The biodistribution of ^{125}I-labeled antiTac dsFv in tumor-bearing mice that had elevated sIL-2Rα levels was evaluated in the presence or absence of a blocking dose of HuTac or a clearing dose of bt-HuTac with avidin. The biodistribution of ^{125}I-labeled antiTac dsFv in mice preinjected with a blocking dose of 20 μg HuTac 15, 30, 60 and 180 min before injection of ^{125}I-labeled antiTac dsFv was determined and compared with the biodistribution in no-preinjection controls (Fig. 7). The biodistribution of ^{125}I-labeled antiTac dsFv in mice preinjected with 20 μg bt-HuTac 15, 30, 60 and 180 min before dsFv injection, followed by a 60-μg avidin chase 15 min later are shown in Figure 8.

Administration of HuTac or bt-HuTac plus avidin chase improved the biodistribution of ^{125}I-labeled antiTac dsFv over the no HuTac (control) group. In groups receiving HuTac or bt-HuTac plus chase, the shorter the interval between

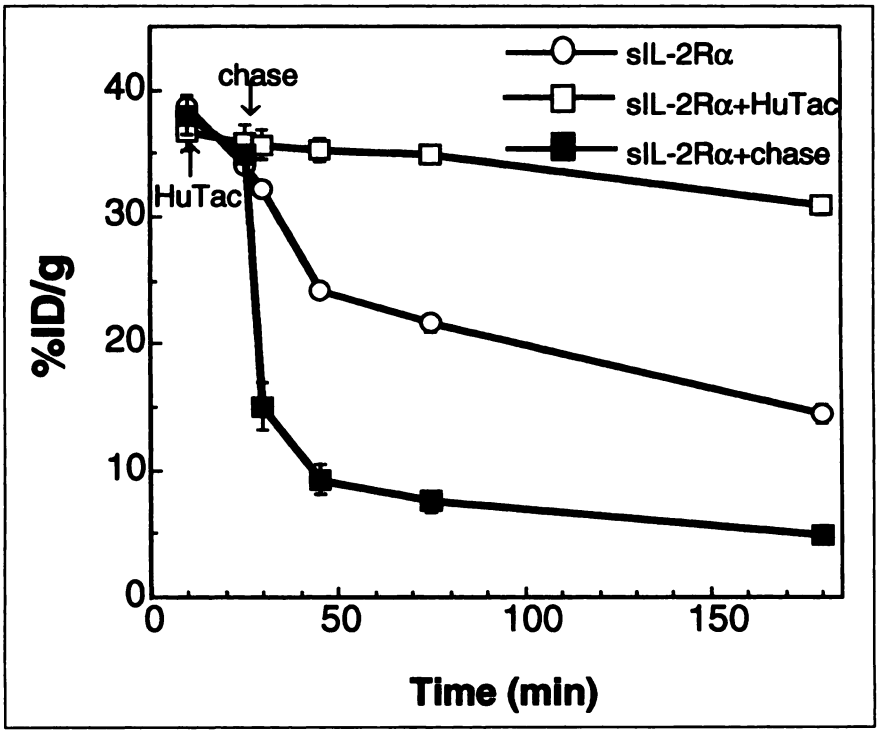

FIGURE 4. Plot of blood clearance of ^{125}I-labeled sIL-2Rα in four normal nude mice 10, 25, 30, 45, 75 and 180 min after injection. Mice received ^{125}I-labeled sIL-2Rα alone (○), followed 10 min later by injection of 20 μg HuTac (□) or followed 10 min later by 20 μg bt-HuTac and then 25 min later 60-μg avidin chase (■). Data are expressed as %ID/g of blood. Values are mean ± SD.

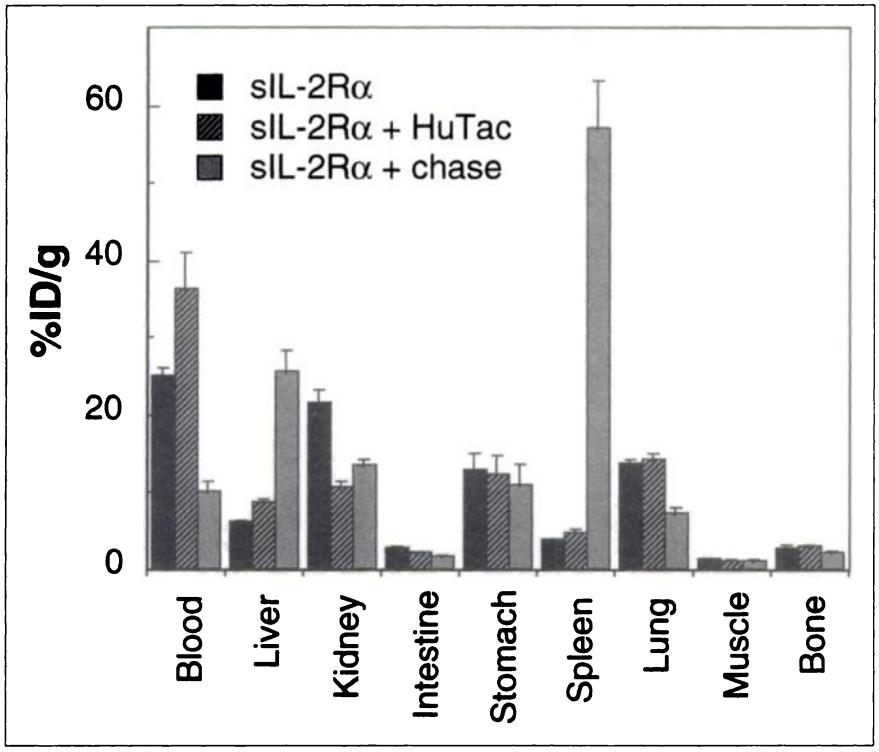

FIGURE 5. Plot of biodistribution of ^{125}I-labeled sIL-2Rα in four normal nude mice per group killed 45 min after injection. Three groups of four mice each received one of the following: ^{125}I-labeled sIL-2Rα alone, ^{125}I-labeled sIL-2Rα followed 10 min later by injection of 20 μg HuTac or ^{125}I-labeled sIL-2Rα followed 10 min later by 20 μg bt-HuTac and 25 min later by 60-μg avidin chase. Data are expressed as %ID/g of blood. Values are mean ± SD.

these antibody injections and the ^{125}I-labeled antiTac dsFv injection, the better biodistribution of ^{125}I-labeled antiTac dsFv. At the shortest time intervals (15 min), the biodistribution was similar with the two methods. The longer the interval between giving the HuTac and bt-HuTac plus chase and ^{125}I-labeled antiTac dsFv injection, the more similar was the biodistribution to the control group. The decreased benefit of the longer intervals was more prominent for the bt-HuTac-plus-chase group than for the HuTac group (Figs. 7 and 8).

HPLC from these mice showed that, when no HuTac or bt-HuTac was preinjected, ^{125}I-labeled antiTac dsFv was predominantly found in complexes. In contrast, when either HuTac or bt-HuTac was preinjected, there was a decrease in the amount of complexes of sIL-2Rα and ^{125}I-labeled antiTac dsFv. The shorter the interval between the HuTac or the bt-HuTac plus avidin chase and dsFv injection, the fewer complexes found in serum. (Figs. 9 and 10).

The tumor-to-normal tissue ratios of mice preinjected with HuTac or bt-HuTac plus chase 15 min before ^{125}I-

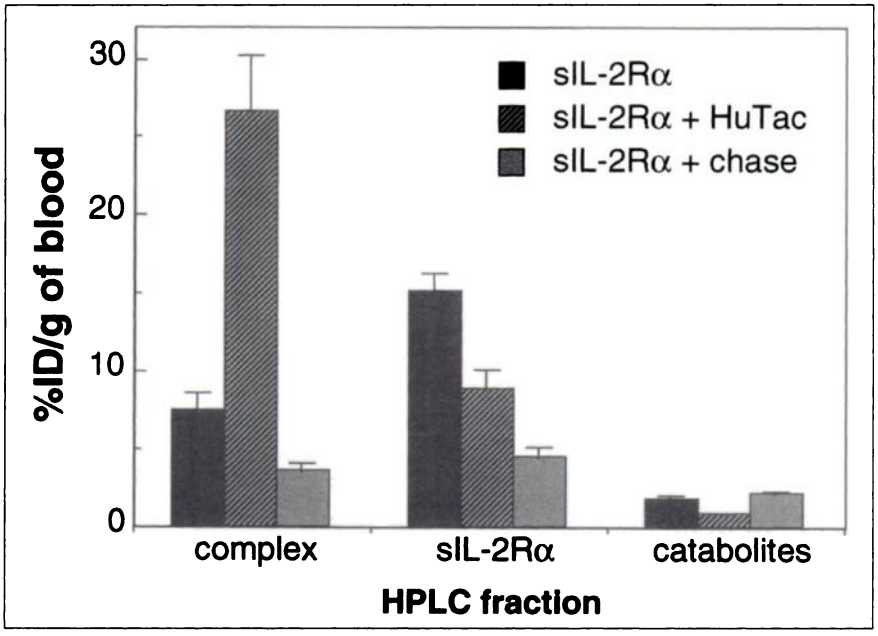

FIGURE 6. Serum from animals described in Figure 5 was obtained for HPLC analyses. Amount of radioactivity in each fraction was plotted for mice receiving ^{125}I-labeled sIL-2Rα or postinjected with HuTac or bt-HuTac plus avidin. Serum was obtained 45 min after sIL-2Rα injection just after kill. Data are expressed as %ID/g of blood of different HPLC fractions. Values are mean ± SD.

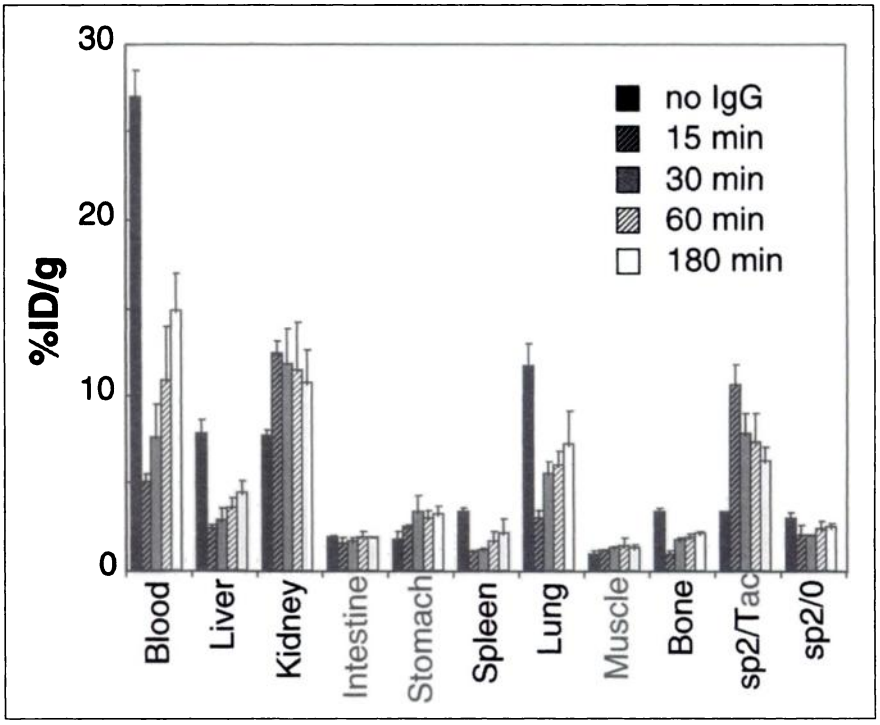

FIGURE 7. Biodistribution of ^{125}I-labeled antiTac dsFv in mice bearing SP2/Tac and SP2/0 tumors was determined. ^{125}I-labeled antiTac dsFv was injected at 15, 30, 60 and 180 min after injection of 20 μg HuTac. Control group of mice was injected with ^{125}I-labeled antiTac dsFv alone. Mice were then killed 15 min after ^{125}I-labeled antiTac dsFv injection; organ biodistribution is shown. n = 4; values are mean ± SD.

labeled antiTac dsFv injection were much higher than the controls, with the exception of tumor-to-kidney ratios (Tables 3 and 4).

DISCUSSION

Circulating antigen may affect the biodistribution of radiolabeled biologic reagents (*7,9,37*). The major advantage of genetically engineered antibody fragments and peptides is their small size, which results in rapid tissue penetration and high tumor-to-nontumor ratios (*38*). However, when these reagents are used in the presence of circulating antigen, the formation of complexes may result in altered biodistribution and longer circulation times (*12*). These findings are analogous to the prolonged circulation of insulin in the presence of anti-insulin antibody (*13*), or to that of insulin-like growth factor in the presence of insulin-

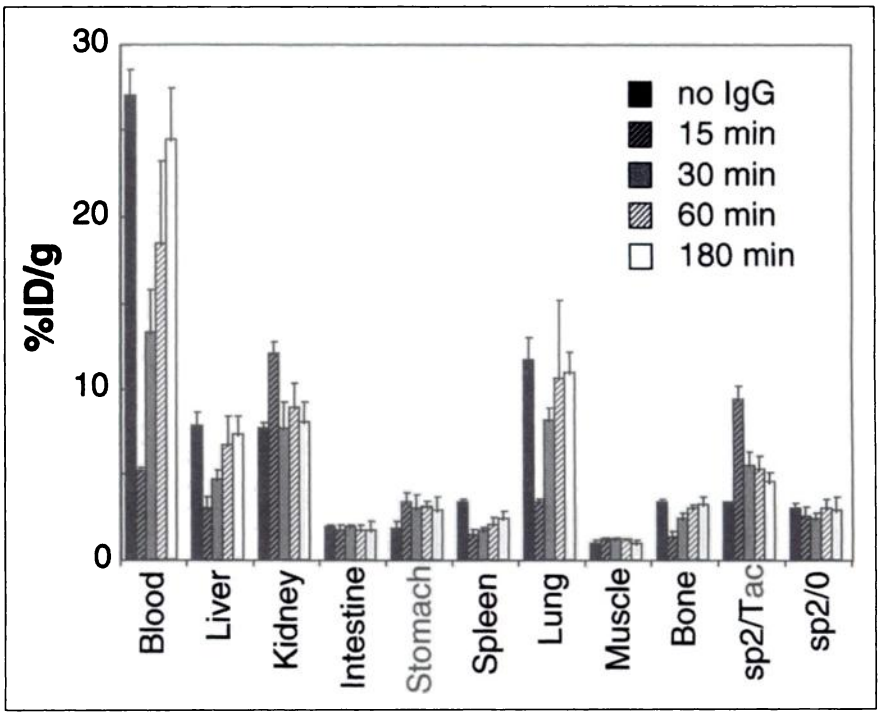

FIGURE 8. Biodistribution of ^{125}I-labeled antiTac dsFv in mice bearing SP2/Tac and SP2/0 tumors was determined. Mice were injected with 20 μg bt-HuTac followed 10 min later by 60-μg avidin chase. Then, ^{125}I-labeled antiTac dsFv was injected at 15, 30, 60 and 180 min after injection of bt-HuTac. Control group of mice was injected with ^{125}I-labeled antiTac dsFv alone. Mice were killed 15 min after ^{125}I-labeled antiTac dsFv, and organ biodistribution was determined. n = 4; values are mean ± SD.

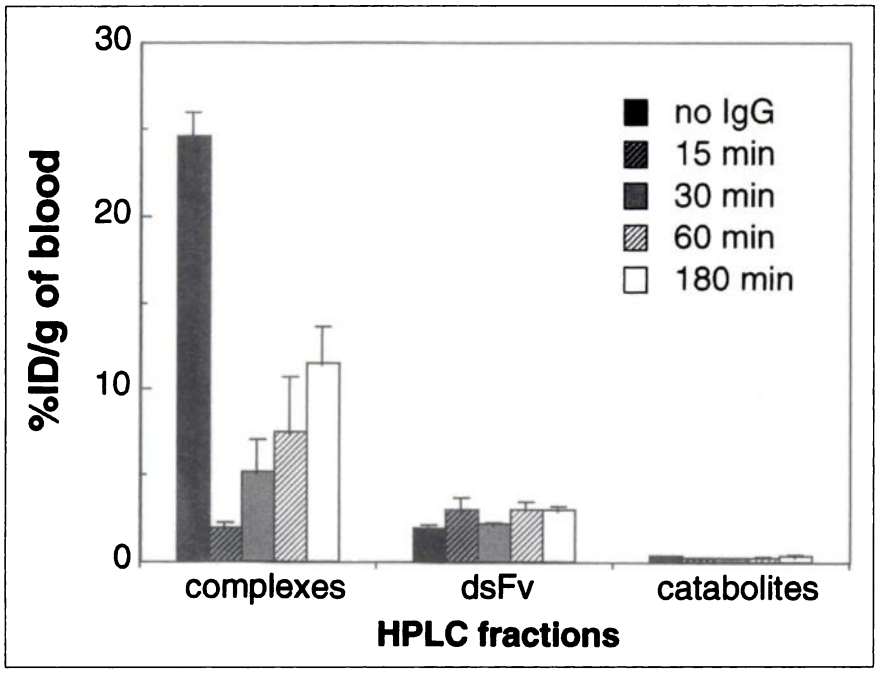

FIGURE 9. Serum from animals described in Figure 7 was obtained for HPLC analyses. Amount of radioactivity in each fraction was plotted for mice that received ^{125}I-labeled antiTac dsFv alone or preinjection of 20 μg HuTac 15, 30, 60 and 180 min before injection of ^{125}I-labeled antiTac dsFv. Serum was obtained 15 min after dsFv injection just after kill. Data are expressed as %ID/g of blood in different HPLC fractions. n = 4; values are mean ± SD.

like growth factor binding proteins (*39*). We have previously shown that when antiTac dsFv is injected in mice with SP2/Tac tumors, the circulating antigen reacts with the dsFv, forming complexes that decrease the clearance rate from the circulation, lower tumor uptake and lower the tumor-to-nontumor ratios (*12*).

When considering dsFv for radioimmunodetection in patients, it would be helpful to overcome the negative effect of circulating antigen to optimize targeting. Our previous animal studies showed that preinjection of intact HuTac, which competes with the dsFv for the same epitope on sIL-2Rα, favorably affects the biodistribution of dsFv (*12*). This strategy was successful because intact antibody is a larger molecule that requires longer to penetrate into tissues than does dsFv, which has rapid tissue kinetics. Therefore, if dsFv is administered shortly after the intact antibody, the binding sites of sIL-2Rα will be blocked, allowing dsFv to enter the tissues. In this study, we compared this blocking technique with the new approach of attempting to clear the sIL-2Rα from the circulation.

Most of the previous studies in the literature using the avidin-biotin method have attempted to clear the biotin-

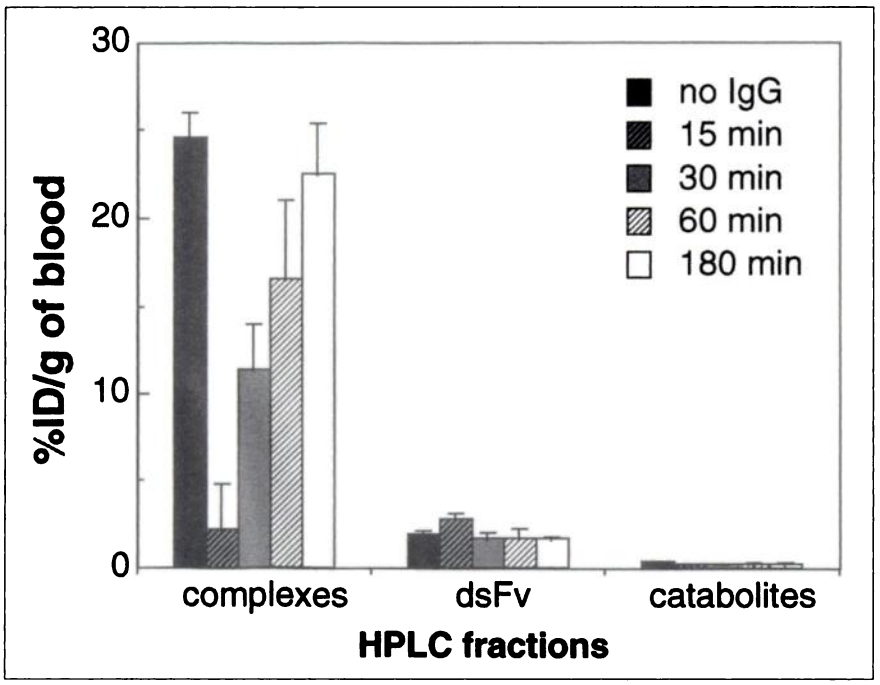

FIGURE 10. Serum from animals described in Figure 8 was obtained for HPLC analyses. Amount of radioactivity in each fraction was plotted for mice that received ^{125}I-labeled antiTac dsFv alone or preinjection of 20 μg bt-HuTac 15, 30, 60 and 180 min before injection of ^{125}I-labeled antiTac dsFv, followed by injection of 60-μg avidin chase. Serum was obtained 15 min after dsFv injection just after kill. Data are expressed as %ID/g of blood of different HPLC fractions. n = 4; mean ± SD.

TABLE 3
SP2/Tac Tumor-to-Normal Tissue Ratio in Tumor-Bearing Mice Preinjected with 20 μg HuTac at Various Intervals Before Injection of ^{125}I-labeled AntiTac dsFv

	No IgG	15 min	30 min	60 min	180 min
Blood	0.12 ± 0.01	2.07 ± 0.26	1.08 ± 0.24	0.75 ± 0.35	0.42 ± 0.09
Liver	0.42 ± 0.04	4.50 ± 0.45	2.77 ± 0.57	2.05 ± 0.54	1.43 ± 0.35
Kidney	0.43 ± 0.03	0.86 ± 0.15	0.94 ± 0.31	0.65 ± 0.05	0.50 ± 0.13
Intestine	1.75 ± 0.10	6.86 ± 1.41	4.69 ± 0.75	3.76 ± 0.86	3.28 ± 0.29
Spleen	0.98 ± 0.05	9.96 ± 2.03	6.34 ± 0.53	4.58 ± 1.31	3.27 ± 1.14
Lung	0.29 ± 0.04	3.59 ± 0.34	1.41 ± 0.11	1.21 ± 0.24	0.91 ± 0.24
Muscle	3.40 ± 0.74	8.82 ± 0.94	6.56 ± 1.00	5.18 ± 1.48	4.62 ± 0.62
SP2/0 tumor	1.12 ± 0.13	5.22 ± 0.90	3.92 ± 0.64	3.05 ± 0.66	2.47 ± 0.40
SP2/Tac tumor weight (g)	1.24 ± 0.34	1.09 ± 0.28	1.34 ± 0.67	1.18 ± 0.64	1.16 ± 0.52

Values are mean ± SD.

labeled protein (*29*). We were primarily interested in clearing the antigen associated with the biotin-labeled HuTac. This study showed that biotinylation did not adversely affect the circulation kinetics of HuTac. We also showed that bt-HuTac efficiently bound to circulating sIL-2Rα. When an avidin chase was administered, this method cleared >90% of the sIL-2Rα within 20 min.

Although the method was effective in clearing sIL-2Rα associated with bt-HuTac from the circulation, our studies showed that this method has some disadvantages compared with the blocking technique of HuTac preinjection in that timing was crucial to obtaining the optimal biodistribution of dsFv. Short times between the avidin chase and administration of dsFv resulted in the best biodistribution. As more time elapsed between avidin chase and the ^{125}I-labeled dsFv, the favorable effect on biodistribution decreased. This time dependence is likely related to reaccumulation of sIL-2Rα in the blood, most likely from redistribution from the extravascular space as well as from new synthesis and release from the tumor (*36*). The half-life of transfer from extravascular tissue into the blood is approximately 3–4 h. Therefore, new synthesis and release of sIL-2Rα must be rapid to maintain its serum concentration, because the half-life of sIL-2Rα is approximately 40 min (*36*). Newly reaccumulated sIL-2Rα in the circulation will bind the injected dsFv. In contrast to the avidin chase, after which bt-HuTac is effectively cleared from the blood and is no longer available to bind any reaccumulating sIL-2Rα, HuTac alone circulates longer and therefore can bind any reaccumulated sIL-2Rα.

CONCLUSION

We have demonstrated two methods that can be used to minimize the adverse effect of circulating antigen on the biodistribution of a low-molecular-weight antibody fragment: a blocking method using intact antibody and a method using a biotinylated antibody plus avidin chase, which clears the circulating antigen. However, rapid reaccumulation of antigen in the circulation makes this more complicated than the blocking technique. In addition, the avidin may evoke an immune response (*40*). Thus, in this system, we conclude that the blocking method is more favorable than the avidin-clearance method.

ACKOWLEDGEMENT

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TABLE 4
SP2/Tac Tumor-to-Normal Tissue Ratio in Tumor-bearing Mice Preinjected with 20 μg Biotinylated (bt) HuTac Followed by 60-μg Avidin Chase 10 Minutes After Injection of bt-HuTac at Various Intervals Before Injection of ^{125}I-labeled AntiTac dsFv

	No IgG	15 min	30 min	60 min	180 min
Blood	0.12 ± 0.01	1.83 ± 0.26	0.43 ± 0.11	0.31 ± 0.12	0.15 ± 0.03
Liver	0.42 ± 0.04	3.20 ± 0.82	1.21 ± 0.27	0.83 ± 0.28	0.49 ± 0.10
Kidney	0.43 ± 0.03	0.78 ± 0.08	0.72 ± 0.14	0.61 ± 0.19	0.59 ± 0.13
Intestine	1.75 ± 0.10	5.64 ± 0.98	2.97 ± 0.69	3.14 ± 0.84	2.29 ± 0.65
Spleen	0.98 ± 0.05	6.59 ± 1.78	3.22 ± 0.73	2.72 ± 0.87	1.51 ± 0.06
Lung	0.29 ± 0.04	2.83 ± 0.26	0.68 ± 0.15	0.58 ± 0.28	0.32 ± 0.05
Muscle	3.40 ± 0.74	7.74 ± 0.48	4.40 ± 0.68	4.49 ± 0.78	3.81 ± 0.83
SP2/0 tumor	1.12 ± 0.13	3.59 ± 1.20	2.39 ± 0.54	1.84 ± 0.53	1.25 ± 0.22
SP2/Tac tumor weight (g)	1.24 ± 0.34	1.12 ± 0.31	1.32 ± 0.52	1.16 ± 0.51	1.28 ± 0.33

Values are mean ± SD.

REFERENCES

1. Carrasquillo JA. Radioimmunotherapy of leukemia and lymphoma. In: Wagner H, ed. *Principles of Nuclear Medicine*. 2nd ed. Philadelphia, PA: WB Saunders;1996: 1117–1132.
2. Larson SM, Divgi CR, Scott AM. Overview of clinical radioimmunodetection of human tumors. *Cancer.* 1994;73:832–835.
3. Gold P, Freedman SO. Specific carcinoembryonic antigens of the human digestive system. *J Exp Med.* 1965;122:467–481.
4. Johnson VG, Schlom J, Paterson AJ, Bennett J, Magnani JL, Colcher D. Analysis of a human tumor-associated glycoprotein (TAG-72) identified by monoclonal antibody B72.3. *Cancer Res.* 1986;46:850–857.
5. Rubin LA, Nelson DL. The soluble interleukin-2 receptor: biology, function, and clinical application. *Ann Intern Med.* 1990;113:619–627.
6. Junghans RP, Carrasquillo JA, Waldmann TA. Impact of antigenemia on the bioactivity of infused anti-Tac antibody: implications for dose selection in antibody immunotherapies. *Proc Natl Acad Sci USA.* 1998;95:1752–1757.
7. Pimm MV. Circulating antigen: bad or good for immunoscintigraphy? *Nucl Med Biol.* 1995;22:137–145.
8. Waldmann TA, White JD, Carrasquillo JA, et al. Radioimmunotherapy of interleukin-2R alpha-expressing adult T-cell leukemia with yttrium-90-labeled anti-Tac. *Blood.* 1995;86:4063–4075.
9. Parker BA, Vassos AB, Halpern SE, et al. Radioimmunotherapy of human B-cell lymphoma with ^{90}Y-conjugated antiidiotype monoclonal antibody. *Cancer Res.* 1990;50:1022s–1028s.
10. Huston JS, Mudgett-Hunter M, Tai MS, et al. Protein engineering of single-chain Fv analogs and fusion proteins. *Methods Enzymol.* 1991;203:46–88.
11. Webber KO, Kreitman RJ, Pastan I. Rapid and specific uptake of anti-Tac disulfide-stabilized Fv by interleukin-2 receptor-bearing tumors. *Cancer Res.* 1995;55:318–323.
12. Kobayashi H, Yoo TM, Drumm D, et al. Improved biodistribution of ^{125}I-labeled anti-Tac disulfide-stabilized Fv fragment by blocking its binding to the α subunit of interleukin-2 receptor in the circulation with preinjected humanized anti-Tac IgG. *Cancer Res.* 1997;57:1955–1961.
13. Berson SA, Yalow RS. Insulin in blood and insulin antibodies. *Am J Med.* 1966;40:676–690.
14. Waldmann TA, Pastan IH, Gansow OA, Junghans RP. The multichain interleukin-2 receptor: a target for immunotherapy. *Ann Intern Med.* 1992;116:148–160.
15. Wolf RE, Brelsford WG. Soluble interleukin-2 receptors in systemic lupus erythematosus. *Arthritis Rheum.* 1988;31:729–735.
16. Nakanishi K, Taniguchi Y, Ohta Y. Increased soluble interleukin 2 receptor levels in autoimmune thyroid disease. *Acta Endocrinol (Copenh).* 1991;125:253–258.
17. Uchiyama T, Broder S, Waldmann TA. A monoclonal antibody (anti-Tac) reactive with activated and functionally mature human T cells. I. Production of anti-Tac monoclonal antibody and distribution of Tac (+) cells. *J Immunol.* 1981;126:1393–1397.
18. Parenteau GL, Dirbas FM, Garmestani K, et al. Prolongation of graft survival in primate allograft transplantation by yttrium-90-labeled anti-Tac in conjunction with granulocyte colony-stimulating factor. *Transplantation.* 1992;54:963–968.
19. Vincenti F, Kirkman R, Light S, et al. Interleukin-2-receptor blockade with daclizumab to prevent acute rejection in renal transplantation. Daclizumab Triple Therapy Study Group. *N Engl J Med.* 1998;338:161–165.
20. Hnatowich DJ, Virzi F, Rusckowski M. Investigations of avidin and biotin for imaging applications. *J Nucl Med.* 1987;28:1294–1302.
21. Goodwin DA, Meares CF. Pretargeting: general principles; October 10–12, 1996. *Cancer.* 1997;80:2675–2680.
22. Casalini P, Luison E, Menard S, Colnaghi MI, Paganelli G, Canevari S. Tumor pretargeting: role of avidin/streptavidin on monoclonal antibody internalization. *J Nucl Med.* 1997;38:1378–1381.
23. Sharkey RM, Karacay H, Griffiths GL, et al. Development of a streptavidin-anti-carcinoembryonic antigen antibody, radiolabeled biotin pretargeting method for radioimmunotherapy of colorectal cancer. Studies in a human colon cancer xenograft model. *Bioconjug Chem.* 1997;8:595–604.
24. Kobayashi H, Sakahara H, Hosono M, et al. Improved clearance of radiolabeled biotinylated monoclonal antibody following the infusion of avidin as a chase without decreased accumulation in the target tumor. *J Nucl Med.* 1994;35:1677–1684.
25. Paganelli G, Stella M, Zito F, et al. Radioimmunoguided surgery using iodine-125-labeled biotinylated monoclonal antibodies and cold avidin. *J Nucl Med.* 1994;35:1970–1975.
26. Webber KO, Reiter Y, Brinkmann U, Kreitman R, Pastan I. Preparation and characterization of a disulfide-stabilized Fv fragment of the anti-Tac antibody: comparison with its single-chain analog. *Mol Immunol.* 1995;32:249–258.
27. Queen C, Schneider WP, Selick HE, et al. A humanized antibody that binds to the interleukin 2 receptor. *Proc Natl Acad Sci USA.* 1989;86:10029–10033.
28. Green NM. Avidin. *Adv Protein Chem.* 1975;29:85–133.
29. Kobayashi H, Sakahara H, Endo K, Yao ZS, Toyama S, Konishi J. Repeating the avidin chase markedly improved the biodistribution of radiolabelled biotinylated antibodies and promoted the excretion of additional background radioactivity. *Eur J Cancer.* 1995;31A:1689–1696.
30. Fraker PJ, Speck JC Jr. Protein and cell membrane iodinations with a sparingly soluble chloroamide, 1,3,4,6-tetrachloro-3α,6α-diphrenylglycoluril. *Biochem Biophys Res Commun.* 1978;80:849–857.
31. Hartmann F, Horak EM, Garmestani K, et al. Radioimmunotherapy of nude mice bearing a human interleukin 2 receptor alpha-expressing lymphoma utilizing the alpha-emitting radionuclide-conjugated monoclonal antibody ^{212}Bi-anti-Tac. *Cancer Res.* 1994;54:4362–4370.
32. Lindmo T, Boven E, Cuttitta F, Fedorko J, Bunn PA Jr. Determination of the immunoreactive fraction of radiolabeled monoclonal antibodies by linear extrapolation to binding at infinite antigen excess. *J Immunol Methods.* 1984;72:77–89.
33. Kobayashi H, Yoo TM, Kim IS, et al. L-lysine effectively blocks renal uptake of ^{125}I- or ^{99m}Tc-labeled anti-Tac dsFv. *Cancer Res.* 1996;56:3788–3795.
34. Behr TM, Sharkey RM, Juweid ME, et al. Reduction of the renal uptake of radiolabeled monoclonal antibody fragments by cationic amino acids and their derivatives. *Cancer Res.* 1995;55:3825–3834.
35. Kobayashi H, Sun BF, Han ES, et al. Epitope blocking: positive and negative effects on the biodistribution of ^{125}I-labeled anti-Tac disulfide-stabilized Fv fragment of two antibodies against different epitopes of the circulating antigen. *Jpn J Cancer Res.* 1998;89:436–444.
36. Junghans RP, Waldmann TA. Metabolism of Tac (IL2Rα): physiology of cell surface shedding and renal catabolism, and suppression of catabolism by antibody binding. *J Exp Med.* 1996;183:1587–1602.
37. Yu B, Carrasquillo J, Milenic D, et al. Phase I trial of iodine 131-labeled COL-1 in patients with gastrointestinal malignancies: influence of serum carcinoembryonic antigen and tumor bulk on pharmacokinetics. *J Clin Oncol.* 1996;14:1798–1809.
38. Yokota T, Milenic DE, Whitlow M, Schlom J. Rapid tumor penetration of a single-chain Fv and comparison with other immunoglobulin forms. *Cancer Res.* 1992;52:3402–3408.
39. Sun BF, Kobayashi H, Le N, et al. Biodistribution of ^{125}I-labeled des(1–3) insulin-like growth factor I in tumor-bearing nude mice and its in vitro catabolism. *Cancer Res.* 1997;57:2754–2759.
40. Chinol M, Casalini P, Maggiolo M, et al. Biochemical modifications of avidin improve pharmacokinetics and biodistribution, and reduce immunogenicity. *Br J Cancer.* 1998;78:189–197.