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

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Prior *in vivo* studies using the ^{125}I-labeled anti-Tac disulfide-stabilized variable region fragment (^{125}I-anti-Tac dsFv) of monoclonal antibody in the presence of the circulating soluble alpha subunit of the interleukin-2 receptor (sIL-2Rα) have shown formation of complexes which interfere with biodistribution. In this study we evaluated the effects of preinjecting HuTac and 7G7/B6, two immunoglobulin Gs (IgGs) that recognize different epitopes of sIL-2Rα, on the biodistribution of ^{125}I-anti-Tac dsFv in mice bearing SP2/Tac tumor xenografts, which produce sIL-2Rα, or on nude mice injected with 500 ng of sIL-2Rα. We also evaluated the biodistribution in mice of ^{125}I-labeled sIL-2Rα injected alone or with HuTac and 7G7/B6. Injection of either HuTac or 7G7/B6 resulted in complexes with the sIL-2Rα in serum. Injection of HuTac before ^{125}I-anti-Tac dsFv, in SP2/Tac tumor-bearing mice, resulted in faster clearance of the dsFv from the blood (7.6%ID/g at 30 min), compared to 23.2%ID/g for the no-antibody control; preinjection of 7G7/B6 prolonged the retention of ^{125}I-anti-Tac dsFv to 35.3%ID/g, with more complexes in serum. In mice pre-injected with 7G7/B6 the concentration of ^{125}I-anti-Tac dsFv in tumor was lower (5.2±0.3%ID/g) than in mice preinjected with HuTac (7.9±1.2%ID/g) or in the control group (5.6±0.7%ID/g). In conclusion, while both IgGs formed complexes with sIL-2Rα and prolonged its retention, preinjection of 7G7/B6 was detrimental, because the increased circulating sIL-2Rα still had the epitope recognized by the dsFv available for binding and neutralized the anti-Tac dsFv upon injection, whereas preinjection of HuTac blocked the epitope.

Key words: Fv fragment — Iodine-125 — Epitope — Radioimmunodetection — Monoclonal antibody

The use of radiolabeled monoclonal antibodies (MoAbs) for radioimmunodetection and radioimmunotherapy of tumors has been extensively evaluated since the initial clinical reports.[1–4] The effect of circulating antigen on tumor targeting has been evaluated in animal models and in clinical trials.[5] Circulating antigen forms complexes with the MoAb in serum.[6–8] These complexes can alter the clearance of radiolabeled antibodies and decrease tumor uptake.[9–13] The effect on clearance may vary depending on the antibody and the target antigen. Whereas some clinical trials have demonstrated no adverse effects on tumor targeting,[14, 15] others have documented a detrimental effect of circulating antigen on radiolabeled antibody biodistribution.[16–18]

The alpha subunit of the interleukin-2 receptor (IL-2Rα) is a cell-surface receptor that is overexpressed in various hematological T cell malignancies [19–22] and in autoimmune diseases.[23–25] Because it is not expressed or is expressed at very low levels in normal lymphocytes, it has been proposed as a target for immunotherapy and radioimmunotherapy.[26] Anti-Tac is an immunoglobulin G2a (IgG2a) murine MoAb that recognizes IL-2Rα[27] and can inhibit the binding of IL-2 to IL-2Rα.[28] This antibody has shown some effectiveness in the treatment of adult T cell leukemia [29] and renal rejection,[30] and prevention of allograft rejection in a monkey heart transplant model.[31, 32] However, trials targeting the IL-2Rα with radiolabeled anti-Tac have shown that the circulating soluble fragment of the alpha subunit of the IL-2Rα receptor (sIL-2Rα) can form complexes that block the antibody-binding site.[29] Our previous experience using radiolabeled anti-Tac disulfide-stabilized variable region fragment (dsFv)[33, 34] in two different tumor-xenograft models showed that circulating sIL-2Rα altered the kinetics and formed complexes that resulted in longer blood retention and lower tumor uptake of anti-Tac dsFv.[35, 36] We have previously demonstrated that blocking the sIL-2Rα with

humanized anti-Tac IgG (HuTac), which competes for the same epitope as the dsFv, resulted in faster and more favorable kinetics of anti-Tac dsFv.[37] These prior studies took advantage of the smaller size of the Fv fragments (~25 kDa), which penetrate tissue much faster and more homogeneously than does intact IgG.[38, 39] Because of these characteristics, Fv fragments are being considered for radioimmunodetection or for therapy with radioisotopes or as immunotoxins.

In this study we tested the hypothesis that the antibody used to bind the sIL-2Rα had to be directed against the same epitope as the dsFv, and that if it did not it would result in longer blood retention of the sIL-2Rα, which would be detrimental to the biodistribution of dsFv, since the sIL-2Rα would still be capable of binding to the radiolabeled dsFv. No studies targeting this antigen or similar circulating antigens have experimentally proven this point. Therefore we compared the effect of preinjection of HuTac to that of 7G7/B6 on the kinetics of sIL-2Rα and anti-Tac dsFv. The 7G7/B6 is an IgG2a murine MoAb that recognizes a different epitope on the IL-2Rα than does HuTac,[40] but does not block the binding of the IL-2 or anti-Tac dsFv to the IL-2Rα.[40]

MATERIALS AND METHODS

MoAbs and receptors   We used radiolabeled anti-Tac dsFv to target tumors xenografted into nude mice. Anti-Tac dsFv is a genetically engineered disulfide-bonded Fv fragment of anti-Tac murine monoclonal antibody.[33] The anti-Tac is an IgG2a, murine-derived MoAb that binds to the C-terminal 1–6 amino acids on the IL-2Rα which overlap with the binding sites of IL-2 ligand.[41] The anti-Tac IgG2a was produced and characterized as previously described.[42] Production of anti-Tac dsFv has been previously described.[33] Briefly, the V_H and V_L domains were expressed in separate *Escherichia coli* cultures. The proteins were recovered as cytosolic inclusion bodies and refolded. The refolded anti-Tac dsFv was purified by ion-exchange and size-exclusion chromatography. The dsFv was >98% pure as determined by size-exclusion high performance liquid chromatography (HPLC) with a UV detector. The binding affinity of dsFv to IL-2Rα was not different from that of the parental murine anti-Tac IgG (9 $\times 10^9/M$)[33] but was greater than that of HuTac (see below).

Two antibodies, HuTac and 7G7/B6, were used to block sIL-2Rα. The HuTac antibody was constructed, as previously described, by combining the complementarity-determining regions of the murine anti-Tac antibody with the human IgG1κ framework and constant regions.[43] HuTac recognizes the same epitope as the anti-Tac dsFv and murine anti-Tac IgG. The 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 to contain >99% IgG. In addition to HuTac, 7G7/B6, a murine MoAb IgG2b[40] that recognizes a different, non-cross-reacting epitope (*N*-terminal 141–144 amino acids) on the IL-2Rα which does not overlap with the binding sites of IL-2 ligand and the anti-Tac epitope,[41] was also used to block the sIL-2Rα.

Iodinated carrier-free sIL-2Rα was provided by Dr. T. A. Waldmann (generous gift of Dr. J. Hakimi); the lyophilized unlabeled sIL-2Rα was purchased from R&D Systems Inc. (Minneapolis, MN).

Radiolabeling   The anti-Tac dsFv and the sIL-2Rα were labeled with ^{125}I using the Iodo-Gen method.[44] Briefly, approximately 1–2 mCi of sodium ^{125}I was added to 50–100 μg of dsFv or sIL-2Rα in 80 μl of phosphate-buffered saline (PBS), pH 7.2, in a conical polypropylene vial coated with 10 μg of Iodo-Gen. After incubation for 10 min at room temperature, the radiolabeled products were purified using a PD-10 column (Pharmacia, Uppsala, Sweden). The specific activities of the ^{125}I-labeled anti-Tac dsFv and ^{125}I-labeled sIL-2Rα were 10–27 mCi/mg and 7–10 mCi/mg, respectively. The radiochemical purity of ^{125}I-labeled anti-Tac dsFv and sIL-2Rα was >98% as confirmed by ITLC and size-exclusion HPLC.

The HuTac and the 7G7/B6 were labeled with ^{125}I using the chloramine-T method.[45] Briefly, 100 μg of HuTac in 0.05 M phosphate buffer (pH 7.5) was mixed with 400–800 μCi of ^{125}I and 12 μg of chloramine-T dissolved in 0.05 M phosphate buffer. The mixture was allowed to react for 5 min, and the radiolabeled products were purified using a PD-10 column (Pharmacia). The specific activities of the ^{125}I-labeled HuTac and the 7G7/B6 were approximately 3–4 mCi/mg and 7 mCi/mg, respectively, with >98% of the protein bound to ^{125}I as confirmed by ITLC and size-exclusion HPLC.

Immunoreactivity   Immunoreactivity was determined by use of a modification of the cell-binding assay of Lindmo *et al.*[46] Aliquots of the ^{125}I-labeled dsFv (1.0 ng), HuTac (5 ng), and 7G7/B6 (3 ng) were incubated for 2 h at 4°C with 2×10^6 to 2×10^7 SP2/Tac (IL-2Rα-positive). Cells were centrifuged, and the cell-bound radioactivity was counted in a gamma counter. Nonspecific binding to the cells was examined under conditions of antibody excess (25 μg of nonradiolabeled anti-Tac antibody). Immunoreactivity of the ^{125}I-labeled anti-Tac dsFv was >78%; the HuTac, >82%; and the 7G7/B6, >68%.

Scatchard plot and cross-inhibition   A Scatchard analysis was performed. A fixed dose of either ^{125}I-labeled HuTac (5 ng/100 μl) or ^{125}I-labeled 7G7/B6 (3 ng/100 μl) and increasing doses of unlabeled HuTac or 7G7/B6 (5 to 5000 ng/50 μl) were incubated with 2×10^5 SP2/Tac cells in 100 μl of phosphate buffer, without calcium and magnesium, for 2 h at 4°C. After centrifugation, the supernatant was aspirated, and the cell-bound radioactivity

was counted in a gamma counter. The affinity constants and epitope densities of both antibodies were calculated by Scatchard plot analysis.[10]

To evaluate the cross-inhibition between HuTac and 7G7/B6, ^{125}I-labeled HuTac (5 ng/100 μl) or ^{125}I-labeled 7G7/B6 (3 ng/100 μl) and a 4000-fold molar excess of the unlabeled opposite antibody were incubated with 6×10^6 SP2/Tac cells in 100 μl of phosphate buffer, without calcium and magnesium, for 2 h at 4°C. After centrifugation, the supernatant was aspirated. The cell-bound radioactivity was then counted in a gamma counter.

Binding of ^{125}I-labeled sIL-2Rα to HuTac or 7G7/B6 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 or 7G7/B6 was mixed with ^{125}I-labeled sIL-2Rα and analyzed with size-exclusion HPLC using a TSK G2000SW column (TosoHaas, Philadelphia, PA; 0.067 M PBS, 0.01 M KCl; pH 6.8; 0.5 ml/min) equipped with an on-line NaI gamma detector (γRAM, IN/US Systems, Inc., Fairfield, NJ). The incubation with HuTac resulted in >90% of the ^{125}I-labeled sIL-2Rα complexing into a high-molecular-weight fraction. However, when 7G7/B6 was incubated with ^{125}I-labeled sIL-2Rα, only >45% of the radioactivity in the ^{125}I-labeled sIL-2Rα fraction shifted into the high-molecular-weight fraction; the rest migrated with the original activity.

Cell lines and animal models Tumor xenografts were generated with an IL-2Rα-positive SP2/Tac cell line.[47] This line was generated by transfecting the gene that encodes for the IL-2Rα into the receptor-negative SP2/0 cells, which are a non-immunoglobulin-secreting murine myeloma line that does not express IL-2Rα (American Type Culture Collection, CRL 1581, Rockville, MD). The cells were provided by Dr. Thomas Waldmann of the Metabolism Branch of the National Cancer Institute and by Protein Design Labs. 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. We used female athymic nude mice (*nu/nu*), 5–7 weeks old and 15 to 20 g (Harlan Sprague-Dawley, Frederick, MD). Tumor xenografts were established by s.c. inoculation of 4×10^6 SP2/Tac and SP2/0 cells. Experiments on tumor-bearing mice were performed 11–14 days after implantation, when SP2/Tac tumors weighed a mean of 1128 mg (443–2008 mg). All mice were killed with CO_2 inhalation and exsanguinated by cardiac puncture prior to dissection. The organs were harvested, blot dried, and weighed on an analytical balance, and the radioactivity was then counted in a gamma counter.

Amino acid preparations We blocked renal uptake of ^{125}I-labeled anti-Tac dsFv with L-lysine, as previously described.[36] Not doing so would have complicated the pharmacokinetic analyses due to the otherwise high renal accumulation and the rapid renal metabolism, which would result in elevated free ^{125}I in the blood.[35, 36] A stock solution of L-lysine (300 mg/ml) was prepared in 0.1 M phosphate buffer, pH 7.5, using L-lysine monohydrochloride (Pierce Chemical Co., Rockford, IL), and coinjected with ^{125}I-labeled anti-Tac dsFv at a final concentration of 250 mg/ml as previously described.[36]

Effect of HuTac or 7G7/B6 on blood clearance and biodistribution of ^{125}I-labeled sIL-2Rα For biodistribution studies, three groups of normal athymic mice (*n*=4) were injected with 250 ng (2 μCi) of ^{125}I-labeled sIL-2Rα i.v. either alone or followed 10 min later by i.v. injection of 20 μg of HuTac or of 20 μg of 7G7/B6. At 45 min after administration of the ^{125}I-labeled sIL-2Rα, all mice were killed by CO_2 inhalation, followed by exsanguination. Their organs were removed and weighed, and the radioactivity was counted. The data were expressed as %ID/g of tissue. An aliquot of serum from these mice was also analyzed by size exclusion HPLC, as described above. To characterize the effects of both IgGs on the rate of blood clearance of the ^{125}I-labeled sIL-2Rα, two additional normal athymic mice per group were studied. An i.v. injection of 500 ng /4 μCi of ^{125}I-labeled sIL-2Rα was followed by serial tail vein bleeding using 5-μl micropipettes 10, 25, 45, 90, and 180 min after injection.

Effect of HuTac and 7G7/B6 on the biodistribution of ^{125}I-labeled anti-Tac dsFv in the presence of sIL-2Rα We evaluated the effect of blocking two different epitopes of sIL-2Rα on the biodistribution of ^{125}I-labeled anti-Tac dsFv. Two groups of normal athymic mice (*n*=4) were first injected with 500 ng of recombinant sIL-2Rα (R&D Systems Inc.) i.v., followed 10 min later by i.v. injection of either 20 μg of HuTac or 7G7/B6, then they received i.v. injection of 5 μCi/ 300 ng/ 200 μl of ^{125}I-labeled dsFv, containing 50 mg of L-lysine 30 min after the administration of the sIL-2Rα. All mice were killed 45 min after administration of sIL-2Rα. Their organs were removed, and weighed, and the radioactivity was counted. In addition, aliquots of their serum were analyzed using size-exclusion HPLC.

Biodistribution in tumor-bearing mice Blood pharmacokinetics and biodistribution studies were performed in tumor-bearing mice. Three groups of mice (*n*=4) bearing SP2/Tac tumor xenografts of approximately 1 g received i.v. injections of 20 μg of HuTac or 20 μg of 7G7/B6 30 min before i.v. injection of 5 μCi/ 300 ng/ 200 μl of ^{125}I-labeled anti-Tac dsFv, containing 50 mg of L-lysine. The mice were then killed 15 min after the ^{125}I-labeled dsFv injection. Their organs were removed and weighed, and the radioactivity was counted. Data were expressed both as %ID/g of tissue and as tumor-to-normal tissue ratios.

Statistical analysis All the data was analyzed by means

of one-way analysis of variance (ANOVA) and Bonferroni's *t* test.

RESULTS

Scatchard plot and cross-inhibition The Scatchard plot analysis showed that HuTac and 7G7/B6 had a binding affinity to SP2/Tac cells of 4.3×10^9 and $5.8 \times 10^8/M$, respectively. As expected, a cross-inhibition test of the monoclonal antibodies showed that 7G7/B6 had almost no effect on the binding of HuTac to SP2/Tac cells at antigen excess. The HuTac had very little effect on binding of 7G7/B6 to SP2/Tac cells (Table I), with 55% of ^{125}I-labeled 7G7/B6 bound in the presence of a 4000-fold

Table I. Cross-inhibition and Binding Assays of HuTac versus 7G7/B6

Cold antibody (×4000)	% Bound	
	^{125}I-labeled HuTac	^{125}I-labeled 7G7/B6
None	84.1[a]	69.0[a]
HuTac	1.4[b]	55.1
7G7/B6	80.3	3.3[b]

a) Represents maximal binding.
b) Represents non-specific binding (antibody excess).

molar excess of HuTac compared with 69% without HuTac.

Effect of HuTac or 7G7/B6 on blood clearance and biodistribution of ^{125}I-labeled sIL-2Rα The ^{125}I-labeled sIL-2Rα survived significantly longer in the blood in mice injected with either HuTac ($36.3 \pm 4.6\%$ ID/g) or 7G7/B6 ($33.0 \pm 0.8\%$ ID/g) than in mice in the no-injection control group ($25.0 \pm 1.3\%$ ID/g) (*P*<0.001 from ANOVA) (Fig. 1). The HPLC analysis of the serum of mice injected with either HuTac or 7G7/B6 showed a large proportion of the ^{125}I in a high-molecular-weight fraction (complexes). In animals receiving HuTac, $82 \pm 6\%$ of the radioactivity was in complexes, whereas with 7G7/B6, $50 \pm 8\%$ was in complexes. Similar blood pharmacokinetics were also seen in the serial tail vein blood draws at all time points up to 3 h (data not shown).

Effect of HuTac and 7G7/B6 on the biodistribution of ^{125}I-labeled anti-Tac dsFv in the presence of sIL-2Rα We evaluated the biodistribution of ^{125}I-labeled dsFv in three groups of mice: normal mice which had been injected with 500 ng of recombinant sIL-2Rα i.v., followed by an i.v. injection of 20 μg of HuTac or an i.v. injection of 20 μg of 7G7/B6, and a no-antibody control group. The blood clearance of ^{125}I-labeled anti-Tac dsFv was significantly different in all three groups (*P*<0.0001 from ANOVA) (Fig. 2). Blood retention of ^{125}I-labeled
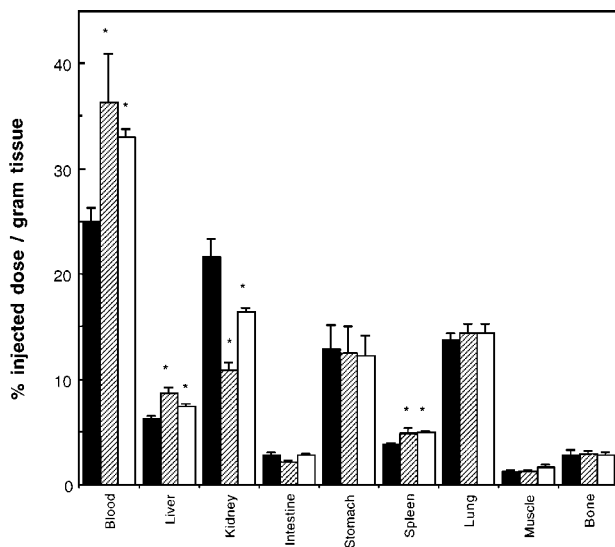

Fig. 1. Normal nude mice (*n*=4) received 2 μCi/250 ng of ^{125}I-labeled sIL-2Rα alone (■) or followed 10 min later by i.v. injection of either 20 μg of HuTac (▨) or 20 μg of 7G7/B6 (□). Mice were killed 45 min after injection of the ^{125}I-labeled sIL-2Rα. A biodistribution study was performed. Data are plotted as means±SD. ∗ *P*<0.01 compared with the no IgG control group.
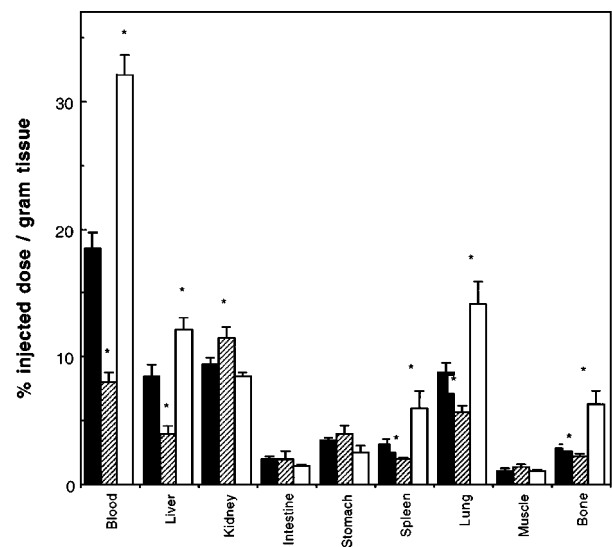

Fig. 2. Three groups of normal athymic mice (*n*=4) were first injected with 500 ng of recombinant sIL-2Rα i.v. alone (■) or followed 10 min later by i.v. injection of 20 μg of HuTac (▨) or 7G7/B6 (□). All mice then received 5 μCi (300 ng/ 200 μl) of ^{125}I-labeled dsFv i.v. 30 min after the administration of the sIL-2Rα and were then killed 15 min afterwards. The biodistribution of ^{125}I-labeled anti-Tac dsFv was plotted (mean±SD). ∗ *P*<0.01 compared with the no-IgG control group.

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anti-Tac dsFv in the group of mice injected with 7G7/B6 was higher than that in the control group, which in turn was higher than that in the HuTac group. The values were 32.1±1.5%ID/g, 18.5±1.3%ID/g, and 8.0±0.6%ID/g, respectively, retained at 15 min postinjection of the ^{125}I-labeled anti-Tac dsFv. The accumulations of ^{125}I in the kidney and the stomach were higher in the HuTac group than in the control or 7G7/B6 group. This was consistent with the finding of a higher fraction of ^{125}I-labeled anti-Tac dsFv present as intact dsFv in the group receiving HuTac (4.1±1.0%ID/g) than in the other two groups (1.9±0.2 and 1.8±0.3%ID/g, respectively). An HPLC analysis of serum (Fig. 3) showed that the molecular weights of the complexes in these three groups were different. In the 7G7/B6 group, complexes were the largest (>200 kDa) and were consistent with the binding of 7G7/B6 with one or two sIL-2Rα and one or two ^{125}I-labeled anti-Tac dsFv (Fig. 4). In the HuTac group most ^{125}I-labeled anti-Tac dsFv appeared intact and uncomplexed, whereas in the no-treatment control (sIL-2Rα only), two separate high-molecular-weight fractions were predominant.

Blood clearance and biodistribution of ^{125}I-labeled anti-Tac dsFv with HuTac or 7G7/B6 in SP2/Tac tumor-bearing mice At 30 min postinjection, the group of mice preinjected with 7G7/B6 showed significantly higher blood retention (35%ID/g) of ^{125}I-labeled anti-Tac dsFv than the no-preinjection control group (23%ID/g) or the HuTac group (8%ID/g) (Fig. 5). In addition, the tumor accumulation in the HuTac group was significantly higher than that in the other two groups. The HPLC analyses from these three groups showed results similar to those seen in non-tumor-bearing mice that had been preinjected with sIL-2Rα. Tumor-to-normal tissue ratios in the HuTac group were significantly higher than those in the other two groups (Table II).

DISCUSSION

Previously[37] we showed that elevated concentrations of circulating sIL-2Rα (45 kDa) resulted in formation of complexes with ^{125}I-labeled anti-Tac dsFv (25 kDa). This resulted in slower blood clearance of the dsFv, lower renal uptake, lower absolute tumor uptake, and lower tumor-to-nontumor ratios than those in mice without circulating sIL-2Rα.[36, 37] This was not surprising, since clinical trials had shown that the presence of sIL-2Rα resulted in formation of complexes with anti-Tac IgG, which did not result in faster clearance of the IgG, but did decrease its immunoreactivity.[29] Similar results were found by Junghans and Waldmann in studies evaluating the catabo-
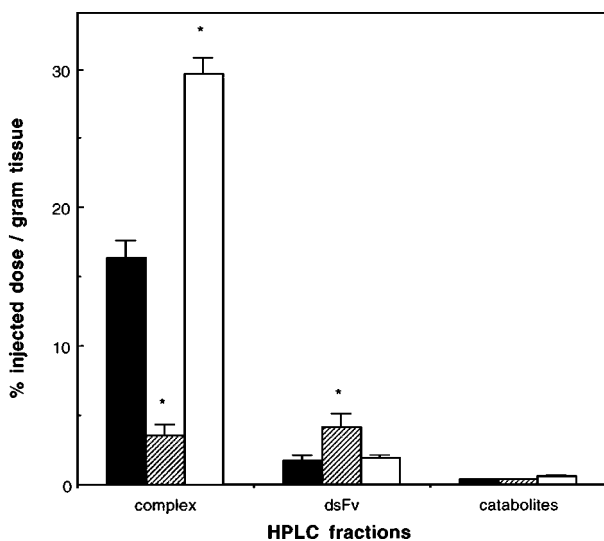

Fig. 3. Serum from the animals killed as described in the legend to Fig. 2 was subjected to HPLC analyses. The amount of radioactivity in each fraction was plotted for mice receiving ^{125}I-labeled dsFv alone (■), for those preinjected with HuTac (▨), and for those receiving 7G7/B6 (□). Serum was obtained 15 min after dsFv injection. The percentages of the injected dose per gram of blood of different HPLC fractions are plotted (means±SD). ∗ $P<0.01$ compared with the no-IgG control group.
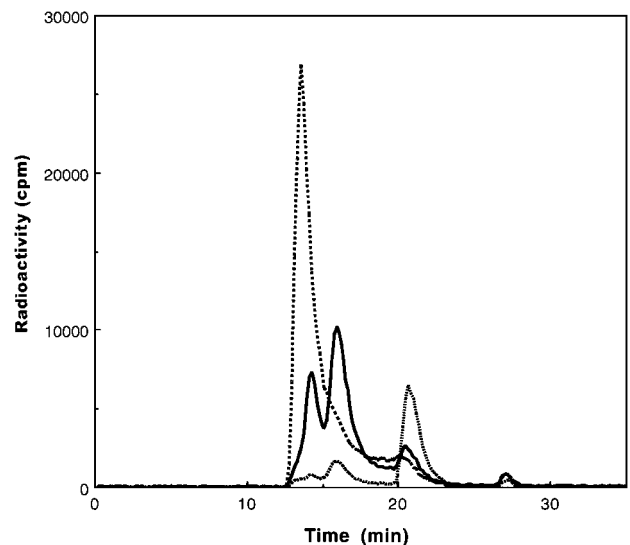

Fig. 4. HPLC of serum from groups of normal mice injected with 500 ng of sIL-2Rα i.v., followed by i.v. ^{125}I-labeled anti-Tac dsFv alone (——), or ^{125}I-labeled anti-Tac dsFv after preinjection with either 7G7/B6 (·······) or HuTac (———) 10 min before the sIL-2Rα injection. All mice were injected with 5 μCi (300 ng/200 μl) of ^{125}I-labeled dsFv i.v. and were killed 15 min afterwards. The same amount of serum (100 μl) from each mouse was subjected to the HPLC. Each curve represents the average of chromatograms from 4 individual mice in each group.

Table II. SP2/Tac Tumor-to-normal Tissue Ratio of ^{125}I-Labeled Anti-Tac dsFv in Mice Bearing about 1 g of SP2/Tac Tumor

Organ	No IgG	HuTac	7G7/B6
Blood	0.25±0.06[a]	1.08±0.24[b]	0.15±0.01[c]
Liver	0.80±0.10	2.77±0.57[b]	0.53±0.03[b]
Kidney	0.67±0.21	0.94±0.31	0.58±0.10
Intestine	3.52±0.51	4.70±0.75[c]	2.38±0.55[c]
Spleen	1.94±0.31	6.39±0.53[b]	1.24±0.10[b]
Lung	0.72±0.18	1.41±0.11[b]	0.36±0.04[b]
Muscle	5.31±1.37	6.26±1.00	4.28±0.97
Bone	3.73±0.54	4.26±0.57	1.48±0.28[b]
SP2/0 tumor (IL-2Rα-)	1.66±0.32	3.92±0.64[b]	0.98±0.04[b]
Tumor weight (g)	1.24±0.34	1.34±0.67	0.96±0.36

a) The values are SP2/Tac tumor-to-normal tissue ratio (means±SD).
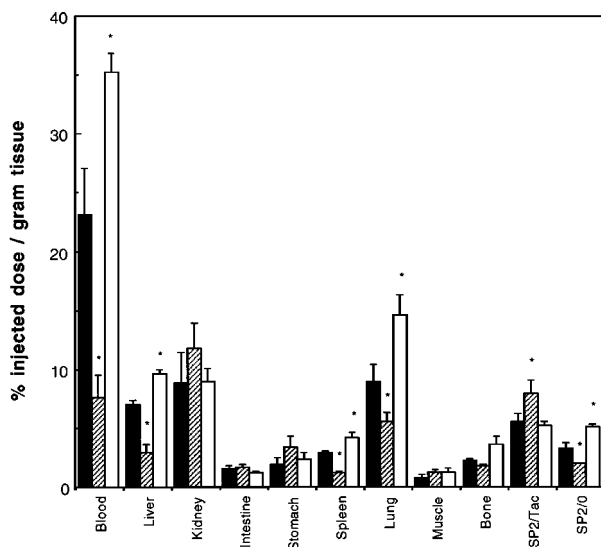b) $P<0.01$ and c) $P<0.05$ compared with the no IgG control group.

Fig. 5. Three groups of mice bearing SP2/Tac tumors ($n=4$) were injected i.v. with ^{125}I-labeled dsFv (5 μCi, 300 ng/ 200 μl) alone (■) or 15 min after preinjection of 20 μg of HuTac (▨) or 20 μg of 7G7/B6 (□). All mice were killed 15 min after administration of ^{125}I-labeled dsFv. The biodistribution of ^{125}I-labeled anti-Tac dsFv was plotted (mean±SD).

lism of sIL-2Rα,[48] in which administration of anti-Tac to mice with increased sIL-2Rα levels in serum resulted in longer retention of the sIL-2Rα, but did not decrease the half-life of the anti-Tac IgG. Furthermore, in our previous study we demonstrated that preinjection of HuTac resulted in formation of complexes with sIL-2Rα that competed with and prevented the binding of the radiolabeled dsFv to sIL-2Rα. These actions resulted in more favorable kinetics in the blood and in tumors.[37]

The current study confirms and builds on the original observations. As expected, the 7G7/B6 also binds the sIL-2Rα and prolongs its retention in blood. Nevertheless, because the epitope that is bound is not the same one recognized by the dsFv, when the dsFv is injected it is still capable of binding to the sIL-2Rα. Because the amounts of sIL-2Rα in serum are elevated due to the slower clearance of the complex of 7G7/B6 and sIL-2Rα, the radiolabeled dsFv is then bound to a large complex that cannot be excreted through the kidney and has more difficulty penetrating the tumor and other tissues. In many respects, these findings are similar to those of Berson and Yalow. They found that anti-insulin antibodies bound injected insulin and prolonged its survival in the circulation.[49] The complexes formed between ^{125}I-labeled sIL-2Rα and HuTac survived longer than those with 7G7/B6. This may have been related to some selective damage caused during the radioiodination of the epitope recognized by 7G7/B6, since sIL-2Rα labeled at higher specific activities showed less binding to the 7G7/B6 (data not shown). A less likely explanation is that the differences were due to the five-fold lower affinity of the 7G7/B6 for sIL-2Rα than that of HuTac.[43, 50] It is unlikely that the differences observed were related to the fact that HuTac is a humanized IgG while 7G7/B6 is a murine IgG, since the amounts retained in the blood were in the same range for both antibodies.

The timing of antibody administration is likely to be critical: if there is too long a period between administration of cold intact anti-Tac IgG and anti-Tac dsFv, there could be significant changes in the results. We chose to compare the biodistribution of the ^{125}I-labeled HuTac and 7G7/B6 at early times, since the kinetics of dsFv are rapid and would thus allow advantage to be taken of the slower penetration of the IgGs out of the vascular system and into the tumor. Our study showed that the distributions of the two IgGs used into tissues were very similar, and

therefore a differential binding to tumor, competing with dsFv, was not responsible for the differences observed in uptake of ^{125}I-labeled anti-Tac dsFv.

In this study, tumor-bearing mice pretreated with HuTac prior to ^{125}I-labeled anti-Tac dsFv showed the most favorable pharmacokinetics as demonstrated by faster clearance, higher absolute tumor uptake, and higher tumor-to-nontumor ratios. In the case of 7G7/B6, the pharmacokinetics were worse than the no-antibody, pretreatment control groups (Fig. 5), as demonstrated by higher blood pool retention, lower tumor accumulation, and smaller tumor-to-nontumor ratios (Table II). The effects of 7G7/B6 on blood pool kinetics seen in the tumor xenograft model were also seen in our more artificial model, in which we preinjected the mice with sIL-2Rα. Some variability in blood pool retention of radiolabeled dsFv was observed among mice used in the biodistribution study. These differences were most likely related to the amount of sIL-2Rα present in the serum.[37] Our previous study is consistent with these findings. We showed that the 1:12 molar ratios of sIL-2Rα to blocking antibody had the maximum effect on the biodistribution of ^{125}I-labeled anti-Tac dsFv.

The injection of 7G7/B6 was detrimental to ^{125}I-labeled anti-Tac dsFv biodistribution. Although not tested here, this approach of binding an IgG against an epitope that is not recognized by the radiolabeled dsFv or a radiolabeled peptide may be generalized to other systems where there are circulating antigens or receptors. Nevertheless, in other situations this phenomenon may be advantageous when the intent is to maintain higher blood levels of certain peptides. For example, if the IL-2 rather than anti-Tac dsFv were administered i.v., it might allow the IL-2 to bind to the complex, resulting in a higher blood retention, and enhancing the biological effects of IL-2, as has been reported in another study.[51]

In conclusion, the presence of sIL-2Rα as a circulating antigen had detrimental effects on the biodistribution of ^{125}I-labeled anti-Tac dsFv. Blocking the circulating antigen resulted in more favorable pharmacokinetics when the antibody used was directed against the same epitope as the radiolabeled dsFv. This strategy takes advantage of the larger size of the IgG and its slower kinetics, which do not allow time for IgG to compete for extravascular tumor sites with dsFv. In contrast, we hypothesize that use of an antibody directed against a different epitope than the dsFv resulted in prolonged retention in the serum of circulating antigen which was still recognized by the dsFv. This antigen decreased the amount of free dsFv available to penetrate the tissues and bind to the tumor. This effect that we describe for the first time in our model system may be a general phenomenon when dealing with small antigen and antibody fragments.

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