Favorable Effects of Glycolate Conjugation on the Biodistribution of Humanized AntiTac Fab Fragment

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One of the major limitations of using intact immunoglobulins for targeting tumors is poor penetration into tissues. Although Fab fragments have been used because of their improved kinetics, they have undesirable high renal accumulation. In this study we tested a new approach to block renal accumulation of Fab. **Methods:** We conjugated humanized antiTac Fab fragments, which are directed against the interleukin-2 receptor, with glycolate. The biodistribution, pharmacokinetics and catabolism of glycolated Fab (glyco-Fab) were evaluated at two different levels of substitution (heavy and light) compared with nonglycolated Fab in Tac-antigen-positive (ATAC4) and -negative (A431) tumor-bearing nude mice. The mice received coinjections of ^{125}I-labeled glyco-Fab (3 μCi/1 μg) and ^{131}I-labeled nonglycolated Fab (5 μCi/1 μg). In addition, groups of mice receiving these reagents were also coinfused with 50 mg L-lysine. **Results:** Significantly less glyco-Fab than nonglycolated Fab accumulated in the kidney (21 versus 189 %ID/g; $P < 0.001$). A higher proportion of glyco-Fab was excreted into the urine in its intact form. The glyco-Fab survived longer in circulation than nonglycolated Fab. The peak tumor accumulation of glyco-Fab was 2.3-fold greater than that of nonglycolated Fab. Furthermore, the ATAC4 tumor-to-normal tissue ratio of glyco-Fab was much higher in all organs than that of nonglycolated Fab. The heavily glyco-Fab accumulated less in the kidney than the lightly glyco-Fab. The coinjected lysine reduced the renal accumulation of both nonglycolated Fab and glyco-Fab. **Conclusion:** Glyco-Fab is a promising agent because of its lower renal accumulation, higher tumor uptake and higher tumor-to-normal tissue ratio.

Key Words: Fab fragment; glycolate; radioimmunodetection; monoclonal antibody

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The use of radiolabeled monoclonal antibodies (MoAbs) for radioimmunodetection and radioimmunotherapy has been extensively evaluated (*1*) since the initial clinical report of Mach et al. (*2*). The major limitations of intact immunoglobulins for targeting tumor are their poor penetration into tissues (*3,4*) and their tendency to evoke human antimouse immune responses (*5*). As an alternative, Fab fragments have been used, because of their improved kinetics and their lower immunogenicity (*5–8*). The Fab fragment of an antibody against myosin has been evaluated (*9–11*) and recently approved for clinical use by the Food and Drug Administration (FDA). The FDA has also approved two antibody fragments, ^{99m}Tc-labeled Fab′ (^{99m}Tc-arcitumomab; Immunomedics, Morris Plains, NJ) and ^{99m}Tc-nofetumomab Merpentan (Dupont Radiopharmaceutical, Billerica, MA), for detection of colorectal cancer and small cell lung cancer, respectively (*12,13*). Although the use of ^{99m}Tc-arcitumomab allows earlier imaging than whole IgG, a major disadvantage is the high renal uptake common to Fab fragments (*14–18*). Our imaging experience, as well as that of others, using ^{99m}Tc-arcitumomab is that the high renal uptake can interfere with tumor imaging, resulting in image reconstruction artifacts that can hamper tumor visualization at the level of the kidneys. Naturally, this high renal accumulation results in higher renal radiation doses than would otherwise occur if lower accumulations were present. For these two reasons (interference with imaging and dosimetry), the developers of this reagent have published an attempt to block the kidney uptake using amino acid administration (*14,19,20*). Several studies have evaluated methods to decrease renal accumulation of radiolabeled Fab (*17,19,21*), Fv (*22*) and peptides (*23,24*). These studies have used positively charged amino acids, in particular lysine. However, because the clearance of lysine is fast, its blocking effects require repeated injections or continuous infusion (*19,22*).

Although preclinical studies have shown that administration of lysine or amino acid solutions may effectively block renal uptake of radiolabeled antibody fragments (*14,17,19, 21,22,24*), a clinical trial has demonstrated marginal success (*25*). Therefore, evaluation of different strategies to minimize renal uptake should be pursued. We reported previously that conjugating glycolate to a disulfide stabilized Fv fragment decreased renal uptake and postulated that this was the result of changes in isoelectric point (*26*). In this study,

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we evaluate the biodistribution of a glycolate [$CH_2(OH)CO$-] conjugated radioiodine-labeled Fab fragment of humanized antiTac (HuTac) MoAb (*27*) and evaluate its ability to reduce renal radioactivity accumulation. Moreover, because the use of lysine is being proposed as a method to decrease renal uptake of small radioactive antibody fragments (*14,17,19,24*), we evaluated whether coinjecting L-lysine with the radiolabeled, glycolated Fab fragment would further decrease renal uptake.

MATERIALS AND METHODS

Monoclonal Antibodies

As our model system, we used antibody fragments that recognize the alpha subunit of the interleukin-2 receptor (IL-2Rα) or the soluble form of the IL-2Rα (sIL-2Rα). The Fab fragment was prepared from the HuTac IgG (*27*), using the papain digestion method. HuTac antibody was constructed, as previously described, by combining the complementary determining region of the murine antiTac antibody with the human IgG1κ framework and constant regions (*27*). The HuTac IgG 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.

The Fab fragment was produced with papain-agarose gel (Pierce Chemical Co., Rockford, IL). Briefly, 0.5 mL HuTac IgG (<20 mg/mL) were incubated with papain-agarose gel in phosphate-buffered saline (PBS) with 0.02 mmol cysteine at pH 7.0 for 14 h at 37°C. The papain-agarose gel was filtered out from the digested sample. The Fab fragments were purified with Protein A Sepharose gel (Pierce Chemical Co.) followed by dialysis in PBS using a dialysis membrane (Slide-A-Lyzer; Pierce Chemical Co.) to remove the undigested IgG, Fc fragment and cysteine. The purity of HuTac Fab was >99%, as determined by an ultraviolet elution profile on a size-exclusion high-performance liquid chromatography (HPLC) using a TSK G2000SW column (TosoHaas, Philadelphia, PA; 0.067 mol sodium PBS, 0.1 mol KCl; pH 6.8; 0.5 mL/min).

Conjugation with Glycolate

First, we synthesized 2,3,5,6,-tetrafluorophenyl (TFP)-glycolate as follows. Briefly, 100 mmol TFP and 10 mmol glycolic acid were reacted in 10 mL acetonitrile using 12 mmol dicyclohexylcarbodiimide at room temperature for 4 h. The yield was 57%. The ester derivatives were purified with normal-phase HPLC equipped with a μPorasil PrepPak cartridge (Waters, Milford, MA; hexane:ethyl acetate:acetic acid = 150:50:1; 1 mL/min). Melting point was measured on the electrothermal digital melting point apparatus (Electrothermal, Southend, UK), ^1H nuclear magnetic resonance (NMR) spectra were determined at 300 MHz on a UNITY plus-300 (Varian Associates, Palo Alto, CA), and elemental analysis was performed by Quantitative Technologies (Whitehouse, NJ) and General Activation Analysis (San Diego, CA). The melting point was 90.8–91.2°C, as determined by ^1H NMR deuterated chloroform (CDCl3): γ 7.12 (m, 1, aromatic H), 4.59 (s, 2, CH2), 3.49 (s, 1, OH). Elemental analysis calculated for $C_8H_4O_3F_4$ was C, 42.87%; H, 1.8%; and F, 33.9%. The values found were C, 43.15%; H, 1.90%; and F, 35.1% (*26*).

To obtain HuTac Fabs with different isolectric points (pIs), two formulations of glycolate were used: one lightly glycolated, with a 36:1 molar ratio of TFP-glycolate to HuTac Fab, and the other heavily glycolated, with a ratio of 73:1. Conjugation with glycolated Fab was performed by reacting HuTac Fab (1.5 mg/mL) with TFP-glycolate dissolved in dimethyl formamide in 0.1 mol sodium carbonate buffer (pH 9.5) on ice for 30 min. The products were purified on a PD-10 column (Pharmacia Biotech AB, Uppsala, Sweden) with 0.05 mol PBS. The conjugations to glycolate were performed on four different occasions and showed similar pI profiles.

Radiolabeling

The HuTac Fab was labeled with either ^{125}I or ^{131}I by a modified chloramine-T method (*28*). The glycolated Fabs were labeled with ^{125}I by the same method. Briefly, purified HuTac Fab or glycolated Fab (100 μg) in 0.05 mol phosphate buffer (pH 7.5) and 600 μCi ^{125}I or ^{131}I were mixed with 12 μg of chloramine-T dissolved in 0.05 mol phosphate buffer pH 7.4. After reacting for 5 min, the radiolabeled product was purified using a PD-10 column without addition of reducing agents. The specific activity of the preparations ranged from approximately 3 to 6 mCi/mg, and their purity was >98% as confirmed by instant thin-layer chromotography and size-exclusion HPLC.

Isoelectric Focusing and Autoradiography

Isoelectric focusing was used to compare the pI of intact HuTac Fab to that of the glycolated Fab. Aliquots containing 0.3–1.0 μg nonradiolabeled, nonglycolated Fab or the lightly or heavily glycolated Fab, as well as known standard markers with pI values ranging from 3.5 to 9.3, were applied on a PhastGel IEF 3–9 (Pharmacia Biotech AB). Isoelectric focusing was performed with the Phast System (Pharmacia Biotech AB). Gels were stained with Coomassie blue R 350 (Pharmacia Biotech AB), and the pIs of the different protein bands were determined by extrapolation to a standard curve from the pI markers. The three ^{125}I-labeled Fab preparations (~50,000 cpm), as well as known pI standards, were isoelectrically focused using the same PhastGel system and autoradiographed with a bioimaging analyzer, BAS-150 (Fuji Medical System USA, Inc., Stamford, CT). The pIs of the different radioactivity bands were determined by comparing them with the known standards (Fig. 1).

Immunoreactivity

Immunoreactivity was determined using a modification of the cell-binding assay of Lindmo et al. (*29*) and by assessing the percentage of complexes formed after mixing radiolabeled HuTac Fab or glycolated Fab with the sIL-2Rα (R&D Systems Inc., Minneapolis, MN) using size-exclusion HPLC.

Aliquots of the ^{125}I-labeled HuTac Fab or glycolated Fab (3 ng/100 μl) were incubated for 2 h at 4°C with 1×10^5 to 4×10^7 SP2/Tac cells. The cell-bound radioactivity was separated by centrifuge and counted in a gamma counter. Nonspecific binding to the cells was examined under conditions of antibody excess (25 μg of nonradiolabeled antiTac antibody).

One hundred nanograms (2 pmol) ^{125}I-labeled HuTac Fab or glycolated Fab were incubated with 3 μg (60 pmol) sIL-2Rα for 15 min at room temperature and analyzed by size-exclusion HPLC, using a TSK G2000SW column (0.067 mol sodium PBS-0.1 mol KCl; pH 6.8; 0.5 mL/min) equipped with an on-line NaI gamma detector (γ RAM; IN/US Systems, Inc., Fairfield, NJ), to evaluate the amount of complexes formed. The HPLC elution profile clearly separated ^{125}I-labeled intact HuTac Fab or glycolated Fab from the complexes (data not shown). The fraction of total activity eluting at

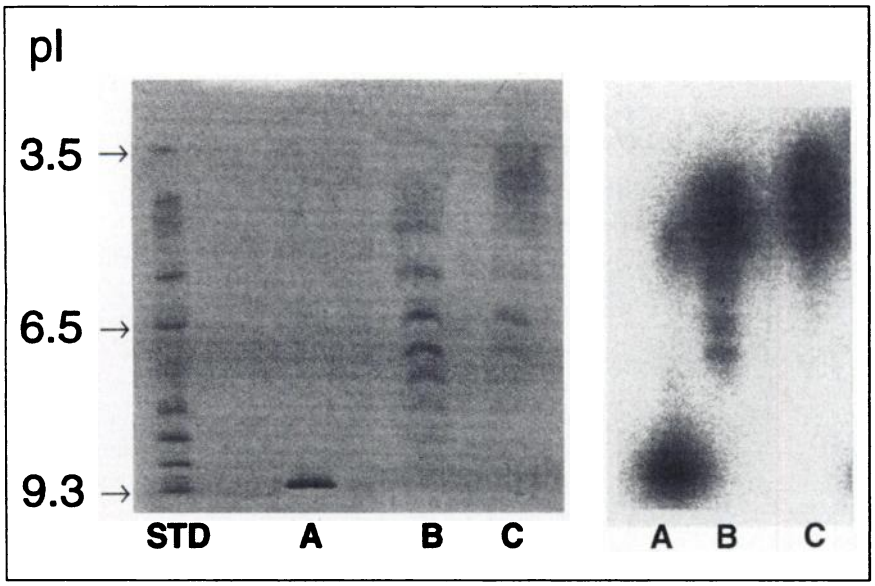

FIGURE 1. Gels for isoelectric focusing. Left: Coomassie Blue staining. Right: autoradiogram of three ^{125}I-labeled preparations. STD = protein standard sample; A = unmodified HuTac Fab. B = lightly glycolated HuTac Fab. C = heavily glycolated HuTac Fab. Numbers are isoelectric points obtained from known standards.

a higher molecular weight than the ^{125}I-labeled Fabs was considered to be the immunoreactive fraction.

Table 1 shows the immunoreactivity of the ^{125}I-labeled HuTac Fab and the lightly or heavily glycolated Fab evaluated by either method.

Scatchard Plot Analysis

A Scatchard analysis was performed. A fixed dose of either ^{125}I-labeled HuTac Fab (5 ng/50 μL) or ^{125}I-labeled heavily glycolated HuTac Fab (5 ng / 50 μL) and increasing doses of unlabeled HuTac Fab or glycolated Fab (5–1000 ng/50 μL) were incubated with 1×10^6 SP2/Tac cells in 100 μL 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 (*28*).

Cell Lines and Animal Models

The binding assay was performed with the IL-2Rα-positive SP2/Tac cell line, a genetically engineered cell line developed by Protein Design Labs (Fremont, CA) (*30*). This cell line was generated by transfecting SP2/0 cells, a nonimmunoglobulin-secreting murine myeloma line (CRL 1581; American Type Culture Collection, Rockville, MD), with the gene that encodes for the IL-2Rα. The cells were provided by Dr. Thomas Waldmann of the Metabolism Branch of the National Cancer Institute and Protein Design Labs. Both cell lines were grown in Dulbecco's modified Eagle's medium (GIBCO Laboratories, Grand Island, NY) containing 10% fetal calf serum (GIBCO Laboratories) and 0.03% L-glutamine at 37°C in 5% CO_2.

Tumor xenografts were generated with the ATAC4 cell line, which expresses the IL-2Rα receptor but does not secrete it into the circulation. This cell line was generated by genetically transfecting the plasmid encoding the IL-2Rα and a neomycin-resistant gene into the receptor-negative A431 cells (*31*). The A431 cells, obtained originally from G. Todaro (National Institutes of Health, Bethesda), are a human epidermoid carcinoma line that does not express IL-2Rα. Both cells were grown in RPMI 1640 medium (GIBCO Laboratories), containing 10% fetal calf serum and 0.03% L-glutamine, at 37°C in 5% CO_2, which was occasionally supplemented with 750 mg/mL sulfate Geneticin (GIBCO Laboratories) to avoid the growth of transformed ATAC4 cells.

Animal studies were performed under an approved Institutional Animal Care and Use Committee protocol. Female athymic nude mice (nu/nu), 5–7 wk old and weighing 15–20 g, were used (Harlan Sprague-Dawley, Frederick, MD). Bilateral tumor xenografts were established by subcutaneous inoculation of 3×10^6 ATAC4 and 5×10^6 A431 cells. Experiments on tumor-bearing mice were performed 10–14 d after implantation, when ATAC4 and A431 tumors weighed a mean of 0.3 ± 0.1 g and 0.2 ± 0.1 g, respectively.

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. The radioactivity was then counted in a gamma counter.

TABLE 1
Immunoreactivity and Affinity Constants of ^{125}I-labeled Antibody Fragments

Method/preparation	^{125}I-labeled HuTac Fab	^{125}I-labeled lightly glycolated HuTac Fab	^{125}I-labeled heavily glycolated HuTac Fab
Immunoreactive fraction by cell-binding assay	76.4%	75.1%	69.8%
Immunoreactive fraction by HPLC	85.2%	84.1%	86.4%
Affinity constant (M^{-1}) measured by Scatchard plot	2.2×10^9	ND	2.2×10^9

HPLC = high-performance liquid chromatography; ND = not determined.

Amino Acid Preparations

A stock solution of L-lysine (270 mg/mL) was prepared in 0.1 mol phosphate buffer, pH 7.5, using L-lysine monohydrochloride

(Pierce Chemical Co.) and 1N NaOH. This solution was coinjected with ^{125}I-labeled glycolated HuTac Fab and ^{131}I-labeled HuTac Fab at a final concentration of 250 mg/mL (*22*).

Biodistribution and HPLC Analysis of Serum and Urine

Six groups of five mice bearing A431 and ATAC4 tumor xenografts were injected intravenously with 1 μg/3 μCi) ^{125}I-labeled heavily glycolated HuTac Fab and 1 μg/5 μCi of ^{131}I-labeled HuTac Fab. The mice were killed at 15, 45, 90, 180, 360 or 960 min after injection. Their blood was drawn, and the serum was separated and analyzed by size-exclusion HPLC. The organs were removed and weighed on an analytical balance, and radioactivity was counted in a gamma counter (Packard Auto-Gamma , Meriden, CT) together with aliquots of blood. To determine the whole-body retention, carcasses were also counted. Data were expressed as percentage of injected dose per gram (%ID/g) of tissue and as ATAC4 (receptor-positive) tumor-to-normal tissue ratios. In addition, when the mice were killed, their urine was collected.

Serum and urine samples from individual mice were analyzed by size-exclusion HPLC using a TSK G2000SW column (Toso-Haas, Philadelphia, PA; 0.067 *M* sodium PBS-0.1 *M* KCl; pH 6.8; 0.5 mL/min) equipped with an on-line NaI gamma detector (γ RAM, IN/US Systems, Inc., Fairfield, NJ). The fractions of activity present as intact Fab or glycolated Fab (retention time 19.4 min), as high molecular-weight complexes or as catabolites were quantified from the HPLC tracing. In the case of serum, these fractions were multiplied by the amount of radioactivity (%ID/g) retained in the blood, which allowed us to determine the total amount of the injected dose in intact Fab or glycolated Fab, complexes or catabolites per gram of the blood.

Comparison of Lightly and Heavily Glycolated HuTac Fab

Four groups of five mice bearing A431 and ATAC4 xenografts were injected intravenously with 1 μg/5 μCi ^{125}I-labeled lightly glycolated HuTac Fab or 1 μg/3 μCi ^{125}I-labeled heavily glycolated HuTac Fab and 1 μg/5 μCi ^{131}I-labeled HuTac Fab. The mice were killed 15 or 45 min after injection. Biodistribution studies and HPLC of serum and urine were performed as described above.

Effects of L-lysine Coinjection

Four groups of four mice bearing A431 and ATAC4 tumor xenografts were injected intravenously with 1 μg/3 μCi ^{125}I-labeled heavily glycolated HuTac Fab and 1 μg/5 μCi ^{131}I-labeled HuTac Fab either in PBS or in PBS containing 50 mg of L-lysine. The mice were killed 15 or 90 min after injection. All procedures of biodistribution and HPLC analysis of serum and urine were performed as described above.

Effects of HuTac Fab or Glycolated Fab on Glomerular Filtration Rate

To evaluate the effects of HuTac Fab or glycolated HuTac Fab on glomerular filtration rate (GFR) two groups of four normal nude mice were injected intravenously with 2 μg/7 μCi ^{125}I-labeled heavily glycolated HuTac Fab or 2 μg/8 μCi ^{125}I-labeled HuTac Fab and 12 μCi ^{99m}Tc-diethylenetriamine pentaacetic acid (DTPA). Serial tail vein blood was collected at 15, 45, 90 and 180 min after injection with 10-μL micropipettes. Data were expressed as percentage of injected dose per gram of blood.

Statistical Analysis

Statistical analysis used the one-way analysis of variance, with pairwise comparison using the Bonferroni method (Sigmastat; Jandel Scientific, San Rafael, CA).

RESULTS

Isoelectric Focusing and Molecular Size

pIs of nonradiolabeled or ^{125}I-labeled HuTac Fab, lightly glycolated Fab and heavily glycolated Fab were >9.3, <8.4 and <7.4, respectively, according to the Coomassie blue staining, and >9.3, <7.4 and <5.9, respectively, according to autoradiography (Fig. 1). The peak retention times of ^{125}I-labeled HuTac Fab, lightly glycolated Fab and heavily glycolated Fab through size-exclusion HPLC using a TSK G2000SW column were 19 min 44 s, 19 min 35 s and 19 min 22 s, respectively.

Immunoreactivity and Affinity of HuTac Fab and Glycolated Fab

There were no obvious differences of immunoreactivity and affinity constants between HuTac Fab and glycolated HuTac Fab (Table 1). Affinity constants of both HuTac Fab (2.2×10^9 mol/L) and glycolated HuTac Fab (2.2×10^9 mol/L) were still high but were a little lower the than intact HuTac IgG (4.3×10^9 mol/L) reported previously (*32*).

Biodistribution and HPLC Analysis of Serum and Urine

The renal accumulation of ^{125}I-labeled heavily glycolated HuTac Fab was significantly lower than that of ^{131}I-labeled HuTac Fab at 15, 45 and 90 min ($P < 0.01$), but was not significantly different at the later time points (180, 360 and 960 min; $P > 0.06$) (Table 2). The tumor uptake of ^{125}I-labeled glycolated HuTac Fab was significantly higher than that of ^{131}I-labeled HuTac Fab at all time points ($P < 0.01$) and was a maximum of 8.5 times higher than the HuTac Fab at 960 min after injection (Table 2). Higher blood retention of the glycolated HuTac Fab was seen at all time points. In spite of the higher blood levels of ^{125}I-labeled glycolated HuTac Fab, the ATAC4 tumor-to-normal tissue ratios or tumor-to-control tumor ratios were higher than those of the nonglycolated HuTac Fab at 90 min after injection and later ($P < 0.03$) (Fig. 2). These ratios were greater than those for most tissues at 180 min or longer after injection of the glycolated HuTac Fab. The ATAC4 tumor-to-blood ratio of ^{125}I-labeled glycolated HuTac Fab was also significantly higher than that of ^{131}I-labeled HuTac Fab at 180 min after injection and later ($P < 0.01$).

Serum fractions analyzed by HPLC demonstrated that in the case of ^{125}I-labeled glycolated HuTac Fab, most of the radioactivity in the serum was found in the intact glycolated HuTac Fab fraction at all time points. However, in the case of ^{131}I-labeled HuTac Fab, more than half of the radioactivity in the serum was found in the catabolite fraction at all time points except 15 min (Fig. 3).

In mice receiving glycolated HuTac Fab, a greater proportion of the excreted radioactivity was excreted as intact Fab

TABLE 2
Serial Biodistribution of ^{125}I-labeled Heavily Glycolated HuTac Fab and ^{131}I-labeled HuTac Fab in Nude Mice Bearing Both ATAC4 (Antigen Positive) and A431 (Antigen Negative) Tumors

Time (min)	Blood	Liver	Kidney	Intestine	Stomach	Spleen	Lung	A431	ATAC4	Whole-body*
					^{125}I-labeled heavily glycolated HuTac Fab					
15	32.2 ± 3.3†	6.9 ± 0.5†	17.6 ± 4.9†	2.7 ± 0.2†	2.4 ± 0.6	4.9 ± 0.6†	14.0 ± 2.7†	2.3 ± 0.5	4.7 ± 0.6†	85.6 ± 2.7†
45	18.3 ± 1.0†	4.2 ± 0.3†	20.8 ± 1.6†	2.6 ± 0.1‡	5.4 ± 1.1†	3.1 ± 0.4‡	9.4 ± 1.1†	3.6 ± 0.2†	9.1 ± 1.3†	76.2 ± 2.4†
90	12.1 ± 1.0†	2.8 ± 0.1†	15.5 ± 0.7†	2.5 ± 0.2‡	9.1 ± 2.2†	2.4 ± 0.2‡	6.5 ± 0.6†	3.8 ± 0.4†	10.7 ± 0.8†	67.0 ± 2.1
180	7.5 ± 0.3†	2.0 ± 0.2†	11.6 ± 1.5	2.0 ± 0.1	10.8 ± 2.5†	1.8 ± 0.2	5.0 ± 0.5†	3.6 ± 0.4†	12.4 ± 1.5†	57.2 ± 2.6
360	4.1 ± 0.3†	1.3 ± 0.1†	7.1 ± 2.6	1.5 ± 0.1	8.6 ± 1.0‡	1.1 ± 0.1†	2.8 ± 0.2†	2.2 ± 0.2†	10.2 ± 1.5†	39.6 ± 2.8†
960	0.8 ± 0.1†	0.3 ± 0.0†	3.6 ± 0.7	0.4 ± 0.1	2.9 ± 1.0	0.3 ± 0.0†	0.7 ± 0.1†	0.7 ± 0.3‡	4.9 ± 1.1†	10.6 ± 1.7
					^{131}I-labeled HuTac Fab					
15	12.2 ± 0.9	3.5 ± 0.4	188.5 ± 8.4	1.9 ± 0.1	3.0 ± 1.3	2.9 ± 0.4	6.2 ± 1.0	1.7 ± 0.4	3.1 ± 0.4	102.6 ± 1.6
45	5.7 ± 0.7	2.0 ± 0.2	105.3 ± 38.0	2.3 ± 0.2	27.8 ± 5.7	2.3 ± 0.4	4.1 ± 0.5	2.9 ± 0.2	5.4 ± 0.9	90.2 ± 5.0
90	4.2 ± 0.5	1.6 ± 0.2	38.2 ± 11.7	2.0 ± 0.4	42.6 ± 9.9	2.0 ± 0.3	3.8 ± 1.0	2.4 ± 0.2	4.7 ± 0.6	67.5 ± 3.0
180	3.9 ± 0.5	1.5 ± 0.2	14.2 ± 5.1	1.9 ± 0.1	27.3 ± 11.0	1.9 ± 0.5	3.2 ± 1.1	2.0 ± 0.5	3.7 ± 0.4	51.9 ± 5.6
360	2.0 ± 0.1	0.8 ± 0.0	6.1 ± 2.1	1.2 ± 0.1	10.9 ± 1.5	0.9 ± 0.1	1.5 ± 0.2	1.0 ± 0.1	2.3 ± 0.4	31.5 ± 1.9
960	0.3 ± 0.1	0.2 ± 0.0	4.0 ± 1.0	0.3 ± 0.1	2.0 ± 1.0	0.2 ± 0.0	0.3 ± 0.1	0.3 ± 0.2	0.6 ± 0.3	8.3 ± 1.9

*Percentage injected dose.
†$P < 0.01$ compared with ^{131}I-labeled HuTac Fab.
‡$P < 0.05$ compared with ^{131}I-labeled HuTac Fab.
All values are %ID/g ± SD, except whole-body.

at all time points than that seen in mice receiving the nonglycolated HuTac Fab ($P < 0.001$). The relative amounts of catabolites in both the glycolated and nonglycolated HuTac Fab groups increased with time but increased much more in the nonglycolated group ($P < 0.001$) (Fig. 4).

The whole-body retention of ^{125}I-labeled glycolated HuTac Fab was significantly less than that of ^{131}I-labeled HuTac Fab at 15 and 45 min ($P < 0.01$), but was similar at 90 min and later.

Comparison Between Heavily and Lightly Glycolated HuTac Fab

Heavily and lightly glycolated ^{125}I-labeled HuTac Fab showed similar distributions in blood, tumor and all organs

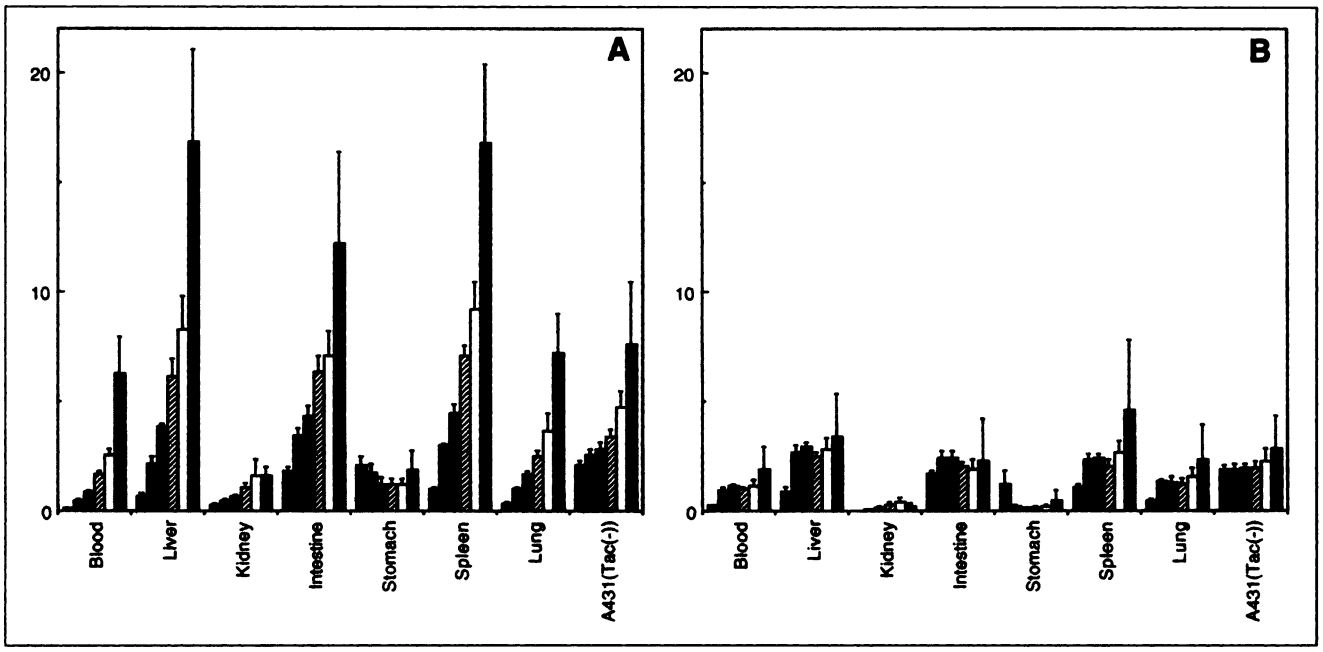

FIGURE 2. Plot of ATAC4 tumor-to-normal tissue ratios of ^{125}I-labeled heavily glycolated HuTac Fab (A) and ^{131}I-labeled HuTac Fab (B) at 15 (■), 45 (▨), 90 (▩), 180 (▨), 360 (□) and 960 (▤) min after injection (n = 5; mean ± SD).

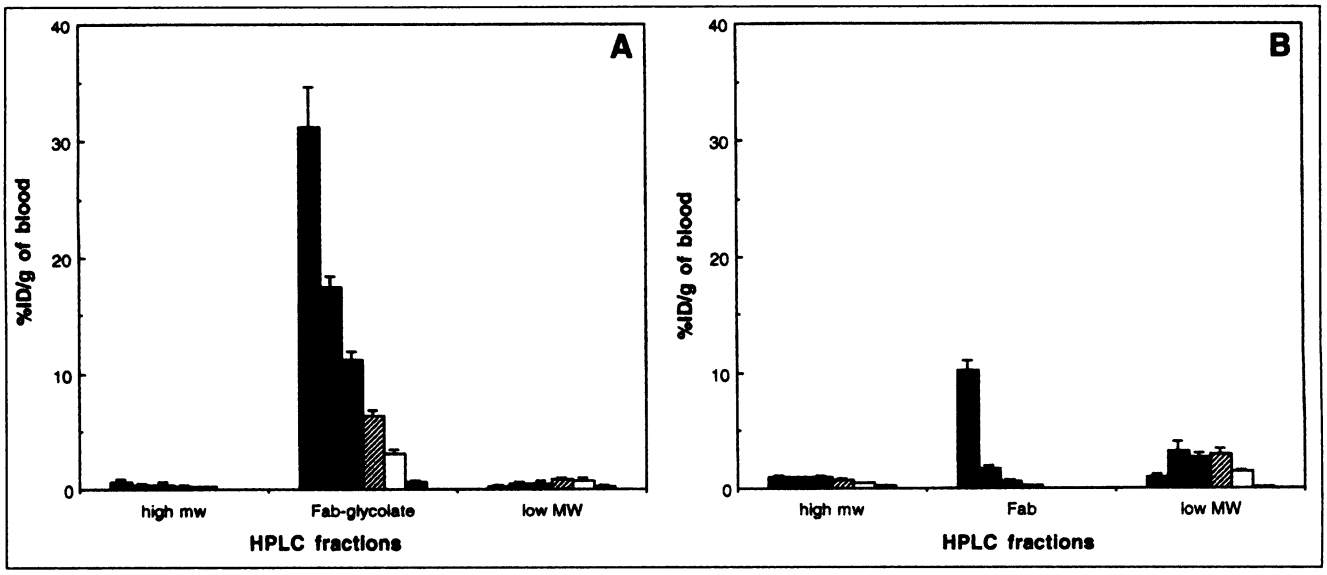

FIGURE 3. Plot of serial serum HPLC fractions of ^{125}I-labeled heavily glycolated HuTac Fab (A) and ^{131}I-labeled HuTac Fab (B) at 15 (■), 45 (▨), 90 (▩), 180 (▨), 360 (□) and 960 (▤) min after injection. (Retention time, high mol wt: 12.5–18 min; Fab (or glycolated Fab): 18–23 min; low mol wt: 25–32 min) Data were normalized to %ID/g of blood (n = 5; mean ± SD).

except the kidney. In the kidney, the accumulation of heavily glycolated ^{125}I-labeled HuTac Fab (18 ± 5 %ID/g at 15 min) was significantly less than that of lightly glycolated ^{125}I-labeled HuTac Fab (34 ± 4 %ID/g at 15 min), and this significant difference ($P < 0.001$) persisted until the 45 min point (Fig. 5).

Effects of L-lysine Coinjection

In the case of ^{125}I-labeled heavily-glycolated HuTac Fab, coinjection of 50 mg L-lysine significantly reduced renal accumulation at 15 min and gastric accumulation at 90 min. Coinjection of L-lysine decreased kidney accumulation at 15 min from 23 ± 4 to 11 ± 1 %ID/g and at 90 min from 16 ± 0 to 14 ± 2 %ID/g ($P < 0.05$) (Table 3). As a result of the lysine coinjection, the percentage of ^{125}I in the urine catabolite fraction decreased from 41% ±3% to 24% ±1% at 90 min ($P < 0.05$) (Table 4). At 15 min after injection, the glycolate conjugated Fab levels in the kidney were similar to the nonglycolated Fab coinjected with lysine (23 ± 4 and 21 ± 4 %ID/g, respectively).

In the case of ^{131}I-labeled HuTac Fab, lysine blocked renal uptake as expected. Coinjection of L-lysine decreased kidney accumulation at 15 min from 175 ± 13 %ID/g to 21 ± 4 %ID/g, and at 90 min from 47 ± 9 %ID/g to 28 ± 4 %ID/g (Table 3). The percentage of radioactivity in intact

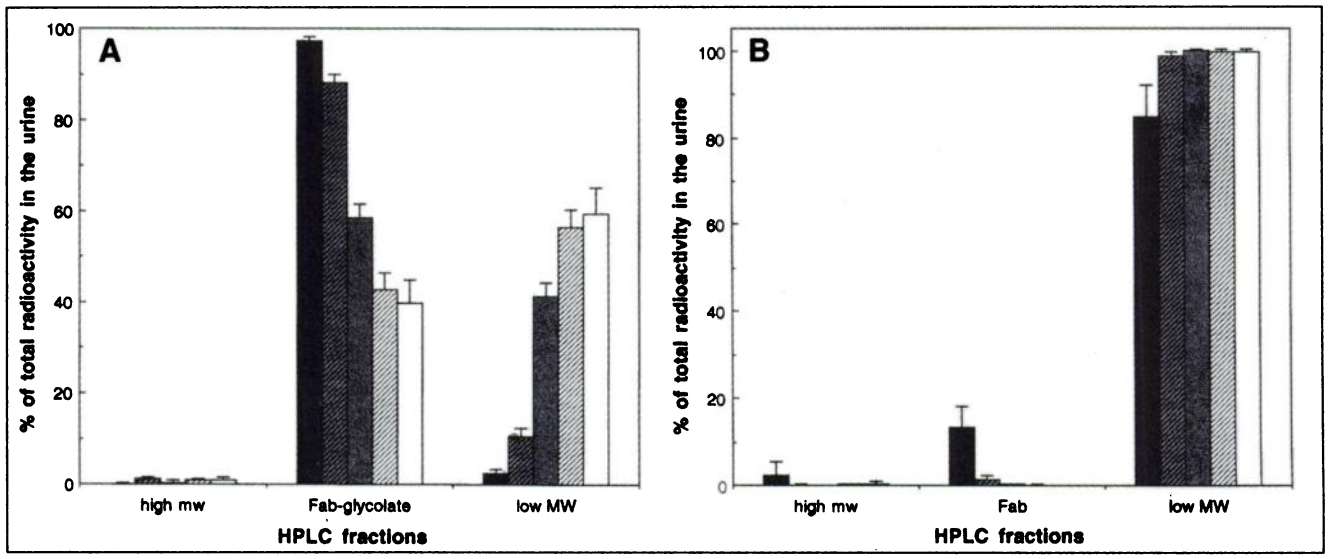

FIGURE 4. Plot of serial urine HPLC fractions of ^{125}I-labeled glycolated HuTac Fab (A) and ^{131}I-labeled HuTac Fab (B) at 15 (■), 45 (▨), 90 (▩), 180 (▨) and 360 (□) min after injection. (Retention time, high mol wt: 12.5–18 min; Fab (or glycolateed Fab): 18–23 min; low mol wt: 25–32 min) Data were expressed as percentage of radioactivity in urine in different fractions (n = 3–4; mean ± SD).

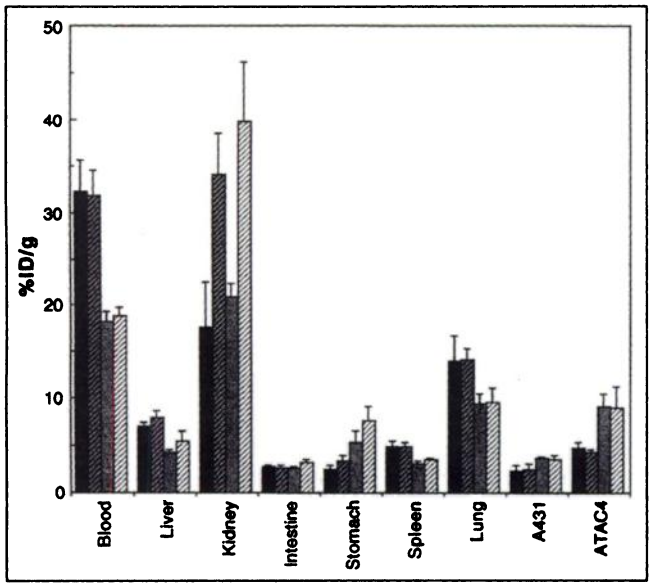

FIGURE 5. Plot of biodistribution of ^{125}I-labeled HuTac heavily glycolated Fab at 15 min (■) and 45 min (■) and ^{125}I-labeled HuTac lightly glycolated Fab at 15 min (▨) and 45 min (▨). Data are given as %ID/g tissue (n = 5; mean ± SD).

Fab excreted in the urine increased from 6% ± 5% to 98% ± 1% at 15 min (Table 4).

Whole-body retention of radioactivity 15 min after injection was largest for the group receiving ^{131}I-labeled HuTac Fab without lysine (104 ± 3 %ID/g), and was smallest in the group receiving ^{131}I-labeled HuTac Fab with L-lysine (53 ± 3 %ID/g).

Effects of HuTac Fab or Glycolated Fab on Glomerular Filtration Rate

There were no differences in the blood clearance of ^{99m}Tc-DTPA between two groups of mice coinjected with ^{125}I-labeled HuTac Fab and ^{125}I-labeled heavily glycolated HuTac Fab (data not shown).

DISCUSSION

The faster tumor localization and higher tumor-to-nontumor ratios associated with Fab fragments (preclinical and clinical) have made them preferred agents for radioimmunodetection. To date, three formulations have been approved by the FDA for clinical use in cancer diagnosis. Nevertheless, higher renal accumulation causes problems in imaging the area around the kidney and in renal dosimetry (*12,14,17,19*). Furthermore, when toxins were conjugated to small fragments of MoAb, renal toxicity was reported (*33*) and thought to be most likely related to high renal accumulation, as reported for Fv-immunotoxin (*34,35*) or neocarcinostacin conjugated with Fab (*33*). In this study, we have shown that by conjugating glycolate to Fab we could favorably alter its biodistribution by decreasing renal uptake, increasing its residence time in the blood and increasing tumor accumulation.

We chose humanized antiTac Fab as our model antibody, because its potential clinical application has been demonstrated in previous trials with intact murine antiTac IgG (*36*). Those trials were limited by development of an immune response. An advantage of this preparation, aside from its being a Fab and therefore less immunogenic, is that it is a humanized fragment and therefore even less likely to elicit an immune response.

The mechanism for this renal blockade with glycolate conjugation is most likely related to a change in pI of the Fab. This mechanism appeared to have two components: (a) a decrease in the filtration rate, which resulted in higher Fab concentration in the blood; and (b) a decrease in proximal tubular uptake, which resulted in higher urinary excretion of

TABLE 3
Biodistribution of ^{125}I-labeled Heavily Glycolated HuTac Fab and ^{131}I-labeled HuTac Fab in Nude Mice Bearing Both ATAC4 and A431 Tumors With or Without Coinjection of 50 mg L-lysine

Time (min)	Lysine (mg)	Blood	Liver	Kidney	Intestine	Stomach	Spleen	Lung	A431	ATAC4	Whole-body*
					^{125}I-labeled heavily glycolated HuTac Fab						
15	50	32.8 ± 0.8	7.0 ± 0.4	10.8 ± 0.4†	2.5 ± 0.4	2.3 ± 0.3	4.7 ± 0.4‡	14.3 ± 1.2	2.4 ± 0.3	4.6 ± 1.1	83.6 ± 1.9
15	0	32.4 ± 1.5	6.4 ± 0.4	22.9 ± 3.7	2.3 ± 0.3	2.0 ± 0.1	4.2 ± 0.0	14.0 ± 0.5	2.6 ± 0.1	3.7 ± 0.6	86.5 ± 2.0
90	50	12.0 ± 0.5	2.6 ± 0.3	13.9 ± 1.6‡	2.1 ± 0.1	4.9 ± 1.3†	1.7 ± 0.1	7.1 ± 0.3‡	3.4 ± 0.2	11.0 ± 0.9	62.7 ± 2.2†
90	0	11.6 ± 0.4	2.6 ± 0.2	16.3 ± 0.1	2.1 ± 0.1	8.0 ± 0.9	1.8 ± 0.0	6.6 ± 0.2	3.6 ± 0.4	10.7 ± 2.3	70.0 ± 2.4
					^{131}I-labeled HuTac Fab						
15	50	13.9 ± 1.1†	3.3 ± 0.3	21.2 ± 3.9†	1.6 ± 0.2	2.3 ± 0.3†	2.5 ± 0.2†	7.1 ± 0.7	1.8 ± 0.2‡	2.9 ± 0.5	52.8 ± 2.7†
15	0	16.7 ± 0.3	3.9 ± 0.5	174.8 ± 13.5	1.8 ± 0.2	3.1 ± 0.1	3.0 ± 0.1	7.5 ± 0.0	2.1 ± 0.1	2.6 ± 0.3	104.4 ± 2.8
90	50	3.4 ± 0.4†	1.3 ± 0.2†	28.2 ± 4.2‡	1.2 ± 0.1†	9.9 ± 2.1†	1.2 ± 0.1†	3.3 ± 0.1‡	1.9 ± 0.3‡	4.0 ± 0.7	41.0 ± 1.7†
90	0	4.5 ± 0.0	1.9 ± 0.2	46.5 ± 9.4	1.8 ± 0.0	46.7 ± 2.2	1.9 ± 0.0	3.8 ± 0.3	2.4 ± 0.1	4.5 ± 0.7	75.0 ± 5.4

*Percentage injected dose.
†$P < 0.01$ compared with no lysine control.
‡$P < 0.05$ compared with no lysine control.
All values are %ID/g ± SD, except whole-body.

TABLE 4
Urine HPLC Fraction of ^{125}I-labeled Heavily Glycolated HuTac Fab and ^{131}I-labeled HuTac Fab With or Without Coinjection of L-lysine

HPLC fractions	^{125}I-labeled heavily glycolated HuTac Fab				^{131}I-labeled HuTac Fab			
	15 min		90 min		15 min		90 min	
	Lysine	No lysine	Lysine	No lysine	Lysine	No lysine	Lysine	No lysine
High MW	1.1 ± 0.0	0.1 ± 0.1	1.1 ± 0.3	0.3 ± 0.5	0.0 ± 0.0	0.4 ± 0.7	0.0 ± 0.0	0.0 ± 0.0
Fab(-glycolate)	97.0 ± 4.3	96.7 ± 1.9	74.2 ± 2.2	58.5 ± 3.1	98.2 ± 1.0	5.9 ± 4.9	3.7 ± 1.8	0.2 ± 0.3
Catabolites	1.9 ± 1.8	3.2 ± 1.9	24.7 ± 2.1	41.1 ± 3.1	1.7 ± 1.0	93.7 ± 4.9	96.2 ± 1.8	99.8 ± 0.3

HPLC = high-performance liquid chromotography; MW = molecular weight.

the glycolated Fab. The change of pI appeared to be important, since the lower pI had lower renal accumulation. This change in charge, which prevented renal tubular uptake, would be analogous to the proposed mechanism of lysine inhibition of renal uptake of small proteins (*37,38*). We speculate that decrease in filtration rate of the glycolated Fab resulted in its higher blood retention. This finding may be similar to that observed with albumin and dextran when they are modified and negatively charged. In that case, the size is similar (*39*) but the charge differences result in different filtration efficiency (40). Our study showed that a general decrease in GFR was not responsible for the changes observed, since administration of both the glycolated Fab and nonglycolated Fab were associated with similar glomerular clearance of ^{99m}Tc-DTPA. We believe it is unlikely that a change in size by glycolation was responsible for these differences in renal and blood accumulation, because the expected size differences were small. There are 28 lysine molecules in the HuTac Fab (*27*), and, if all 28 are conjugated with glycolate, the size increase would be only 1624 daltons (3.2% of Fab mol wt). The higher tumor uptake is probably the result of the higher time integral in the blood (input function). A less likely mechanism that could explain the higher concentration of the glycolated Fab and lower catabolite formation in serum would be if glycolation resulted in stabilization of the radiolabeled Fab.

The differences in renal uptake of glycolated and nonglycolated Fab are particularly pronounced at early times, when the glycolated Fab is excreted intact in the urine. At later times, the kidney is effective in breaking down the Fabs, which are catabolized into iodide or small molecular-weight products that are reabsorbed by the tubules and then excreted in the urine, as shown in our HPLC studies. This rapid catabolism resulted in early differences in whole-body retention as a result of glycolated Fab clearing faster through the kidney than unmodified Fab. Although at 90 min and later the whole-body retention is similar for both reagents, this is a coincidence caused by the rapid breakdown of unmodified Fab in the kidney and clearance of its catabolites.

Although we used ^{125}I as the radiolabel for our Fab because of the convenience of preparation, similar benefits are expected from radiometal isotopes, since the major differences in the kidney were observed at early times with ^{125}I when catabolite formation would be small. It is expected that with ^{99m}Tc or other radiometals, which persist intracellularly for much longer than ^{125}I, the renal blocking effect of the glycolate would have a greater improvement in dosimetry.

Although the glycolate-conjugated Fab showed a decrease in renal uptake similar to that of the nonglycolated Fab coinfused with lysine, this was seen only at early times after infusion. At later times the glycolated Fab showed lower renal uptake. Therefore, the advantage of this glycolate-conjugate approach versus the use of lysine alone is that it does not require multiple injections or a continuous infusion. Nevertheless, it appears to be of some additional benefit to coadminister lysine with the glycolated Fab.

CONCLUSION

We were able to modify chemically HuTac Fab with glycolate without affecting its immunoreactivity or affinity. Furthermore, the biodistribution of the glycolated Fab was more favorable, with lower renal accumulation, longer survival in the circulation, higher tumor uptake and a higher tumor-to-nontumor ratio than the original HuTac Fab. The glycolate conjugation, which may be used in conjunction with lysine administration, is a promising approach that may be applicable to other small fragments to decrease renal accumulation and increase tumor uptake without loss of immunoreactivity.

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