



Lysine-Directed Conjugation of Ethidium Homodimer to B72.3 Antibody: Retention of Immunoreactivity but Altered Tumor Targeting

Ravi S. Harapanhalli, Khalid Z. Matalka, Peter L. Jones, Ashfaq Mahmood, S. James Adelstein and Amin I. Kassis

DEPARTMENT OF RADIOLOGY (NUCLEAR MEDICINE), BRIGHAM AND WOMEN'S HOSPITAL AND HARVARD MEDICAL SCHOOL, BOSTON, MASSACHUSETTS 02115 USA

ABSTRACT. Ethidium homodimer (EHD) was conjugated to B72.3 monoclonal antibody using a method whereby 85–90% of the conjugated EHD remains available for DNA intercalation. Antibody was thiopropionylated by reaction with *N*-succinimidyl 3-(2-pyridyldithio)propionate and reduction of pyridyldithio groups with dithiothreitol. EHD was maleimido-functionalized with succinimidyl-4-(*N*-maleimidoethyl)-cyclohexane-1-carboxylate and treated with thiopropionylated antibody to obtain a conjugate containing ~3.4 EHD per antibody molecule. For biologic studies, ¹⁴C-labeled EHD was synthesized by reductive amination and conjugated as above. *In vitro* the conjugate maintained chemical integrity and immunoreactivity, while *in vivo* its targeting of LS174T tumors was reduced compared with that of iodinated antibody. A decrease in isoelectric point of the immunoconjugate was also observed. NUCL MED BIOL 25;3:267–278, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. B72.3 antibody, Ethidium homodimer, Biodistribution

INTRODUCTION

Immunoconjugates of monoclonal antibodies (MAbs) with drugs (15–18), toxins (1, 6) and radionuclides (8, 14) have gained considerable attention for the treatment of human cancers. The underlying concept is the use of MAbs with their remarkable specificity and selectivity in targeting tumors as carriers of the intended probes to desired sites. The anticipation that such targeting will enhance tumor cell killing while minimizing damage to normal tissues has been shown to be true for various antibody conjugates, including those containing DNA-binding agents such as adriamycin, doxorubicin (17) and vindesine (15, 18).

In addition to the role antibodies play as agents that enhance targeting, many of them are internalized by tumor cells. Consequently, if such antibodies are conjugated to toxic molecules to which the plasma membrane is impermeable, they can be used to bring into the cell agents that are otherwise excluded. Such an approach would enable the assessment of the therapeutic potential of such molecules.

To this end, we have conjugated to the B72.3 MAb the DNA intercalator ethidium homodimer (EHD), a molecule that cannot permeate mammalian cell membranes. Although Wagner *et al.* (20) developed a method for chemically conjugating EHD through its carbohydrate moiety to proteins such as transferrin, their results showed that the conjugate-bound EHD did not intercalate with DNA, presumably owing to some inadvertent chemical change. In the conjugation approach presented here, the protein-bound EHD molecules retain their ability to intercalate with DNA. However, our data reveal that, although the immunoreactivity and the DNA-binding ability of the B72.3(EHD)₃ immunoconjugate were

preserved, its ability to target tumors declined substantially and that this appears to have been caused by an inadvertent change in its isoelectric point (pI).

MATERIALS AND METHODS

General

Ethidium homodimer (EHD-I), *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) and *N*-succinimidyl 4-(*N*-maleimidoethyl)cyclohexane-1-carboxylate (SMCC) were obtained from Molecular Probes (Eugene, OR). All other chemicals were purchased from Aldrich Chemical Company (Milwaukee, WI). [¹⁴C]Formaldehyde at a specific activity of 54 mCi/mmol was obtained from NEN Research Products, DuPont Company (Boston, MA). High performance liquid chromatographic (HPLC) analyses were carried out on a Waters 510 HPLC system (Milford, MA) equipped with a tuneable UV detector. Autogamma 500 (Packard Instruments, Downers Grove, IL) and LS 8000 (Beckman Instruments, Irvine, CA) instruments were used for counting all radioactive samples. Thin-layer chromatographic (TLC) analyses of EHD and its derivatives were carried out on silica plates (mobile phase, *n*-butanol:trifluoroacetic acid:water:acetonitrile:ethyl acetate, 8:0.5:1:16:20).

Production, Purification and Characterization of B72.3 Antibody

CULTURING OF B72.3 HYBRIDOMA CELLS. B72.3 hybridoma cells (#F-8950) from the American Type Culture Collection (ATCC, Rockville, MD) were grown in RPMI-1640 medium supplemented per 500 mL with 10% fetal bovine serum (heat-inactivated), 5 mL of 200 mM L-glutamine and 5 mL of 5000 µg/mL penicillin-streptomycin. The hybridomas were recultured twice a week, and the medium was freshly made every 15 days as L-glutamine degrades with a 15-day half-life.

Address correspondence to: Amin I. Kassis, Ph.D., 220 Longwood Avenue, Goldenson Building, B242, Boston, Massachusetts 02115; e-mail akassis@hms.harvard.edu

Accepted 15 June 1997.

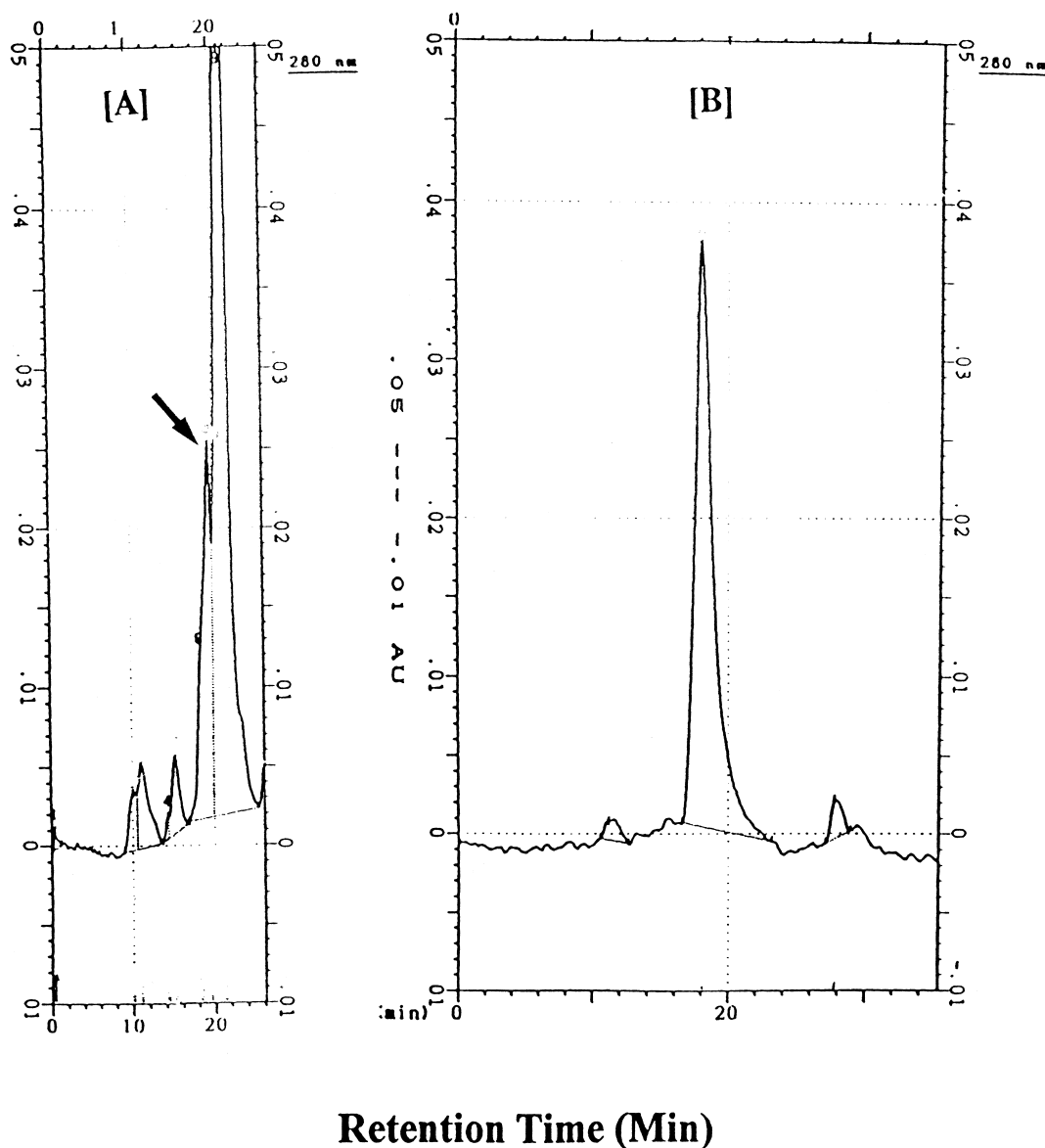


FIG. 1. BioSep-Sec-S 3000 size-exclusion HPLC profile. (A) Crude B72.3 ascites. Note two major peaks corresponding to molecules with molecular weight of albumin and IgG (shown by arrow). (B) Purified IgG peak.

ASCITES PRODUCTION IN MICE. Pristane-primed BALB/c male mice (18–20 g) were obtained from Charles River Laboratories (Wilmington, MA). The hybridomas were injected intraperitoneally (2×10^6 per mouse in 0.5 mL of phosphate-buffered saline [PBS]). If needed, a second dose of hybridomas was injected 7 days later. The ascites was collected on days 18, 22 and 25, and the combined taps were clarified by centrifugation ($10,000 \times g$) and stored at -20°C . The MAbs were isolated on a protein A/G affinity column (No. 20422, Pierce, Rockford, IL) per the manufacturer's procedure. In essence, the fluid was mixed with binding buffer (100 mM TRIS, pH ~ 8) in 1:1 ratio and centrifuged at $10,000 \times g$ to separate lipoproteins. The supernatant was purified on a protein A/G column using elution buffer (0.1 M glycine buffer, pH 2–3). The eluate was immediately neutralized, dialyzed against PBS, pH 7.4, and concentrated by centrifugal membrane filtration (Centricon-30, Amicon, Incorporated, Beverly, MA). With this procedure, $>95\%$ of the IgG was recovered from the column, and this was then

analyzed by size-exclusion HPLC (Fig. 1) on a BioSep-Sec-S 3000 column (7.8×600 mm; Phenomenex, Torrance, CA) using 0.1 M PBS, pH 7.2, as an eluant (1 mL/min). The purified IgG MAb was stored at -20°C in PBS containing 0.1% azide.

RADIOIODINATION OF B72.3. The MAb ($200 \mu\text{g}/100 \mu\text{L}$ PBS) was transferred to a vial precoated with $10 \mu\text{g}$ of Iodogen and $\sim 300 \mu\text{Ci}$ of Na^{125}I was added. The closed vial was kept at ambient temperature for 10 min with occasional shaking. The reaction was stopped by transferring the vial contents to a PD-10 Sephadex column (Sigma Chemical Company, St. Louis, MO) which had been presaturated with 1% bovine serum albumin (BSA) in PBS. The eluates (1 mL each) were collected and checked for absorption at 280 nm as well as assayed for radioactivity in a dose calibrator. The protein eluting in the void volume accounted for 88% of the radioactivity. It was pooled and concentrated on a Centricon-30 and analyzed on instant TLC plates (ITLC SG, Gelman Sciences

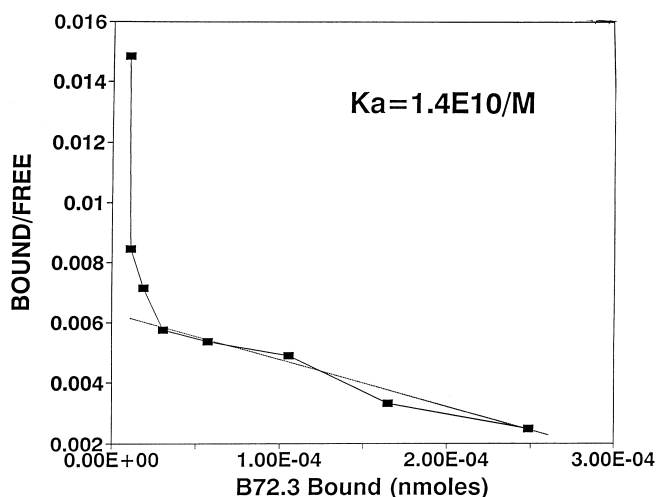


FIG. 2. Scatchard plot of radioimmunoassay of ^{125}I -B72.3 tested against BSM. Note high association constant for purified B72.3 antibody. Dotted line is linear fit through data.

Incorporated, Ann Arbor, MI) with PBS as the mobile phase. The presence of $\geq 98\%$ radioactivity at the origin indicated the purity of this radiochemical. The specific activity of $2.5 \mu\text{Ci}/\mu\text{g}$ of IgG corresponded to an ^{125}I :IgG ratio of 1:6.

ASSESSMENT OF IMMUNOREACTIVITY OF ^{125}I -B72.3 BY DIRECT RADIO-IMMUNOASSAY. Since B72.3 is known to react strongly with bovine submaxillary mucin (BSM), the immunoreactivity of the ^{125}I -B72.3 was assessed in 96-well microtiter plates (Costar, Cambridge, MA) coated with $1 \mu\text{g}$ BSM (4). In brief, serial dilutions of ^{125}I -B72.3 ($15 \mu\text{g}$, $7.5 \mu\text{g}$, etc.), prepared in 0.1 M PBS containing 1% BSA and 0.1% Tween 80, were added to each well and the plates were incubated at 37°C for 1 h. The plates were then washed five times with PBS in a plate washer (model 1250 Immunowash, Bio-Rad Laboratories, Incorporated, Hercules, CA), and the radioactivity associated with each well was counted on a γ -counter. The data were analyzed and are presented as a scatchard plot (Fig. 2).

Synthesis of [N-Methyl- ^{14}C]EHD from [^{14}C]Formaldehyde

EHD (1 mg , $1.17 \mu\text{mol}$) was dissolved in $200 \mu\text{L}$ of methanol, and $250 \mu\text{Ci}$ of an aqueous solution of [^{14}C]HCHO¹ ($10 \mu\text{L}$, $54 \text{ mCi}/\text{mmol}$) was added (Fig. 3). After 30 min at room temperature (RT), $200 \mu\text{L}$ of bicarbonate buffer (0.1 M bicarbonate, 0.5 M NaCl, pH 8.3), $63 \mu\text{L}$ of aqueous sodium cyanoborohydride ($10 \text{ mg}/\text{mL}$, $3.4 \mu\text{mol}$) and $200 \mu\text{L}$ of methanol were added. The tightly closed vial was kept at RT for 48 h and monitored by radio-ITLC in PBS, wherein free formaldehyde and its oxidation products move with the solvent front and EHD remains at the origin. The product (52% yield) was purified by preparative ITLC in PBS. The strip containing EHD was eluted with *n*-butanol:trifluoroacetic acid:water:methyl cyanide:ethyl acetate (8:1:1:16:20) and evaporated to dryness by nitrogen purge. Three coevaporations with dry acetonitrile ensured the product in pure form, free of any organics and moisture.

¹ The purity of commercially procured [^{14}C]formaldehyde was determined by converting it to a precipitable dimedon adduct and estimating the radioactivity (8). A purity of 89% was found.

Formation and Characterization of the EHD-B72.3 Conjugate

SYNTHESIS.² A gently stirred solution of 16 mg of B72.3 in 4 mL of PBS was treated with slow addition of 1 mg of SPDP in $150 \mu\text{L}$ of ethanol at RT for 40 min (Fig. 4); unreacted SPDP was removed by gel filtration on a PD-10 column, and the eluate was concentrated on a Centricon-30. To determine the number of conjugated 2-pyridyl disulfide groups (3), we subjected the immunoconjugate to a buffer exchange with 0.1 M sodium acetate buffer, pH 4.4, containing 0.1 M NaCl using Centricon-30.

To a portion of diluted protein solution at RT, $100 \mu\text{L}$ of 50 mM dithiothreitol (DTT) was added, and absorption at 343 nm and 280 nm was assessed on a UV-VIS spectrophotometer after 30 min. Based on the observed OD_{343} of the solution and the molar absorptivity of 2-thiopyridone (2-PT) $\epsilon_{343} = 8080$, the concentration of 2-PT released by DTT reduction from the MAb was obtained. Knowing the ϵ_{280} of 2-PT (5100), the OD_{280} of the protein = total $\text{OD}_{280} - [\text{concentration } 2\text{-PT} \times 5100]$. To the main stock, 10 mg of DTT was added and incubated for 45 min at RT. The thiolated antibody was purified on a PD-10 column pre-equilibrated with PBS, and the protein eluates were concentrated (*vide infra*) and stored under argon.

To 1 mg of EHD ($1.17 \mu\text{mol}$), a solution of one equivalent (0.143 mg , $1.17 \mu\text{mol}$) of 4-dimethylaminopyridine (DMAP) in $25 \mu\text{L}$ of dry dimethylformamide (DMF) was added. The mixture was vortexed and SMCC (0.39 mg , $1.17 \mu\text{mol}$) in $25 \mu\text{L}$ of dry DMF was added, the vial tightly capped and vortexed again, and the sample was incubated in the dark at RT overnight. TLC analysis revealed a sharp spot ($R_f = 0.41$) above the EHD spot ($R_f = 0.34$) that accounted for more than 85% of the fluorescence absorption. PBS (1 mL) was added, and the solution was extracted with ethyl acetate ($3 \times 1 \text{ mL}$) to remove unreacted SMCC and DMAP. The EHD-containing aqueous phase was purged with argon and added to the thiopropionylated MAb solution in PBS (pH 7.4) under argon. The mixture was gently vortex-mixed and incubated in the dark at RT overnight; 2 mg of *N*-ethylmaleimide in $50 \mu\text{L}$ of DMF was then added and the mixture was incubated for 1 h. Dialysis against PBS ($3 \times 0.5 \text{ L}$) resulted in a bright-red clear solution. Based on the ϵ_{490} of EHD (8.34) and the observed OD_{490} for the conjugate, the concentration of bound EHD was determined. Knowing the ϵ_{280} of EHD (66.94), we calculated its contribution to the absorptivity at OD_{280} . Based on the OD_{280} for protein alone, the protein concentration could also be computed. Routinely three to four EHD molecules were conjugated per molecule of protein.

ASSESSMENT OF THE INTEGRITY OF B72.3(EHD)₃ BY SIZE-EXCLUSION HPLC. A BioSep-Sec-S 3000 size-exclusion HPLC column ($600 \times 7.8 \text{ mm}$) was equilibrated with phosphate buffer (0.1 M , pH 7.2, $1 \text{ mL}/\text{min}$) and calibrated by injecting protein standards of varying molecular weights (Bio-Rad Laboratories, Incorporated): bovine thyroglobulin (MW 670 kDa), bovine γ -globulin (MW 158 kDa), chicken ovalbumin (MW 44 kDa), horse myoglobin (MW 17 kDa) and vitamin B12 (MW 1350 Da). A calibration plot of retention time versus molecular weight was constructed. The retention times of B72.3 antibody, B72.3(EHD)₃ and ^{14}C -labeled immunoconjugate were noted, and from the calibration plot the molecular weights of native and modified antibody were calculated. For the

² The presence of any dissolved oxygen is undesirable during all chemical manipulations involving a thiolated antibody. Therefore, all buffers were degassed *in vacuo* and purged with argon. It was also important to maintain an inert atmosphere during the chemical reactions.

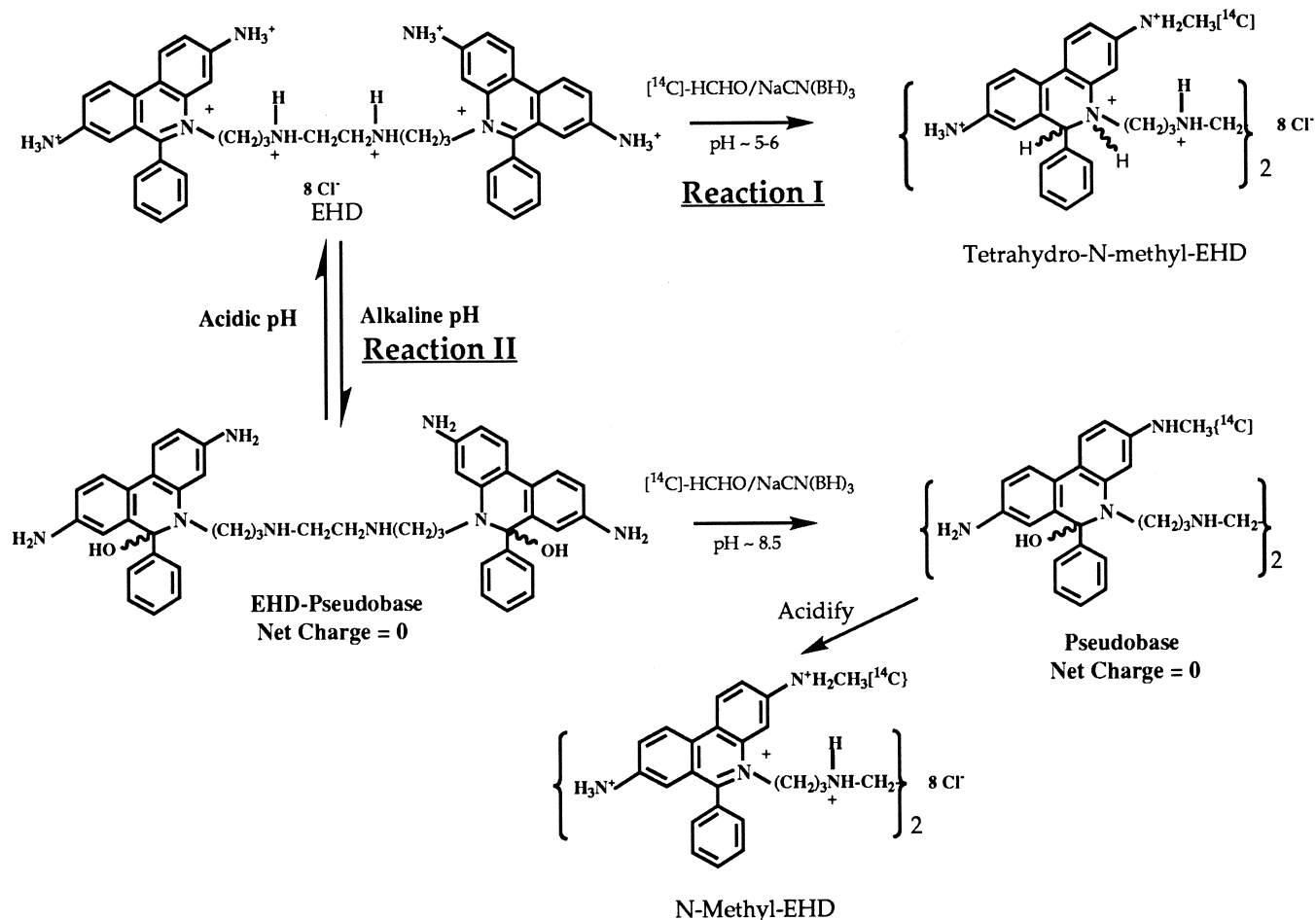


FIG. 3. Schematic for radiolabeling of ethidium homodimer with ^{14}C . Reaction I demonstrates effect of pH on undesirable reduction of cyclic Schiff-base structure. In reaction II, note that at pH 8.8 internal Schiff-base structure tautomerizes to pseudobase form and, following labeling reaction, it reverts back to its original form upon acidification.

construction of a radio-HPLC profile, the eluates from ^{14}C -labeled immunoconjugate were collected every minute and the activity counted in a scintillation counter using Aquasol (20 mL).

ASSESSMENT OF IMMUNOREACTIVITY OF B72.3(EHD)₃ BY INDIRECT RADIOIMMUNOASSAY. The procedure of Carney *et al.* (4) developed for ELISA was adapted with some modifications for radioimmunoassay (RIA). The BSM-coated plates were prepared as described above. Stock solutions of B72.3 and B72.3(EHD)₃ were prepared at 10 $\mu\text{g/mL}$ and were serially diluted down to 0.1 $\mu\text{g/mL}$ in PBS containing 1% BSA and 0.1% Tween 80. A 100- μL aliquot of the antibody solution was added, and the plates were incubated at 37°C for 1 h and then washed five times with PBS in a microplate washer. ^{125}I -rabbit antimouse IgG (NEN Research Products, $\sim 150,000$ cpm/100 $\mu\text{L/well}$) prepared in the same dilution buffer was added, the plates were incubated for 1 h at 37°C and washed five times with PBS in the plate washer before counting.

Determination of pI of MAb by Isoelectric Focusing

The electrophoretic mobilities of proteins were determined in an isoelectric focusing chamber (EC-Apparatus Corporation, St. Petersburg, FL) equipped with a thermal control unit. The samples were focused on a 1% agarose focusing gel (pI 3–10; Iso-Gel, FMC Bioproducts, Rockland, ME) that was prefocused for 10 min at 15°C

at a constant voltage (500 V) with the current and power set at 20 mA and 10 W, respectively. The samples (15 $\mu\text{g}/10$ μL) were loaded at the cathode pole (1 M NaOH) along with pI markers (FMC Bioproducts, Rockland, ME). After focusing at 15°C for 90 min, the gels were fixed for 20 min in a solution containing 18 g of sulfosalicylic acid, 30 g of trichloroacetic acid and 180 mL of methanol diluted to 500 mL with water and then dried, washed and stained with Coomassie brilliant blue R-250.

Preparation of LS174T Tumor-Bearing Mice

All experiments were carried out in compliance with the National Institutes of Health standards and the Public Health Service policy on the use of laboratory animals. Human colonic adenocarcinoma LS174T cells ($\neq\text{F-11130}$, ATCC) were maintained as monolayers in Dulbecco's modified Eagle's medium (GIBCO BRL, Life Technologies, Grand Island, NY) containing 10% fetal bovine serum (50 mL total) and enriched with 100 μM minimum nonessential amino acids, 2 mM L-glutamine and 50,000 U/L of penicillin–streptomycin. The cell cultures were passaged twice a week. To provide single-cell suspensions for injection, the cells were gently pipetted and repeatedly passed through a syringe with a 22-gauge needle. Athymic *nu/nu* female mice 4–5 weeks old (Harlan Sprague Dawley, Indianapolis, IN) were injected subcutaneously in the flank

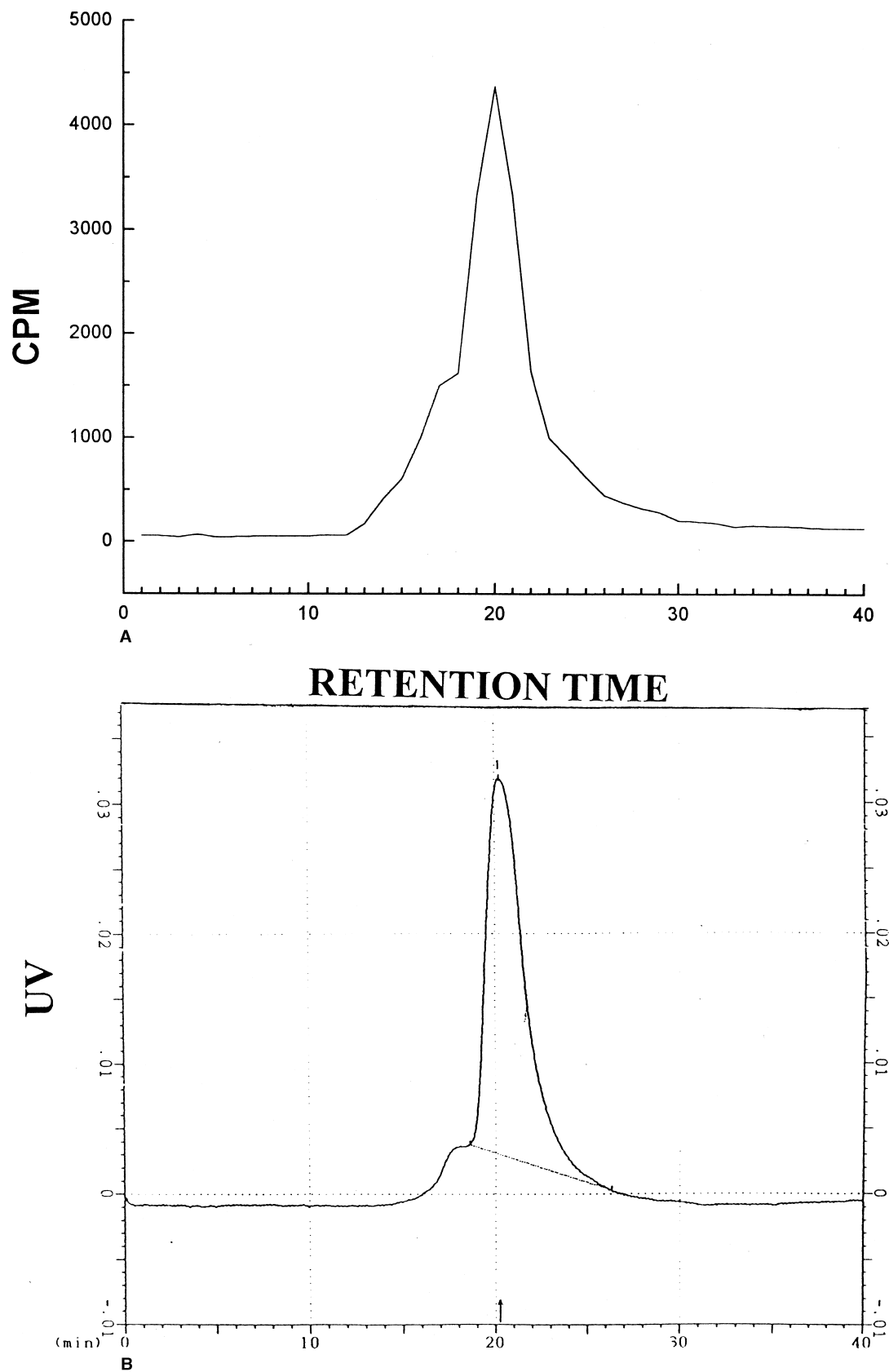


FIG. 5. Size-exclusion HPLC profile of B72.3[^{14}C -EHD] $_3$ conjugate. (A) Monitored by UV at 280 nm. (B) Monitored by liquid scintillation counting. Note chemical and radiochemical homogeneity of immunoconjugate. Also note that there was 1-min delay in measurement of radioactivity from eluates.

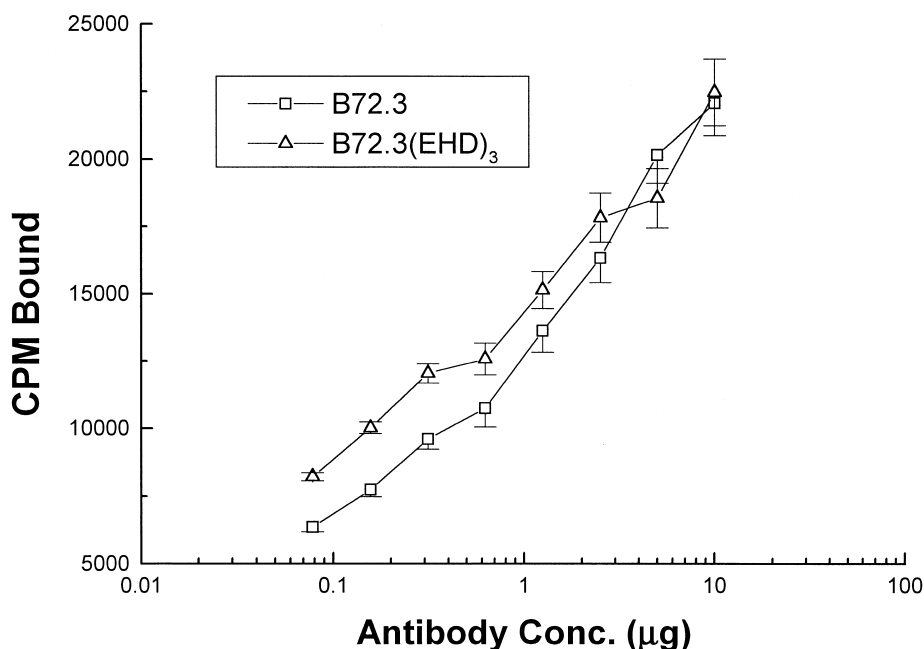


FIG. 6. Indirect RIA of B72.3 and B72.3(EHD)₃ against BSM using ¹²⁵I-rabbit antimouse IgG. Antibody concentration at half-maximum for both proteins is around 1 µg/mL.

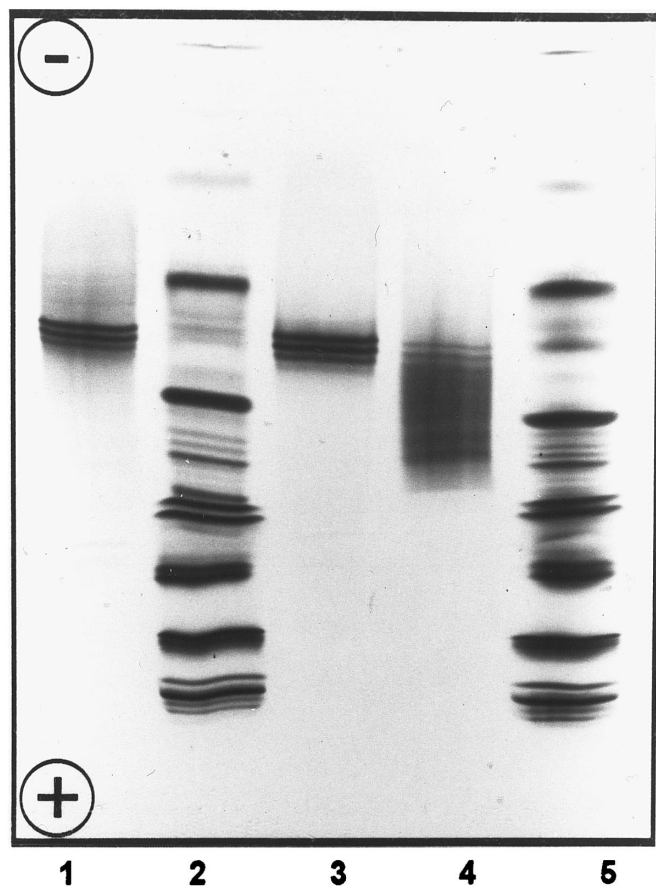


FIG. 7. Isoelectric focusing gel profile of native B72.3, B72.3(EHD)₃ and pI markers. Note presence of three isoforms of native B72.3 (entries 1 and 3) and of several isoforms of B72.3(EHD)₃ (entry 4).

profile (Fig. 1B) demonstrates the presence of a single 150 kDa molecular weight species (19.2 min). Its immunoaffinity is evident from the Scatchard analysis (Fig. 2; $K_a = 1.4 \times 10^{10} \text{ M}^{-1}$) of its binding to antigen BSM and is somewhat higher than the value reported using LS174T antigen TAG-72 (10, 11).

In the synthesis of [*N*-methyl-¹⁴C]EHD from [¹⁴C]formaldehyde by reductive amination (Fig. 3), if the Schiff-base form of EHD is not protected by conversion to its pseudobase, a tetrahydro-*N*-methyl EHD is formed as evidenced by the change in fluorescence from red to blue (reaction I). However, reacting the pseudobase of EHD with formaldehyde prior to its back conversion to the Schiff-base form (reaction II) bypasses this problem. The labeling resulted in a specific activity of 13.5 mCi/mmol from [¹⁴C]HCHO (54 mCi/mmol), indicating that, on average, approximately two molecules of formaldehyde had reacted per molecule of EHD. Radio-TLC showed a single highly fluorescent spot ($R_f = 0.34$) that accounted for >98% of the ¹⁴C activity.

In the procedure for conjugation of EHD to B72.3 (Fig. 4), the thiol groups were introduced by the method of Carlsson *et al.* (3) using SPDP, a heterobifunctional cross-linking agent that reacts with ϵ -amino groups of lysine residues in a nonsite-specific manner. Pyridyldithio groups thus introduced (6–8/IgG) were reduced with DTT at pH 4.4 (acetate–EDTA) wherein only the extrinsic disulfides were prone to reduction. EHD was made thiol-reactive by the introduction of maleimide groups upon reaction with the heterobifunctional agent SMCC. The thiolated antibody reacted with this chemical in oxygen-free PBS (RT, overnight, argon) yielding a conjugate containing three to four EHD molecules. In the same manner [*N*-methyl-¹⁴C]EHD was conjugated to B72.3. In the size-exclusion HPLC (UV/radio) of this radioimmunoconjugate (Fig. 5), a small shoulder at the base prior to the IgG peak may be seen because of the dimeric aggregate (~10–15%) formed during conjugation. Chemical and radiochemical homogeneity of the preparation are confirmed by the observation that all injected activity was accounted for in the IgG peak. A plot of calibration of the HPLC column with various protein standards revealed a good correlation between protein molecular weights and retention times (data not shown). The specific activity of the immunoconjugate was

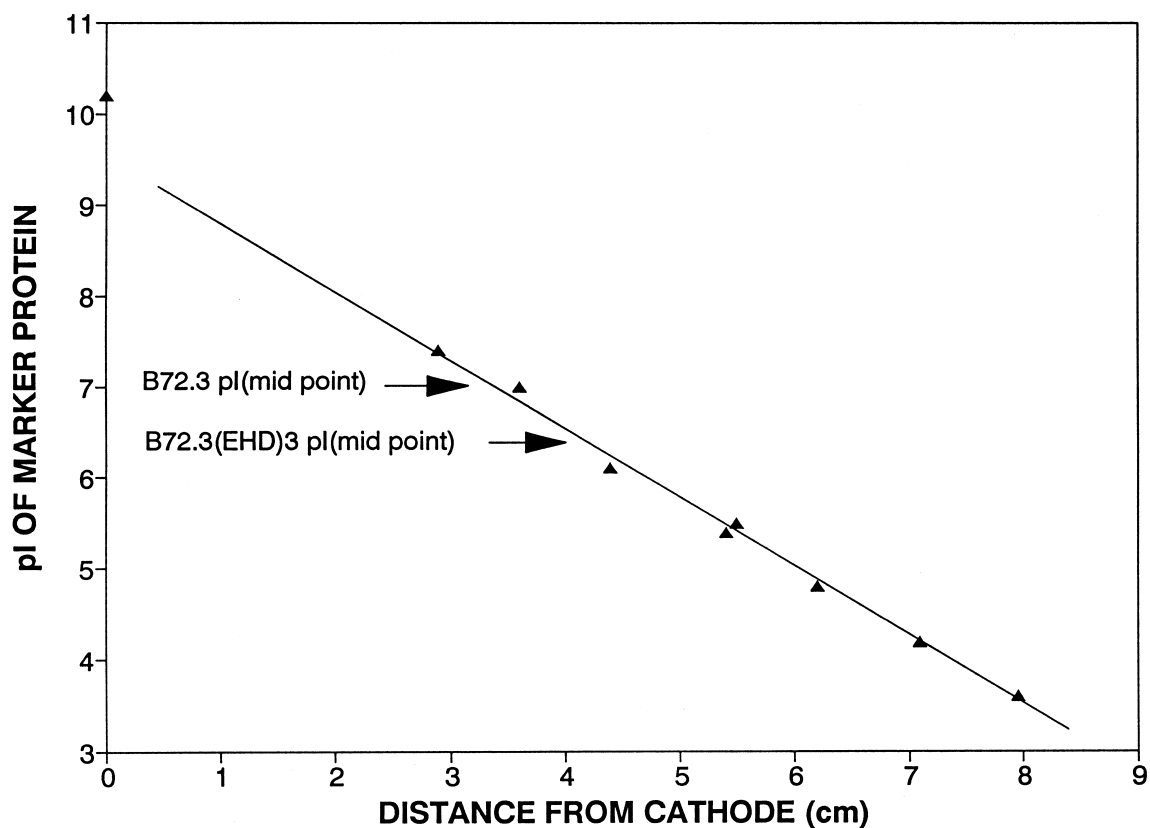


FIG. 8. Calibration plot of pI of known proteins (entries 2 and 5, Fig. 7) versus distance (cm) migrated from cathode pole.

353.8 mCi/mmol; a 3-fold increase from 113.5 mCi/mmol for [N-methyl- ^{14}C]EHD is consistent with the conjugation ratio of 3:1 determined by spectrophotometry. Based on fluorescence titration studies, the ability of protein-bound EHD to intercalate DNA was found to be nearly 90% (data not shown). RIA of B72.3(EHD) $_3$ (Fig. 6) indicates that conjugation of three to four EHD per B72.3 molecule by the SPDP-SMCC method had no effect on its immunoreactivity. The isoelectric focusing data (Fig. 7) show that the native antibody consists of three close moving isoforms (pIs of 7.0, 7.1 and 7.2) with a midpoint pI of 7.1 (lanes 1 and 3). The conjugate (lane 4), on the other hand, has a profile that contains several isoforms of decreased pI from 5.9 to 7.0 (midpoint pI 6.4). The pI of these antibody preparations was deduced from the calibration plot of the pI of the standard markers versus the distance traveled from the cathode (Fig. 8). The marker proteins resolved into distinct bands representing the proteins of varying electrophoretic mobilities (Fig. 7, lanes 2 and 5; pI range 3.6–10.2), and diluting the markers in 10 μL of PBS (equaling that for the antibody samples) did not affect their mobilities (lane 5).

The tumor uptake of ^{125}I -B72.3 (Fig. 9) increased with time, reaching 28–30% of injected dose per gram organ (ID/g) by 24 h, and the activity remained close to these levels thereafter. The radioactivity was not retained in normal tissues and maximum tumor-to-nontumor ratios were seen at 72 h. However, the biodistribution profile of B72.3[^{14}C -EHD] $_3$ (Fig. 10A) reveals a tumor uptake of 7–9% by 12 h that remained steady. This is nearly 3-to-3.5-fold less than the uptake of unconjugated ^{125}I -B72.3. Concurrently, a high percentage of activity was found in the liver (40–60% ID/g), spleen (10–15% ID/g), and kidneys (5–8% ID/g). The radiolabeled ^{125}I -B72.3(EHD) $_3$ shows a similar profile (Fig.

10B) in that tumor activity gradually rose to 8–9% and remained unchanged thereafter. However, its localization within the liver by 1 h ($\sim 20\%$ ID/g) was nearly 2.5-fold less than that of the [^{14}C -EHD]-labeled MAb, and this activity declined thereafter. A concurrent rise of activity in the thyroid was also noted with time. The blood pharmacokinetics of all three agents are summarized in Table 1. While the blood clearance of all preparations was biphasic, the clearance of conjugates was nearly twice as rapid as that of the native antibody.

DISCUSSION

[^{14}C]-formaldehyde (2, 7, 12) was reductively methylated to ethidium homodimer (Fig. 3). Initially labeling at pH 5–6 resulted in a change of the native fluorescence from red to blue, and the silica TLC analysis showed that compared to the R_f of EHD (0.55), the newly formed product had a much higher value of 0.8. Although primary amines are converted into secondary amines during reductive amination, this is not likely to modify the fluorescence properties of EHD. The change in color meant the native chromophore had been modified. The two ring nitrogens in the form of cyclic Schiff bases (*i.e.*, $\text{C} = \text{N}^+\text{R}_1\text{R}_2$), an essential part of the chromophore, may have been reduced inadvertently during reductive amination with cyanoborohydride, and this may have led to the fluorescence color change. To avoid this, the vulnerable chromophore was converted to its pseudobase form under alkaline conditions, reductive amination was carried out, and the original red fluorescence was regenerated by acidification. A radiochemical yield of $\sim 52\%$ and purity of $\geq 98\%$ were achieved following

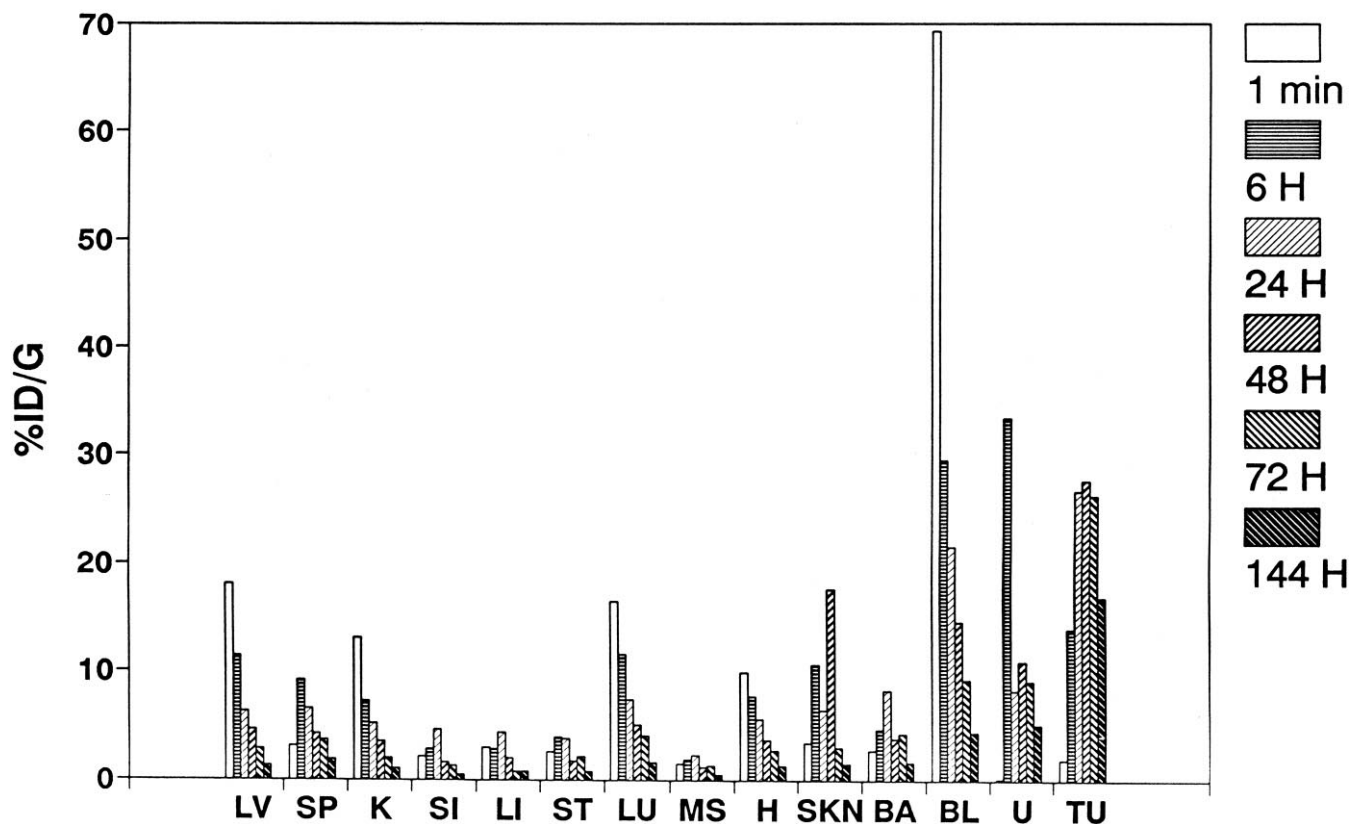


FIG. 9. Biodistribution profile of ^{125}I -B72.3 in LS174T tumor-bearing athymic nude mice. Note that tumor uptake remained steady for up to 72 h after injection and then cleared at very slow rate. Maximum T/NT ratio was seen at 72 h ($n = 5$ and standard deviations between 5–11% of mean). Abbreviations: LV, liver; SP, spleen; K, kidney; SI, small intestine; LI, large intestine; ST, stomach; LU, lungs; MS, muscle; SKT, skeleton; H, heart; SKN, skin; BA, bladder; NK, neck contents; BL, blood; U, urine; TU, LS174T tumor.

purification by preparative ITLC. In essence, a facile and mild method of labeling EHD with ^{14}C has been developed.

In our early attempts to conjugate EHD to B72.3, the maleimido-functionalization of EHD was carried out in PBS using stoichiometric amounts of SMCC. The isolated yield of the immunoconjugate was only ~25%, the remaining being aggregated protein. Realizing that this was due to the lack of solubility of SMCC in aqueous buffers, we then carried out the reaction of EHD with SMCC in DMF, and any unreacted SMCC was extracted out of the solution with ethyl acetate before adding the maleimido-functionalized EHD to the thiopropionylated antibody. This modification improved the yield of immunoconjugate to 60% and emphasizes the need to avoid formation of aggregates associated with the use of SMCC in aqueous solutions.

The biodistribution results of ^{125}I -B72.3 and B72.3[^{14}C -EHD] $_3$ immunoconjugate indicate that while the immunoreactivity of the EHD conjugate was similar to the native iodinated MAb (Fig. 6), its tumor uptake was 3- to 3.5-fold less (7–9% versus 28–30% ID/g). Since the injected activity was recovered in the IgG peak (Fig. 5), this decrease is not due to aggregation of the conjugate as demonstrated by the size-exclusion HPLC analysis of the conjugates. Interestingly, our earlier work (19) had demonstrated that while the radioiodination of the anti-insulinoma MAb A1D2 at iodine:protein ratios of 15:1 and 25:1 minimally affected *in vitro* immunoreactivity assessed by RIA, it nevertheless led to an ~3-fold reduction in tumor uptake *in vivo*; in fact, the higher the iodine:MAB ratio, the lower the tumor uptake. It is clear, therefore, that

such *in vitro* testing of immunoconjugates does not necessarily predict their *in vivo* behavior. Other investigators have also reported such discrepancies between *in vitro* immunoassay and *in vivo* biodistribution results. For example, Sakahara *et al.* (13), assessing the *in vitro* affinity of two MAb undergoing identical labeling procedures and of the same MAb radiolabeled with two different isotopes, found that the data did not reflect the *in vivo* behavior of these conjugates. Rodwell *et al.* (9) compared the *in vivo* behavior of R9.75 MAb iodinated by either conventional tyrosine-directed electrophilic labeling or an iodinated tyrosine-containing peptide site-specifically attached to the oxidized oligosaccharides present on the F_c region of the antibody. In these studies, the site-specifically radiolabeled antibody localized in tumors with an 18-fold greater efficiency than the corresponding conjugate modified nonselectively on its tyrosines, despite the fact that *in vitro* both radioimmunoconjugates had comparable binding properties with the antigen.

To address whether this difference in tumor uptake was a consequence of the dissociation of the ^{14}C label, biodistribution experiments were also performed using an ^{125}I -B72.3(EHD) $_3$ conjugate. A low tumor uptake (8.5% ID/g) corroborated the above results. Interestingly, the liver uptake of ^{125}I -B72.3(EHD) $_3$ was not so high as that seen with [^{14}C -EHD]-labeled immunoconjugate. However, since high radioactivity (^{125}I) was seen in the neck contents and urine, presumably owing to free iodide, the data seem to indicate that while both immunoconjugates were rapidly taken up and retained by the liver, the radioiodine became dissociated

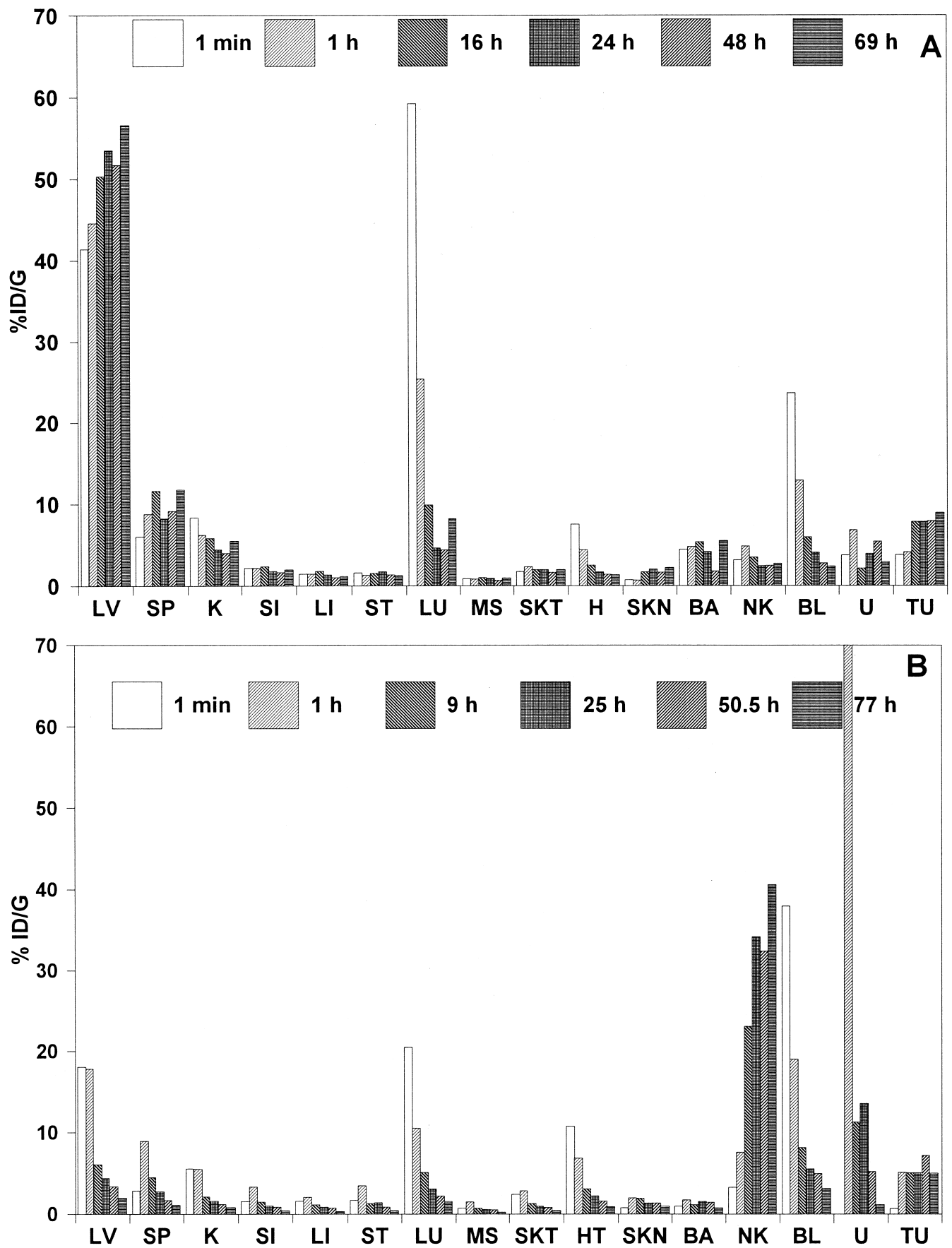


FIG. 10. Biodistribution profile of B72.3(EHD)₃ immunoconjugate. (A) Labeled with ¹⁴C. (B) Labeled with ¹²⁵I. Low tumor uptake (approximately 7–9%) may be noted for both immunoconjugates (*n* = 5 and standard deviations between 6–11% of mean). Abbreviations: LV, liver; SP, spleen; K, kidney; SI, small intestine; LI, large intestine; ST, stomach; LU, lungs; MS, muscle; SKT, skeleton; H, heart; SKN, skin; BA, bladder; NK, neck contents; BL, blood; U, urine; TU, LS174T tumor.

TABLE 1. Blood Pharmacokinetics of ^{125}I -B72.3, B72.3[^{14}C -EHD] $_3$, and ^{125}I -B72.3(EHD) $_3$

Antibody	$t_{1/2}$ (h)		%ID/g					
	α	β	1 min	1 h	6–9 h	24–25 h	48–50 h	69–77 h
^{125}I -B72.3	2.3	57	69.2	ND	29.5	21.5	14.5	9.1
B72.3[^{14}C -EHD] $_3$	1	32	23.6	12.9	ND	4.1	2.71	2.4
^{125}I -B72.3(EHD) $_3$	1	35	38.0	19.0	8.2	5.6	5.0	3.1

from its immunoconjugate (probably by the action of various liver dehalogenases) and was then either excreted or taken up by the thyroid. This argument is supported by various studies demonstrating that the tyrosine-directed iodination of MAb usually leads to *in vivo* deiodination and, therefore, a high thyroid activity. For example, using ^{125}I -81C6 MAb in tumor (D-54 MG)-xenografted nude mice, Zalutsky *et al.* (21) reported that the antibody directly iodinated by Iodogen resulted in high thyroid uptake and increased urinary excretion of radioiodine as compared to the same antibody labeled with a small radioiodinated molecule. It is probable that such *in vivo* deiodination is due to the structural similarity between the iodotyrosines created in conventional protein iodinations and thyroid hormones, compounds known to undergo extensive enzymatic dehalogenation (5).

Since the biodistribution experiments (Figs. 9 and 10) demonstrate a significant reduction in the tumor-targeting capacity of the B72.3(EHD) $_3$ that was due to neither alteration in the immunoreactivity nor molecular weight changes of MAb (Figs. 5 and 6), the pIs of these immunoconjugates were assessed. The midpoint pI of the native B72.3 MAb decreased from 7.1 to 6.4 following EHD conjugation (Figs. 7 and 8). The conjugate also showed a series of closely related bands, indicating a heterogeneity associated with the formation of several isoforms during such lysine-directed conjugation. Such a decrease in pIs could indicate either the acquisition of negative charges by the antibody or the neutralization of its native positive charges during the following three phases of conjugation. (1) *SPDP–antibody conjugate*. Since the SPDP–SMCC-based conjugation method directs the EHD molecules to the ϵ -amino groups of the lysine residues on the antibody, these amino groups will be converted into amides, and for every SPDP molecule conjugated, one positive charge will be neutralized. In our studies, six to eight SPDP molecules were conjugated on average per MAb molecule during its thiopropionylation and, consequently, six to eight lysyl residues were neutralized, an overall loss of six to eight positive charges. (2) *The EHD molecule*. Only one of the eight nitrogen atoms in this molecule was used in its conjugation to the antibody. Thus, the MAb can potentially carry seven positive charges per conjugated EHD molecule. However, the pK_a of the amino groups of EHD differ greatly and, as such, all of them will not be positively charged at physiologic pH. For example, the pK_a of the four aromatic amino groups is ~ 4.6 , and at pH 7.4 they will lose a proton and hence their positive charge. In addition, the two nitrogen atoms within the phenanthridine rings of EHD in the form of Schiff bases, each carrying a single positive charge, could revert to the pseudobase form and also lose their charge at pH 7.4. Because the two amino groups in the aliphatic side chain of EHD are the most reactive, it is highly likely that either one of them could interact with the SMCC cross-linking reagent during the conjugation reaction and, therefore, lose its charge when an amide link is formed. Since the pK_a of such amino groups is in the range of 9–10, the remaining aliphatic amino group would be expected to retain its positive charge at physiologic pH of 7.4 and contribute a single

positive charge to the MAb–EHD immunoconjugate. (3) *MAb–EHD conjugate*. As mentioned above, the conjugation of six to eight SPDP molecules per MAb leads to an overall loss of an equal number of positive charges. In addition, a single positive charge is contributed to each MAb molecule per EHD molecule conjugated. Since three to four EHD molecules were conjugated per MAb in our studies, the change in the antibody pI should therefore reflect an overall loss of three to four positive charges. The observed decrease of the MAb pI from 7.1 to 6.4 (Figs. 7 and 8) agrees with these expectations. Both the differences in the blood pharmacokinetics of ^{125}I -B72.3 and ^{125}I -B72.3(EHD) $_3$ (Table 1) and their biodistribution profiles (Figs. 9 and 10) appear, therefore, to be related to the decrease in pI of the EHD-labeled antibody, although it should be mentioned that changes in hydrophobicity due to conjugation of small molecules such as EHD may as well be responsible in part.

As mentioned earlier, tumor localization of the EHD–immunoconjugate was reduced severalfold compared with ^{125}I -B72.3 MAb. In normal tissues, however, both the uptake and retention differed (Figs. 9 and 10). For example, whereas the localization of the iodinated MAb within the liver was $\sim 20\%$ within the first few minutes after injection and declined rapidly with time, $\sim 40\%$ of the MAb–EHD conjugate activity was found in the liver by 1 min and the radioactivity increased to $\sim 60\%$ over the next 3 days. The altered pI may have led to the recognition and rapid uptake of the MAb by the reticuloendothelial system.

The results herein have confirmed that an *in vitro* immunoreactivity assay is not an indication of the *in vivo* behavior of immunoconjugates. They address the need for determining the extent of tumor targeting of immunoconjugates of drugs and toxins prior to their use in cancer therapy experiments. Owing to the limitations of immunoconjugate prepared by lysine-directed conjugation chemistry, it would be fortuitous to conjugate EHD to B72.3 MAb in a manner producing an immunoconjugate with unaltered pI that would target tumors efficiently ($\sim 30\%$ ID/g). We are presently exploring site-specific conjugation of EHD to the MAb via the carbohydrates of the F_c region with the objective of overcoming the drawbacks in the current procedure and of developing pretargeting approaches for radioimmunotherapy.

This work was supported by a grant from Boston Life Sciences, Incorporated, Waltham, MA. R. S. H. is recipient of a National Research Science Award.

References

- Blair A. H. and Ghose T. I. (1983) Linkage of cytotoxic agents to immunoglobulins. *J. Immunol. Methods* **59**, 129–143.
- Borch R. F., Bernstein M. D. and Durst H. D. (1971) The cyanohydroborate anion as a selective reducing agent. *J. Am. Chem. Soc.* **93**, 2897–2904.
- Carlsson J., Drevin H. and Axén R. (1978) Protein thiolation and reversible protein–protein conjugation: *N*-succinimidyl 3-(2-pyridyldithio)propionate, a new heterobifunctional reagent. *Biochem. J.* **173**, 723–737.

4. Carney P. L., Rogers P. E. and Johnson D. K. (1989) Dual isotope study of iodine-125 and indium-111-labeled antibody in athymic mice. *J. Nucl. Med.* **30**, 374–384.
5. Eary J. F., Krohn K. A., Kishore R. and Nelp W. B. (1989) Radiochemistry of halogenated antibodies. In: *Antibodies in Radiodiagnosis and Therapy* (Edited by Zalutsky M. R.), pp. 83–102. CRC Press, Incorporated, Boca Raton, Florida.
6. Ghose T. I., Blair A. H. and Kulkarni P. N. (1983) Preparation of antibody-linked cytotoxic agents. *Methods Enzymol.* **93**, 280–333.
7. Jentoft N. and Dearborn D. G. (1979) Labeling of proteins by reductive methylation using sodium cyanoborohydride. *J. Biol. Chem.* **254**, 4359–4365.
8. Macklis R. M., Kinsey B. M., Kassis A. I., Ferrara J. L. M., Atcher R. W., Hines J. J., Coleman C. N., Adelstein S. J. and Burakoff S. J. (1988) Radioimmunotherapy with alpha-particle-emitting immunoconjugates. *Science* **240**, 1024–1026.
9. Rodwell J. D., Alvarez V. L., Lee C., Lopes A. D., Goers J. W. F., King H. D., Powsner H. J. and McKearn T. J. (1986) Site-specific covalent modification of monoclonal antibodies: *In vitro* and *in vivo* evaluations. *Proc. Natl. Acad. Sci. USA* **83**, 2632–2636.
10. Roselli M., Schlom J., Gansow O. A., Brechbiel M. W., Mirzadeh S., Pippin C. G., Milenic D. E. and Colcher D. (1991) Comparative biodistribution studies of DTPA-derivative bifunctional chelates for radiometal labeled monoclonal antibodies. *Nucl. Med. Biol.* **18**, 389–394.
11. Roselli M., Schlom J., Gansow O. A., Raubitschek A., Mirzadeh S., Brechbiel M. W. and Colcher D. (1989) Comparative biodistributions of yttrium- and indium-labeled monoclonal antibody B72.3 in athymic mice bearing human colon carcinoma xenografts. *J. Nucl. Med.* **30**, 672–682.
12. Roy R., Katzenellenbogen E. and Jennings H. J. (1984) Improved procedures for the conjugation of oligosaccharides to protein by reductive amination. *Can. J. Biochem. Cell Biol.* **62**, 270–275.
13. Sakahara H., Endo K., Koizumi M., Nakashima T., Kunimatsu M., Watanabe Y., Kawamura Y., Nakamura T., Tanaka H., Kotoura Y., Yamamuro T., Hosoi S., Toyama S. and Torizuka K. (1988) Relationship between *in vitro* binding activity and *in vivo* tumor accumulation of radiolabeled monoclonal antibodies. *J. Nucl. Med.* **29**, 235–240.
14. Schlom J., Eggenberger D., Colcher D., Molinolo A., Houchens D., Miller L. S., Hinkle G. and Siler K. (1992) Therapeutic advantage of high-affinity anticarcinoma radioimmunoconjugates. *Cancer Res.* **52**, 1067–1072.
15. Schrappe M., Bumol T. F., Apelgren L. D., Briggs S. L., Koppel G. A., Markowitz D. D., Mueller B. M. and Reisfeld R. A. (1992) Long-term growth suppression of human glioma xenografts by chemoimmunoconjugates of 4-desacetylvinblastine-3-carboxyhydrazide and monoclonal antibody 9.2.27. *Cancer Res.* **52**, 3838–3844.
16. Shih L. B., Sharkey R. M., Primus F. J. and Goldenberg D. M. (1988) Site-specific linkage of methotrexate to monoclonal antibodies using an intermediate carrier. *Int. J. Cancer* **41**, 832–839.
17. Sivam G. P., Martin P. J., Reisfeld R. A. and Mueller B. M. (1995) Therapeutic efficacy of a doxorubicin immunoconjugate in a preclinical model of spontaneous metastatic human melanoma. *Cancer Res.* **55**, 2352–2356.
18. Starling J. J., Maciak R. S., Law K. L., Hinson N. A., Briggs S. L., Laguzza B. C. and Johnson D. A. (1991) *In vivo* antitumor activity of a monoclonal antibody-*vinca* alkaloid immunoconjugate directed against a solid tumor membrane antigen characterized by heterogeneous expression and noninternalization of antibody-antigen complexes. *Cancer Res.* **51**, 2965–2972.
19. Van den Abbeele A. D., Aaronson R. A., Adelstein S. J. and Kassis A. I. (1988) Does the *in vitro* testing of the immunoreactivity of an antibody reflect its *in vivo* behavior? *J. Nucl. Med. Allied Sci.* **32**, 260–267.
20. Wagner E., Cotten M., Mechtler K., Kirlappos H. and Birnstiel M. L. (1991) DNA-binding transferrin conjugates as functional gene-delivery agents: Synthesis by linkage of polylysine or ethidium homodimer to the transferrin carbohydrate moiety. *Bioconjugate Chem.* **2**, 226–231.
21. Zalutsky M. R., Noska M. A., Colapinto E. V., Garg P. K. and Bigner D. D. (1989) Enhanced tumor localization and *in vivo* stability of a monoclonal antibody radioiodinated using *N*-succinimidyl 3-(*tri-n*-butylstannyl)benzoate. *Cancer Res.* **49**, 5543–5549.