Antibodies as Drug Carriers. II. For Proteins

Bruce N. Rehlaender[1,2] and Moo J. Cho[1,3]

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Purpose. To evaluate the potential use of antibodies as a carrier for monovalent protein haptens.

Methods. A single –SH functionality present in the human IgG light chain was fluoresceinated. This conjugate, FL-LC, was treated with pepsin to obtain FL conjugate of half light chain, FL-$(LC)_{½}$, of MW 11 kDa. These two were radiolabeled using [^{3}H]-propionic acid *N*-hydroxysuccinimide ester, and administered via tail vein to FL-immunized or mock-immunized mice. The blood radioactivity was measured over a 72-h period. Attempts were made to measure the affinity constant for the interaction between the conjugates and anti-FL antibodies by fluorescence quenching, surface plasmon resonance spectroscopy, and competitive ELISA.

Results. All of the three methods used produced supportive, if not conclusive, evidence of decreased binding affinity with increased conjugate size. Subsequent to tail-vein injection to FL-immunized mice, FL-LC showed approximately 4-fold smaller volume of distribution than mock-immunized mice: 0.041 ± 0.005 vs. 0.16 ± 0.02 mL/g. Corresponding values for FL-$(LC)_{½}$ were significantly larger: 0.070 ± 0.013 and 0.30 ± 0.02 mL/g, respectively. Compared with a small FL conjugate of ethanolamine, FL-EA, we studied earlier, the dose-normalized concentrations of the protein conjugates started at a higher level but declined more rapidly with time. In mock-immunized mice, the radioactivity disappeared very rapidly after administration, followed by an extremely slow decline with half-life close to 60 h. Evidence is provided to support that the radiolabel dissociated in the kidney, however, binding to anti-FL antibodies greatly stabilized the conjugate.

Conclusions. Based on an entropic principle alone the affinity of monovalent hapten-antibody interaction is expected to diminish with increase in hapten size. As such, the size of a hapten should be an important determinant of its pharmacokinetics in animals harboring antibodies that recognize the hapten. Relative to what was observed with small MW FL-EA, the protein conjugates showed substantially sustained circulation as a result of antibody binding, but this effect was diminished at later time points. Both affinity and pharmacokinetic data are consistent with the hypothesis of reduced affinity with increasing MW for monovalent hapten conjugates, but neither offered overwhelming proof.

KEY WORDS: endogenous/natural antibodies; titer; affinity; pharmacokinetics; protein carrier.

INTRODUCTION

The clinical potential of highly potent recombinant proteins has been severely hampered by rapid clearance from the body (1). Giving higher doses over a sustained period of time is prohibitive in terms of both cost and side effects (2). With rapidly advancing biotechnology, the dilemma associated with high potency but rapid clearance of proteins is likely to become increasingly familiar. Thus delivery systems that can prolong the circulation of this class of drug molecules are urgently needed. Covalent modification of proteins with hydrophilic polymers such as polyethylene glycol protects the protein from degradative enzymes and from other clearance mechanism (3). However, shielding a protein from enzymes and uptake mechanisms without shielding it from its site of action is seldom a simple feat. Although technology is improving in this field (4), it seems unlikely that it will be effective for all potential biomolecular drugs.

As our preceding report implies (5), using endogenous antibodies as carrier proteins would require identifying a single epitope against which a majority of the population has pre-existing antibodies. In theory this universal epitope could be attached to any protein. As with small molecules, the effectiveness of this system would depend, to a great extent, on the strength of the interaction between the endogenous antibody and the protein. In all likelihood, antibodies against any "universal" epitope would have different affinities in different patients, and in some cases, the affinities might be just high enough to shown a carrier effect. Thus a key question is whether and to what extent the strength of the interaction will change the pharmacokinetics of a protein when it is coupled to the hapten. As applied to the model system described in the preceding paper (5), the question becomes whether anti-fluorescein antibodies will bind fluorescein (FL) as strongly when it is attached to a large protein molecule as they do when it is attached to a very small molecule such as ethanolamine.

A low MW hapten has a limited number of orientations with which it can approach an antibody binding site, while a large molecule with a single epitope has a much greater surface area with which it can approach the binding site. Although the hypothesis of reduced affinity with increasing MW based on this basic entropic principle (6) and its implication in antibody drug carrier is inherently simplistic, testing it is deceptively difficult. Rigorous evaluation of the hypothesis requires the determination of affinity constants for monovalent conjugates of the same hapten with proteins of similar structure but different MW. Our approach has been to use light chain (LC) and half light chain [$(LC)_{½}$] of human IgG as model proteins of MW 25 kDa and 11 kDa, respectively. Note that each contains only one –SH functionality (7), to which our hapten FL is chemically attached. The goals of the experiments outlined in this paper are to evaluate the use of antibodies as carriers for these FL-containing proteins and to assess the effect of protein size on their pharmacokinetics as dictated by varying affinity.

MATERIALS AND METHODS

Materials

Human serum (Cat. # H1388), dithioerythritol (DTE), 5-iodoacetamidofluorescein (5-IAF), and pepsin were purchased from Sigma (St. Louis, MO). The bicinchoninic acid (BCA) protein assay kit (Cat. # 23250), methylmethanethiosulfonate (MMTS), and tris(2-carboxyethyl)phospine

[1] Division of Drug Delivery and Disposition, School of Pharmacy, CB # 7360, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7360.

[2] Present address: Cirrus Pharmaceuticals, P.O. Box 14748, RTP, North Carolina 27709.

[3] To whom correspondence should be addressed. (e-mail: m_j_cho@unc.edu)

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(TCEP) · HCl were all purchased from Pierce Chemical Co. (Rockford, IL). [^{3}H]-Succinimidyl propionate at a specific activity of 100 Ci/mmol was obtained from Amersham (Piscataway, NJ).

Sephadex G-75 of different gel sizes and Superdex 75 (Prep Grade) were obtained from Pharmacia (Uppsala, Sweden). Econopac Q anion exchange cartridge columns, and Readi-Gel for sodium laurylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from BioRad (Hercules, CA). Gels were run in a Mini Protean 3 electrophoresis tank (BioRad). Nunc Maxisorp certified 96-well plates were used for the ELISA assay (Nunc, Naperville, IL). Goat anti-mouse peroxidase conjugate (Cat. #A3673), anti-FL monoclonal antibody IgG1 isotype (FL-D6; Cat. #F5636), and *o*-phenylenediamine · 2HCl (OPD) tablets (cat. # P9187) were obtained from Sigma (St. Louis, MO). Plates were read on a BioRad plate reader (BioRad, Hercules, CA). Fisher EconoScint II liquid scintillation fluid was used with a Packard 2100 liquid scintillation counter (Packard Instruments, Downers Grove, IL). In most instances, 5 mL cocktail was added to samples of less than 0.5 mL volume. The counting efficiency was in the range of 45% and the quenching curve, whenever needed, was generated by following the procedure given by the manufacturer.

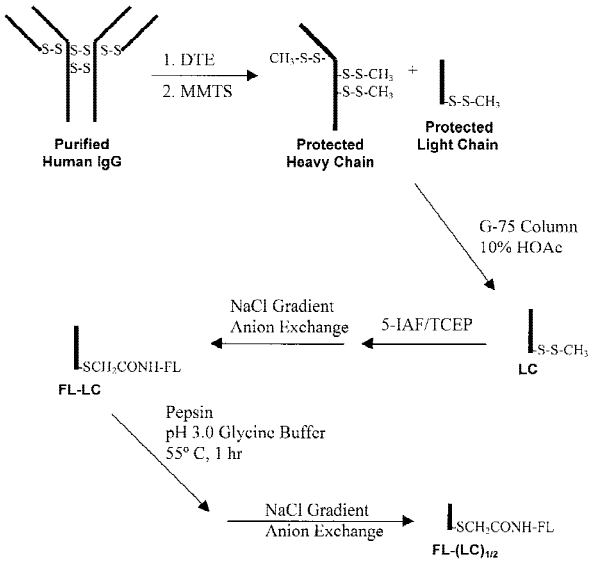

Fig. 1. Preparation of fluoresceinated human IgG light chain (FL-LC) and fluoresceinated human half IgG light chain [FL-(LC)$_{½}$] from purified human IgG. DTE, dithioerythritol; MMTS, methylmethanethiosulfonate; 5-IAF, 5-iodoacetamidofluorescein; TCEP, tris(2-carboxyethyl)phospine; and FL, fluorescein.

Human IgG Light Chain (LC)

Human IgG was purified from serum following a standard procedure (8). Briefly, $(NH_4)_2SO_4$ precipitates were dissolved in tris(hydroxymethyl)aminomethane (Tris) buffer at pH 8.0 and dialyzed overnight. It was then subject to Sepharose Q column separation. Fractions containing pure IgG were pooled, dialyzed against H_2O and lyophilized. Protein purity and MW were routinely estimated with SDS-PAGE using 4-15% gradient on Mini Protean 3 electrophoresis unit (BioRad).

To 2.0 mL of 0.1 M Tris buffer at pH 8.2 that contained 50-100 mg of purified IgG, DTE was added to a concentration of 25 mM (Fig. 1). After 1 h at RT, MMTS was added to a concentration of 75 mM and further stirred for 1 h under argon protection. The often turbid mixture was transferred to a 12–14 kDa MW-cutoff dialysis sac and dialyzed overnight at 5°C against 10% acetic acid (HOAc). It was then loaded on a Sephadex G-75 gel column and eluted with 10% HOAc. The second peak, containing primarily 25-kDa protein as confirmed by SDS-PAGE, was pooled, dialyzed, and lyophilized after pH adjusted to 9.6 with NH_4OH.

Fluoresceinated Human IgG Light Chain (FL-LC)

To 5 to 10 mg of LC in 1-3 mL of 0.2 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer containing 5 mM ethylenediaminetetraacetic acid (EDTA) at pH 7.4, 20 μL of a dimethylformamide (DMF) solution of 5-IAF and 20 μL of HEPES buffered TCEP solution were added such that the final concentrations of 5-IAF and TCEP were 5 and 3 mM, respectively. The reaction mixture was argon-protected and stirred at RT for 1 h. It was then loaded onto a small Sephadex G-25 column and eluted with PBS. After dialysis against 25 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer pH 6.5, the product was further purified by NaCl-gradient anion exchange chromatography on a 1 mL Econopac Q column (BioRad) at a flow rate of 0.5 mL/min. Elution was monitored by the absorbance ratio at 494 and 280 nm after the pH of each fraction was adjusted to 8.5 with Tris base. Fractions with approximately equal A_{280}/A_{494} ratios were pooled and dialyzed against H_2O or PBS depending upon the intended use.

Fluoresceinated Human IgG Half Light Chain [FL-(LC)$_{½}$]

To a solution of 5 mg/mL FL-LC in glycine buffer at pH 3, pepsin was added to a final pepsin-to-LC ratio of 1:500. After 2 h at 55°C, the pH was raised to 9–10 with NaOH, and urea was directly weighed into the solution to a concentration of 6 M. The solution was loaded on a 0.7 × 30 cm Superdex (Prep Grade) 75 column and eluted with 6 M urea in 25 mM bicarbonate buffer at pH 9.6. Fractions containing the 11 kDa fragment were pooled, dialyzed against H_2O, and lyophilized. Product was purified by gradient ion exchange as described for the light chain fluorescein conjugate.

Radiolabeling

The original stock solution of [^{3}H]-succinimidyl propionate in toluene, 400 μL containing 0.4 mCi, was evaporated in a 1-mL glass vial. To this 2 mg of lyophilized FL-LC in 50 μL of PBS was added. The reaction was allowed to proceed for 2 h at RT. Completeness of the reaction was checked by TLC on silica plates developed with a 1:1 mixture of toluene and ethyl acetate. The reaction mixture was loaded on a 2 mL Sephadex G-25 column and was eluted with PBS. Approximately 400 μL of radiolabeled protein with high specific activity was obtained. A stock solution for animal experiments was prepared by adding 200 μL of the above tritiated FL-LC solution to 1.0 mL cold FL-LC in PBS at 0.3 mM. Specific activity was determined by counting the radioactivity of 5-μL aliquots of the final solution and measuring the protein content by BCA assay.

The half light chain conjugate, FL-$(LC)_{½}$, was radiolabeled in the same manner except that a solution of FL-$(LC)_{½}$ was passed through a 2-mL G-25 column equilibrated with PBS prior to radiolabeling. A 100-μL aliquot from the gel-filtration was added to [^{3}H]-propionic acid NHS ester. This procedure resulted in a much higher radiochemical yield (~43%) and specific activity.

Affinity Determination

Fluorescence Quenching

Affinity constants for binding of anti-FL antibodies to FL-LC, FL-$(LC)_{½}$, and a low-MW peptide fragment were determined by a classical fluorescence quenching method (9). The low-MW peptide was conveniently prepared by digesting FL-LC with trypsin at a 1:200 ratio, and then separating the fragment on a G-25 gel. Based on the known amino acid sequence and the known trypsin cleavage site, we estimated its MW to be close to 0.82 kDa. Its concentration was determined by A_{494} with FL-LC as a standard. Fluorescence intensity was measured as a function of anti-FL concentration at a constant 10 nM conjugates in PBS at RT. A Perkin Elmer Model LS50B spectrofluorometer was used for the measurement with excitation and emission wavelengths of 493 and 513 nm, respectively. Antibody used was purified from pooled peritoneal ascites from FL-immunized mice through two rounds of $(NH_4)_2SO_4$ precipitation. The concentration of anti-FL antibodies was estimated based on titer determined by enzyme linked immunosorbent assay (ELISA). Fluorescence intensity data were fitted to a theoretical equation expressing the equilibrium constant (9).

Plasmon Resonance Spectroscopy

On- and off-rates for binding of FL-LC and FL-$(LC)_{½}$ to anti-FL monoclonal antibody FL-D6 was determined on a Biacore 2000 (Pharmacia Biosensor AB, Uppsala, Sweden) using a CM5 sensor chip (10). The antibody was attached to the sensor chip following the procedure recommended by the manufacturer at antibody dilution of 100 μg/mL in 10 mM acetate buffer at pH 5.0. The conjugates diluted to 25, 100, 500, and 1000 nM in PBS were introduced to the sensor chip at a flow rate of 10 μL/min.

Competitive ELISA

The ability of FL-LC and FL-$(LC)_{½}$ to compete with FL-labeled bovine serum albumin (FL-BSA) in binding to anti-FL antibodies was evaluated in a competitive ELISA format. After FL-BSA was plated on a 96-well microplate, different dilutions of the conjugates were introduced along with anti-FL monoclonal antibody, FL-D6, diluted at 1:2500. Then a secondary antibody, goat anti-mouse IgG peroxidase conjugate (Sigma Cat. #A3673), was introduced. Otherwise, the procedure was the same as for normal ELISA.

Animal Experiments

Male BALB/c mice (25–30 gm; from Charles River) were immunized and anti-FL titers were determined as described in the preceding paper (5). Four to seven weeks after the final inoculation, mice were given a 100-μL bolus injection of a conjugate solution via the tail vein. Blood was sampled by nicking the tail vein. In some cases, peritoneal fluid was collected by inserting a 25 G needle into the peritoneal space and withdrawing 25–50 μL of fluid.

Stability of Radiolabel *In Vivo*

Gel filtration was used to establish *in vivo* cleavage of [^{3}H]-propionic acid from the conjugates. Glass Pasteur pipettes with 5-cm Sepharose G-25 were used to fractionate 50 μL samples spiked with high-MW blue dextran (Pharmacia) in PBS. Eluent was collected in fractions of 15 drops beginning when the dextran is about to come out.

RESULTS

Radiolabeled FL-LC and FL-$(LC)_{½}$

As outlined in Fig. 1, the light chain of human IgG was obtained by reducing the immunoglobulin with DTE. The single –SH group present was protected with a mixed disulfide by means of $CH_3SO_2SCH_3$ (MMTS) (11). After size exclusion chromatography, the disulfide bond was once again reduced to free –SH with TCEP (12) and was fluoresceinated with 5-IAF. The reaction was run in several batches, each of which corresponded to 25–50 mg of purified human IgG as starting material. Yield was generally low due to precipitation of product at some stages and the number of steps in the synthesis and purification.

Initial lots were prepared with 1 mM TCEP and 3 mM 5-IAF, but these conditions led to the appearance of a significant amount of a 50 kDa protein, reducing the overall yield. This protein was presumed to be the product of the disulfide linkage of two light chains. Increasing the TCEP and 5-IAF concentrations to 3 mM and 5 mM, respectively, markedly decreased the appearance of the 50 kDa protein without changing the level of fluorescein conjugation in the final product. Successive fractions from ion exchange chromatography for which the A_{280}/A_{494} ratio was at about 0.6 were pooled as product.

A single green fluorescent band was visible after 4–15% SDS-PAGE. As shown in the second marked lane on Fig. 2, it was also the only band visible with Coomassie blue stain, and corresponded to a MW of 25 kDa. Protein concentration of the product was determined by the BCA assay (Pierce) using a standard curve prepared with highly purified human IgG light chain. The FL concentration was estimated from A_{490} against a standard curve prepared from 5-IAF reacted with an excess of cysteine. Fluorescein-to-protein ratios for individual batches of product are given in Table I.

A fluorescein-to-protein ratio close to 0.8 was consistently obtained, even when reaction conditions were changed. Because the ratio did not increase with increasing concentrations of 5-IAF and TCEP in the reaction mixture, it is believed that the measured 0.8 ratio represents a completed reaction with an actual ratio of 1.0. The measured ratio is less than unity probably because the A_{490} of FL is somewhat muted by its conjugation to the protein. Compared with 5-IAF, the UV spectrum of FL-LC indeed underwent a 5-nm bathochromic shift. In the absence of a truly appropriate standard, it seem reasonable to assume that the measured 0.8 ratio represents a 1:1 conjugation. Reaction of FL-LC with

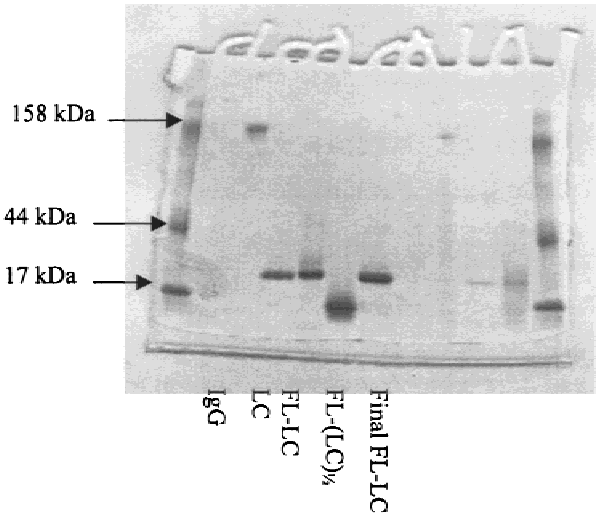

Fig. 2. SDS-PAGE of human IgG, its light chain (LC), fluoresceinated human IgG light chain (FL-LC) prior to ion-exchange chromatography, fluoresceinated human half IgG light chain [FL-$(LC)_{½}$], and final FL-LC used in the experiments. The electrophoresis was run on a 4–15% polyacrylamide gradient gel.

[^{3}H]-propionic acid NHS ester yielded a radiolabeled product with high specific activity. This was mixed with cold FL-LC to give a stock solution for injection with a concentration of 0.14 mM and a specific activity of 133 mCi/mmol.

Fluoresceinated human IgG half light chain, FL-$(LC)_{½}$, was prepared by pepsin digestion of FL-LC at 55°C. The digestion yielded several different fluorescent proteins as visualized on SDS-PAGE. The predominant protein, at about 11 kDa, was purified on a Superdex 75 column. Main fractions formed a broad green fluorescent band on a 15% SDS PAGE Product was further purified by anion exchange chromatography to give a single sharp band corresponding to a MW of about 11 kDa (Fig. 2). Values of 0.80 and 0.81 were obtained for the measured FL-to-protein ratios. Radiolabeling of FL-$(LC)_{½}$ was accomplished using the same procedure as was used for FL-LC except that FL-$(LC)_{½}$ was dissolved in buffer and purified on a 1 mL G-25 gel filtration column prior to reaction with [^{3}H]-propionic acid NHS ester. This change in procedure resulted in a higher radiochemical yield (43%). The radiochemical purity of the final product was determined to be 99.7% by TLC. The sterile-filtered working solution was at a concentration of 0.091 mM and a specific activity of 716 mCi/mmol.

Table I. Fluorescein-to-Protein Ratios in Individual Batches of FL-LC Conjugate

Lot	Protein (mM)	Fluorescein (mM)	FL/Protein
042799	10.9	9.2	0.84
052699	47.9	36.6	0.76
052799	12.0	10.1	0.84
062399	8.3	6.7	0.81
062999	27.7	20.7	0.75
070799	5.4	4.4	0.82
Average ±SD			0.80 ± 0.04

Note: Protein was determined by BCA assay using purified human IgG light chain as a standard, and fluorescein was spectrophotometrically determined using the absorbance peak near 490 nm.

Affinity Determination

The extent of fluorescence quenching was determined as a function of antibody concentration by adding different amounts of purified mouse anti-FL antibody to a series of test tubes containing a constant amount of FL-LC, FL-$(LC)_{½}$, or a low MW fragment of FL-LC. Affinity constants were determined by least squares regression to a theoretical curve (9) using WinNonlin (version 1.1; Scientific Consulting, Apex, NC). The results of three determinations per conjugate are summarized in Table II. The affinity constant for the low MW probe is significantly greater than that of either of the conjugates. However, this assay did not demonstrate any difference between the last two conjugates.

Surface plasmon resonance spectroscopy was used to determine on- and off-rates for the interaction between the FL conjugates and FL-specific monoclonal antibody FL-D6. The antibody was covalently attached to one flow cell of a sensor chip, and appropriate dilutions of FL-LC or FL-$(LC)_{½}$ were passed through the antibody-containing cell and a control flow cell. Considerable effort was put into optimizing the wash procedure to minimize damage to the antibodies while still inducing release of the conjugates. Nonetheless, the number of determinations that could be run was limited by the gradual deterioration of the binding surface with successive washes. On- and off-rates were determined based on fitting of the data by the BIA-Fit program provided by the Biacore manufacturer (version 3.0; Pharmacia Biosensor). Data obtained for FL-LC generally provided a good fit to the theoretical curves while fit was not as good for FL-$(LC)_{½}$ (data not shown). The results obtained for the two conjugates are summarized in Table III. Although the average affinity measurement was higher for FL-$(LC)_{½}$, the difference was not significant. Surprisingly, the off-rate was significantly lower for the 11-kDa conjugate ($p < 0.01$), while there was no difference in average on rate.

Competitive ELISA was also used as a means of comparing binding of FL-LC and FL-$(LC)_{½}$ to a standard monoclonal anti-FL antibody, FL-D6. Here, we measured how well the conjugates interfere with the binding of the antibody to FL-BSA plated on a 96-well microplate. Because of the high avidity involved in the interaction between the antibodies and the FL-BSA conjugate with multiple epitopes, higher concentrations of our monovalent conjugates than were available were required to develop a full binding curve and allow computation of IC_{50} values. Nonetheless, we were able to observe a significant difference in IC_{20} values: 2×10^{-7} M for FL-$(LC)_{½}$ and 10^{-6} M for FL-LC (data not shown).

Pharmacokinetics

A 14-nanomole dose of FL-LC was given in 0.1 mL to each of four FL-immunized and three mock-immunized mice.

Table II. Affinity Constant (in M^{-1}) Measured for the Interaction between Anti-FL Antibody and Three Fluorescein-Protein Conjugates by Fluorescence Quenching

Determination	Low MW	FL-LC	FL-$(LC)_{1/2}$
1	1.28×10^8	6.78×10^7	6.72×10^7
2	1.24×10^8	6.78×10^7	6.47×10^7
3	1.24×10^8	6.72×10^7	6.74×10^7
Average ± SD	$1.25 \pm 0.03 \times 10^8$	$6.76 \pm 0.03 \times 10^7$	$6.64 \pm 0.01 \times 10^7$

Table III. On-Rate, Off-Rate, and Affinity Constants Determined by Surface Plasmon Resonance Spectroscopy for Binding of FL-LC and FL-$(LC)_{1/2}$ to Matrix Affixed anti-FL Monoclonal Antibody, FL-D6

FL-LC	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_A (M^{-1})
25 nM	3.89×10^5	1.36×10^{-3}	2.86×10^8
50 nM	8.16×10^4	1.47×10^{-3}	5.55×10^7
250 nM	1.51×10^5	1.18×10^{-3}	1.27×10^8
500 nM	1.94×10^5	1.03×10^{-3}	1.93×10^8
Average	2.04×10^5	1.26×10^{-3}	1.65×10^8
SD	$\pm 1.32 \times 10^5$	$\pm 0.19 \times 10^{-3}$	$\pm 0.98 \times 10^8$
FL-$LC_{1/2}$	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_A (M^{-1})
25 nM	4.08×10^5	2.85×10^{-4}	1.41×10^9
250 nM	7.64×10^4	2.56×10^{-4}	2.98×10^8
Average	2.42×10^5	2.71×10^{-4}	8.54×10^8

During the first few hours after dosing of the former, the blood was difficult to collect, and the mice were markedly lethargic. This morbidity was similar to that observed when high doses of the low MW FL conjugate of ethanolamine (FL-EA) were administered (5). It is likely related to the triggering of an immune response. The mice appeared to recover fully after a few hours. A 9.1-nanomole dose of FL-$(LC)_{½}$ was administered to each of four FL-immunized and three mock-immunized mice. Morbidity in this series of mice was not as pronounced as it was with FL-LC. Serum concentration-vs-time curves for the two conjugates are shown in Fig. 3 and pharmacokinetic parameters are summarized in Table IV.

The volume of distribution for the largest conjugate studied, FL-LC, was very small, at 0.041 ± 0.005 mL/g in immunized mice. The corresponding value in mock-immunized mice, 0.16 ± 0.02 mL/g, is approximately 4-fold greater and significantly different ($p < 0.05$). The smaller protein conjugate, FL-$(LC)_{½}$, distributed to a significantly higher volume both in immunized ($p < 0.05$) and mock-immunized ($p < 0.01$) mice with volumes of 0.070 ± 0.013 and 0.30 ± 0.02 mL/g in the FL-immunized and mock-immunized mice, respectively. As with FL-LC, the volume of distribution was significantly higher in the mock-immunized mice ($p < 0.01$).

As seen in Fig. 3B, compared with the low MW FL-EA conjugate we studied earlier (5), the dose-normalized concentrations of the two protein conjugates start at a higher level but decline more rapidly with time. To a lesser extent, the same trend is seen with FL-$(LC)_{½}$ relative to FL-LC. Thus, the lower MW protein conjugate distributes more widely and thus has lower serum concentrations at early time points, however, the difference became less significant with time.

In Vivo Instability of Radiolabel in Mock-Immunized Mice

During the course of pharmacokinetic experiment with FL-$(LC)_{½}$ in mock-immunized mice, we noticed a very sharp biphasic disappearance of radioactivity: for the first 3 h, the [^{3}H]-level diminished with a $t_{½}$ of 1.7 h, but after 6 h, it slowed dramatically and the $t_{½}$ was extended to 54–57 h. This observation is consistent with cleavage of the radiolabel to yield a small radiolabeled molecule, probably propionic acid, with very slow clearance. Note that [^{3}H]-propionic acid was attached to the proteins via proteolytically labile amide bond. To specifically address this issue, FL-LC was administered to

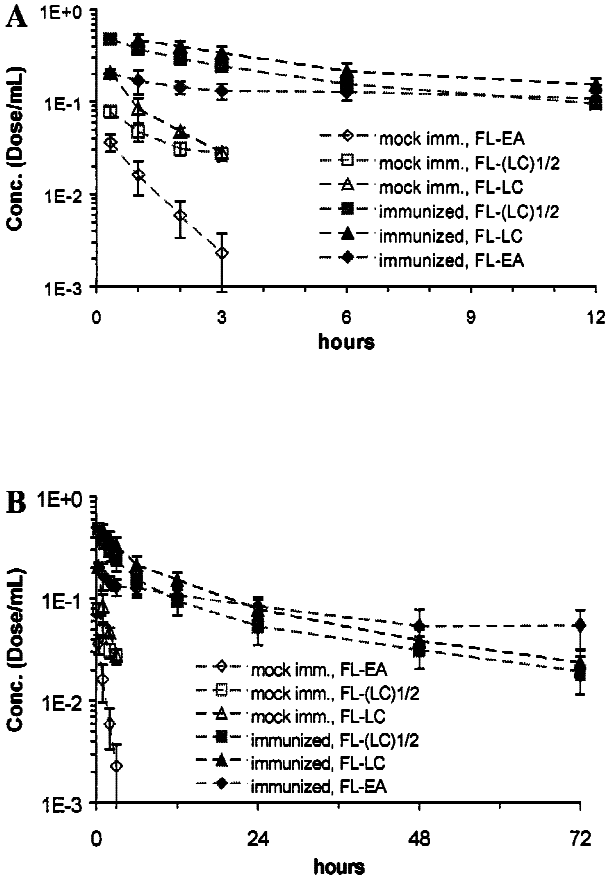

Fig. 3. Time-dependence of dose-normalized concentration of fluorescein conjugate of ethanolamine (FL-EA), fluoresceinated human half IgG light chain [FL-$(LC)_{½}$], and fluoresceinated human IgG light chain (FL-LC) in mock-immunized and fluorescein-immunized mice over the course of 12 (A) and 72 (B) h.

one mock-immunized and one FL-immunized mouse. Blood samples were taken at 24 h, and the animals were exsanguinated at 48 h. Radioactivity in serum or urine aliquots were counted directly as well as after being passed through a 1 mL Sephadex G-25 column to remove low MW material such as [^{3}H]-propionic acid. The radioactivity associated with the protein conjugate remained the same in the FL-immunized mouse serum harvested at 24 h or 48 h, however, it was only 28 and 15%, respectively, in the mock-immunized mouse serum. Data are summarized in Table V. Interestingly, the 48-h urine sample from the FL-immunized mouse showed that only 2% of the total radioactivity in the urine represent intact FL-LC conjugate. When the above procedure was applied to the FL-immunized mouse serum samples from the actual pharmacokinetic experiments, on day 7 approximately 50% of the radioactivity was from intact FL-LC conjugate (data not shown). With mock-immunized mice, the same level of degradation was observed within 6 h.

DISCUSSION

Isolation of light chain from human IgG followed by conjugation on the single free –SH group provided a suitable means of obtaining a 1:1 FL-protein conjugate for evaluation of the effects of anti-hapten antibodies on the pharmacokinetics of a monovalent hapten-protein conjugate. Pepsin di-

Table IV. Pharmacokinetic Parameters for FL-LC and FL-$(LC)_{1/2}$ in Mock-Immunized and FL-Immunized Mice

Mouse	Wt g	αFL Titer *fbu*[a]	Duration of Expt.	Vd mL/g	AUC_{3h}[b] dose · hr/mL	AUC_{12h} dose · hr/mL	AUC_{72h} dose · hr/mL
FL-LC in mock-immunized mice							
LK-1	32.5	n.d.[c]	6 hr	0.15	0.26		
LK-2	30.2	n.d.	6 hr	0.12	0.32		
LK-3	33.0	n.d.	6 hr	0.17	0.24		
Average	31.9			0.16	0.27		
SD	±1.49			±0.02	±0.04		
FL-$(LC)_{1/2}$ in mock-immunized mice							
HK-1	35.9	nd.	48 hr	0.30	0.11		
HK-2	32.3	nd.	48 hr	0.28	0.16		
HK-3	31.8	nd.	48 hr	0.32	0.14		
Average	33.3			0.30	0.14		
SD	±2.2			±0.02	±0.02		
FL-LC in high titer mice							
LI-3	31.6	1.91	168 hr	0.044	1.42	3.59	7.89
LI-4	29.9	2.27	168 hr	0.035	1.67	3.72	7.52
LI-5	34.8	1.73	168 hr	0.040	1.19	2.60	5.07
LI-9	35.6	2.88	168 hr	0.045	1.37	3.47	6.99
Average	33.0	2.20		0.041	1.41	3.34	6.89
SD	±2.68	±0.53		±0.005	±0.20	±0.51	±1.28
FL-$(LC)_{1/2}$ in high titer mice							
HI-1	32.8	1.51	168 hr	0.058	1.08	2.40	4.88
HI-2	34.5	1.92	168 hr	0.060	1.13	2.61	5.31
HI-7	32.0	1.07	168 hr	0.082	0.88	2.08	4.21
HI-8	31.7	2.23	168 hr	0.081	0.96	2.36	5.10
Average	32.8	1.68		0.070	1.01	2.36	4.88
SD	±1.26	±0.48		±0.013	±0.11	±0.22	±0.48
High titer mice with FL-EA[d]							
Average	31.6	1.37	Varying	0.15	0.49	1.65	6.40
SD	±2.4	±0.6		±0.05	±0.09	±0.28	±1.33

Note: A two-compartment model was used in processing the raw data from mock-immunized mice, while those from immunized mice were fitted to the model given in the preceding papers.

[a] One *fbu* (fluorescein binding unit) is defined as being the titer necessary to give the same midpoint in the plot of absorbance at 492 nm against log (dilution) as 1.0 mg/ml of reference monoclonal antibody against FL, FL-D6 (see more in the preceding paper).

[b] AUC is in the unit based on normalized dose, unity being the total initial dose.

[c] not detected.

[d] From the previous studies with fluorescein conjugate of ethanolamine (FL-EA; n = 8) and varying duration of experiment.

gestion of this 25-kDa FL-LC conjugate produced a second conjugate with MW of about 11 kDa, FL-$(LC)_{½}$. A third peptidyl fragment of MW 0.82 kDa was further obtained by treating FL-LC with more trypsin. Since the smaller conjugates were a piece of the larger one, their properties should be quite similar, and the environment of the FL moiety in the conjugate must be the same.

The binding orientation hypothesis put forth earlier predicts that binding affinity will decrease with MW to the power of two-thirds for spherical proteins. If one assumes globular structures for both protein conjugates, FL-$(LC)_{½}$ should bind to the anti-FL antibodies with an affinity constant 1.7 time that of FL-LC. A difference of this magnitude is predictably difficult to measure reproducibly, although the difference in affinity between either of the two conjugates and the low MW probe such as FL-EA (5) should be considerably larger. Because most means of affinity determination are not particularly sensitive, several means of determination were attempted.

Surface plasmon resonance spectroscopy detects changes in light refraction that occur when mass is added to a dextran matrix affixed to a thin film of gold (10). This allows real-time monitor of binding kinetics. To optimize sensitivity, the lower-MW binding partner is usually attached to the matrix

Table V. Stability of FL-LC *In Vivo* as Assessed by Means of Gel Filtration[a]

Sample	Before	After	After/Before
Control	351569	327211	0.93
FL-immunized mouse			
48 h serum, assay 1	7953	7547	0.95
48 h serum, assay 2	5866	5494	0.94
48 h serum, assay 3	7199	7068	0.98
24 h serum	11114	10584	0.95
48 h urine	3855	84	0.02
Mock-immunized mouse			
48 h serum, assay 1	2391	283	0.12
48 h serum, assay 2	2906	543	0.19
48 h serum, assay 3	2705	324	0.12
24 h serum	1524	397	0.26

[a] Immunized and mock-immunized mice were given FL-LC. Serum and urine samples obtained either 24 or 48 hours after the administration were passed through a Sephadex G-25 column to remove small MW compounds such as [^{3}H]-propionic acid. The radioactivity recovered is assumed to be the intact protein conjugate.

and the higher-MW component is allowed to flow across the surface. Since the protein conjugates were in short supply, in the present study the arrangement was reversed: antibody was affixed to the sensor chip into which the conjugates were made to flow. This uncommon setup and the gradual deterioration of the attached antibody after repeat determinations which entailed harsh conditions, such as low pH or high ionic strength, resulted in only supportive evidence for decreased affinity with size of protein. For instance, a decreased affinity was expected for a higher MW conjugate due to a slower on-rate, however, no difference in on-rates was seen between the conjugates. In contrast, the off-rate for FL-$(LC)_{½}$ was significantly slower ($p < 0.05$) than that of FL-LC. No concrete explanation is provided for interpreting these unexpected observations.

The fluorescence of the conjugates was quenched upon binding to anti-FL antibodies. This quenching was used to determine affinity constants for FL-LC, FL-$(LC)_{½}$, and a low-MW FL-containing peptide obtained by digestion of FL-LC with more trypsin. Rather than using a constant concentration of antibody and altering the conjugate concentration, as is done in typical Scatchard analysis, the opposite approach was taken. Although this necessitated non-linear data analysis, it simplified the experimental procedure considerably. A significantly larger affinity constant was determined for the low-MW conjugate, but no difference in affinity constant could be detected between the two protein conjugates. Finally, the results of competitive ELISA were also supportive of lower binding constant for the larger conjugate: compared with FL-LC, approximately 5-fold less FL-$(LC)_{½}$ was needed in preventing the interaction between the standard anti-FL antibody and FL-BSA by 20%.

As shown in Table V, antibody binding greatly stabilizes the serum stability of FL-LC *in vivo*. This together with the poor urinary recovery in the same FL-immunized mouse suggests that cleavage of the radiolabel from the conjugates may occur not in the circulation, but in the kidneys. A likely scenario is that unbound conjugate is partially metabolized in the kidneys after being filtered through the glomerulus. This degradation likely releases [^{3}H]-propionic acid, which can then re-enter the circulation through active re-uptake and/or passive diffusion. Although more stable in FL-immunized mice than in mock-immunized mice, only 50% of the circulating radioactivity on day 7 represented intact FL-LC. For this reason alone, no serious pharmacokinetic modeling was attempted for the protein conjugates.

Both protein conjugates behaved quite differently *in vivo* than did the low-MW conjugate, FL-EA. First, their volume of distribution was very low, especially in the immunized mice. This must reflect their limited ability to extravasate. As with the FL-EA, the lower volumes in the immunized mice were the result of the conjugates being sequestered in the bloodstream by antibody binding. It is clear by its significantly higher volumes of distribution both in immunized and mock immunized mice that FL-$(FC)_{½}$ has greater freedom of movement than FL-LC.

While the conjugates have higher initial dose-normalized concentrations due to their limited volumes of distribution, they are eliminated more rapidly than FL-EA. After a few days, the concentration-vs-time curves cross each other when they are plotted together (Fig. 3). Two factors most likely contribute to the increased disappearance of the protein conjugates relative to FL-EA. First, the hindered redistribution of FL-EA into tissue contributes to its long serum duration, as discussed in the preceding paper (5). Since the protein conjugates cannot redistribute as freely in their unbound state, it should play less of a role in sustaining their circulation. The more important factor in determining the time course of blood level appears to be reduced antibody binding. Since the anti-FL titers were the same in the mice given FL-EA and those given the protein conjugates, the reduced binding must be due to decreased affinity for the antibodies. Thus the comparison of pharmacokinetic behavior between the protein conjugates and FL-EA is consistent with the hypothesis of reduced binding affinity with increased MW.

Similarly, but to a lesser extent, the pharmacokinetic profiles of FL-LC and FL-$(LC)_{½}$ differ from each other. As shown in Fig. 3, the latter disappears from the circulation more quickly at first, but it declines more slowly with time such that at 72 h the difference between FL-LC and FL-$(LC)_{½}$ concentrations becomes no longer significant ($p = 0.19$). The more rapid initial decrease in serum concentration is probably due to more extensive redistribution. The more gentle decline in the later part of the curve could be a factor of this redistribution or it could, in part, indicate a higher binding affinity.

In summary, the two protein conjugates enjoyed substantially sustained circulation as a result of antibody binding, but this effect was somewhat diminished relative to what was observed with the low-MW conjugate, FL-EA. Both affinity and pharmacokinetic data are consistent with the hypothesis of reduced affinity with increasing MW for monovalent hapten conjugates, but neither offered overwhelming proof. A low-MW conjugate was shown to have higher affinity for antibodies than the protein conjugates, although steric hindrance and other local factors cannot be ruled out as the cause of this difference. None of the methods used was conclusive in demonstrating an affinity difference between the two protein conjugates, mainly because of the poor reproducibility of these analyses. To overcome these deficits, future studies will need to investigate a wider range of molecular weights, so that more substantial differences in binding affinity can be anticipated.

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