Anti-Drug Antibodies as Drug Carriers. I. For Small Molecules

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Purpose. To better understand the pharmacokinetics of drugs compounds that bind endogenous antibodies

Methods. Three groups of mice with differing anti-fluorescein (FL) titers were established by empirically developed immunization protocols. These with two control groups were given intravenously [^{3}H]-ethanolamine conjugate of FL (FL-EA). The latter was synthesized using isothiocyanate chemistry. Radioactivity in the circulation, and occasionally in peritoneal ascites, was monitored for 7 days. A group of mice was immunized with eosin Y and given FL-EA. Conversely, eosin Y conjugate of radiolabeled EA (EY-EA) was given to mice immunized with FL. These two groups represented animals of low affinity to probe haptens. The affinity was assessed by a precipitation procedure, while titer was determined by a standard ELISA. Dose of FL-EA varied over a 100-fold.

Results. On average, the three immunized groups showed a 1:13:85 ratio of anti-FL titer, with remarkably consistent levels within each group. Elimination rates of FL-EA from the serum of very high-titer mice and high-titer mice were similar, however, were substantially lower than that found in low-titer mice. The latter was in turn lower than that found in non- or mock-immunized mice. Serum of mice immunized with FL showed approximately 200-fold lower affinity towards EY-EA than FL-EA. In these mice and in mice immunized with eosin Y and given FL-EA, the elimination of the probe haptens was again fast, reminiscent of low-titer mice. Mice of either low titer or low affinity showed more rapid redistribution of the conjugate between serum and peritoneal fluid. In a group of mice with comparable anti-FL titer, elimination from serum was independent of dose over a 100-fold difference. The bi-phasic concentration-time profile observed was accommodated by a physiologically meaningful pharmacokinetic model incorporating two compartments in which antibody binding can occur.

Conclusions. Monovalent antigenic substance cannot trigger immune clearance. As such, endogenous antibodies that recognize the molecule can serve as a carrier to result in a substantial decrease in clearance.

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

INTRODUCTION

The circulation time of a drug molecule is largely determined by its propensity to bind serum proteins. While bound to protein, a drug molecule is generally unavailable to clearance mechanisms as well as to its site of action (1,2). Although protein binding is usually associated with relatively inert blood proteins such as albumin, antibodies specific to a drug provide an interesting case of high affinity, low capacity protein binding. Given that antibodies usually have the function of neutralizing or destroying target ligands, such binding might be expected to simply eliminate drug activity. In reality, antibody binding can have a wide range of effects depending on the size of the drug, the number of binding sites it has for antibody, and the concentration and affinity of the antibody (3).

Antibodies themselves enjoy long half-lives in the circulation. Three of the four subclasses of IgG, for instance, have half-lives of about 20 days in humans (4). When combined at appropriate stoichiometric ratios, multivalent antigens and antibodies tend to form large, extensively crosslinked complexes that precipitate from solution. If the antigen provides fewer binding sites for antibody or if the antibody to antigen ratio is less optimal, smaller soluble immune complexes are formed (5,6). These can be cleared as a result of complement activation (7,8) or Fcγ receptor binding. In either case, clearance mechanisms require that antibodies be crosslinked by antigens to some degree (6).

An antigen with only one or two combining sites for antibody is unlikely to crosslink antibodies and hence would not be subject to the normal immune clearance mechanisms (11). For example, in mice immunized against DNP, human serum albumin had to be conjugated with greater than three DNP epitopes per molecule to exhibit accelerated clearance (12). If binding of a drug to an antibody does not trigger clearance, the antibody should act as a carrier for the drug, similar to any other protein in the blood that binds the drug. Observations made in such a disparity of disciplines as toxicology, diabetes, and cytokine therapy show this to be the case (3 and references therein).

In the present study, we prepared a fluorescein (FL) conjugate of a small radiolabeled probe molecule, ethanolamine (EA), and investigated its pharmacokinetics in mice immunized with FL. To evaluate antibody titer as a variable, we have empirically adjusted the immunization protocol for different groups of mice so as to obtain three groups that differ from one another in average anti-FL titer by about an order of magnitude. To evaluate the effects of reduced binding affinity we have used a fluorescein analog, eosin Y (EY), that binds to anti-FL antibodies, but with a lower association constant. While the present study deals with a small probe molecule, FL-EA of MW 450, the accompanying paper addresses similar issues with protein molecules.

MATERIALS AND METHODS

Materials

Freund's adjuvant, keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA), fluorescein isothiocyanate (FITC), eosin Y (EY) isothiocyanate, Goat anti-mouse peroxidase conjugate (Cat. #A3673), anti-FL monoclonal antibody FL-D6 (#F5636), and *o*-phenylenediamine · 2HCl (OPD) tablets (# P9187) were all obtained from Sigma (St. Louis, MO). [^{3}H]-Ethanolamine (EA) with a specific activity of 27.0 Ci/mmol was from Amersham (Piscataway, NJ). Nunc Maxisorp certified 96-well plates were used for the immunoassay (Nunc, Naperville, IL). Plates were read on a BioRad Model 3550 microplate reader (BioRad, Hercules, CA).

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Vaccines and Vaccination

To 30 mL of 20 mM borate buffer at pH 8.8 that contained 30 mg protein, 25 mg fluorescent labeling agent was added as a powder, and the pH was readjusted to 8.8 with NaOH. The mixture was stirred for 2 h at room temperature (RT). Unreacted reagent was removed by dialyzing against several changes of phosphate-buffered saline (PBS). Vaccines were prepared by emulsifying one part of a solution of 5 mg/mL KLH or a KLH conjugate with 2 parts of Freund's complete or incomplete adjuvant. Vaccines were used within 3 h of preparation.

Male BALB/c mice (25–30 gm; from Charles River) vaccinated under a high-titer protocol were given an intraperitoneal (IP) injection of 500 μL of vaccine in Freund's complete adjuvant, which was followed by a 500-μL IP booster in Freund's incomplete adjuvant two weeks later. Mice vaccinated under a low-titer protocol were given a single IP injection of 50 μL of primary vaccine. Very high-titer mice received three 500-μL injections, one of primary and two of secondary vaccine. The second booster was given 7 or 12 months after the primary vaccination. Otherwise, experiments were performed 6–8 weeks after the initial injection. Mice were allowed to eat and drink freely throughout the study.

Synthesis of Radiolabeled Probe Molecules, Ethanolamine Conjugate of Fluorescein (FL-EA) and Eosin Y (EY-EA), and Serum Stability of FL-EA

Into a 0.7-mL microcentrifuge tube containing 0.9 mCi of dried [^{3}H]-EA, were added 5 μL of 2.5% (V/V) cold EA in *N,N*-dimethylformamide (DMF) and 10 μL of 156 mg/mL FITC in DMF. The mixture was protected from light and allowed to react at RT for 45 min, at which time 6 μL of the 20% cold EA in DMF was added to complete the reaction. After additional 5 min, 500 μL of ethyl acetate (EtOAc) was added to precipitate the product. Precipitates dissolved in 250 μL H_2O was run through a 1 mL Sephadex CM fast-flow cation exchange column to remove any trace of unreacted EA. The eluent was assessed for purity on silica gel TLC using two different developing systems: 5:4:1 $CHCl_3$, CH_3OH, and H_2O for visual analysis and 5:3:2 n-propanol, H_2O, and EtOAc for radioactivity determination. Radiolabeled ethanolamine conjugate of eosin Y (EY-EA) was prepared in the same manner. Both products were pure by TLC with essentially all radioactivity associated with the section of the TLC plate containing the product.

To assess serum stability of the conjugates, a 1:1 mixture of aseptically prepared mouse serum and conjugate stock solution was stored at 37°C. At given time intervals serum proteins were precipitated by adding 80 μL of n-propanol to a 20 μL aliquot of the serum sample. The supernatant was evaluated by TLC developed in 5:4:1 $CHCl_3$, CH_3OH, and H_2O. This procedure was also used in testing serum samples from animal experiments.

Determination of Anti-FL Titers

Titers of anti-FL antibodies were measured by ELISA. Plates were coated with 100 μL per well of 0.2 μg/mL FL-BSA solution and then blocked with 200 μL of 2% BSA in 100 mM bicarbonate buffer at pH 9.6. After three washes, serial dilutions of mouse serum samples, ranging form 10^{-2} to 3.2×10^{-6} at half-log increments, were applied. After a 1-h incubation the plates were washed 3×, and incubated for an hour with a 1:2000 goat anti-mouse IgG peroxidase conjugate. Plates were washed 5× before OPD substrate was added. The reaction was stopped after 20 min by adding 50 μL of 3N-HCl. Plates were read at 492 nm with a reference wavelength of 595 nm. Sigmoid curves were fitted to the A_{492} versus log[concentration] data using WinNonlin (version 1.1; Scientific Consulting Inc., Apex, NC). The midpoint of each curve was taken as the serum dilution, D_{50}, which gives 50% of the maximal response. Titer was calculated by the following equation:

$$titer_{sample} = titer_{std} \frac{D_{50}\,(std)}{D_{50}\,(sample)}$$

Titers are expressed as fluorescein binding units (*fbu*), where 1 *fbu* is a titer giving the same D_{50} value as 1.0 mg/mL of anti-FL monoclonal antibody FL-D6.

Affinity Determinations by Precipitation Method

Affinity of FL-EA to anti-FL antibodies was determined using an antibody precipitation procedure (13) with FL-EA concentration ranging from 2×10^{-8} to 1×10^{-6} M. Mouse IgG was partially purified by two successive precipitations with saturated $(NH_4)_2SO_4$ (14), assayed by ELISA, and diluted to a titer of approximately 7.5×10^{-6} *fbu*. This solution was incubated for 1 h at RT with one of the dilutions of FL-EA, both in 100 μL. Then 50 μL of porcine IgG (25 mg/mL) was added, followed immediately by 300 μL of saturated $(NH_4)_2SO_4$. The tubes were vortexed and then spun for 10 min at 12,000 × G.

Bound FL-EA was determined from the radioactivity of the re-dissolved pellet. Unbound FL-EA was calculated by subtracting radioactivity in the pellet from that in 100 μL of the corresponding FL-EA dilution. Binding parameters were determined by fitting the raw data to a Scatchard equation with two binding sites using WinNonlin.

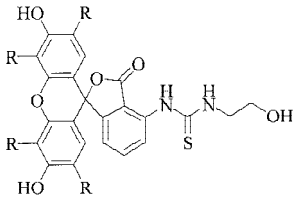
Pharmacokinetic Experiments

Each mouse, under anesthesia with an IP injection of 0.1 mg ketamine and 7 μg xylazine per gram body weight, was given 100 μL of a test solution via tail vein injection. Blood was collected by slitting the tail artery, and serum sample obtained (20 μL) was transferred to a 7 mL scintillation vial containing 5.0 mL scintillation cocktail (ScintiSafe Econo 2; Fisher Scientific). Mice were allowed to eat and drink freely during the course of the experiments. At the final time point, mice were anesthetized and exsanguinated by cardiac puncture.

RESULTS

Preliminary Studies

Structures of the two probe conjugates studied, shown in Fig. 1, were established by means of proton NMR and electrospray mass spectrometry. In all cases, purity was assessed to be greater than 97% by means of TLC as detected by radioactivity and distinct FL color. The specific activity of FL-EA and EY-EA conjugates used in preparing stock solutions at 0.3 mM were 218 and 34.4 mCi/mmole, respectively.



Conjugate	R
FL - EA	—H
EY - EA	—Br

Fig. 1. Structure of FL-EA and EY-EA conjugates.

In the synthesis of EY-EA, a smaller amount of [^{3}H]-EA was used to result in a reduced specific activity. The radiolabel in FL-EA was found to be stable when incubated with serum, as assessed with TLC.

A standard pool of serum from twice immunized mice was used to optimize the ELISA for anti-FL titer and to assess the range of the assay and validate the method of calculation. Measured antibody titer was found to be reasonably proportional to serum concentration to as low as 0.003 *fbu*. For titers higher than 10 *fbu,* additional dilution increased the accuracy of the determination by pulling more of the upper portion of the curve into the range of the assay.

Antibody Titers in Immunized Mouse Groups

Anti-FL titers are summarized in Table I for the three groups of mice given different doses of vaccine. Most of the mock-immunized mice showed some response at the highest serum concentration (1:100 dilution) in the anti-FL ELISA. This might be a result of a low level of cross reactivity between an epitope on KLH and FL or could be due to trace contamination of FL-KLH in the KLH control vaccine. The same double-ended syringe connectors were used to prepare both vaccines, and minute traces might have remained after cleaning.

Selection of a Low-Affinity Fluorescein Analog

Our attempts to determine affinity of FL-EA to sera of immunized mice were only partially satisfactory. Overall, the precipitation-based assay showed itself to be repeatable for determination of the affinity constant for high-affinity antibodies. Other values calculated from Scatchard analysis with two classes of binding site were less reliable. Inability to accurately measure low-affinity binding is thus a drawback of this method. Other methods proved to be impractical for the high number of samples and the small serum volumes available.

With the above limitation, the effects of antibody affinity on the pharmacokinetics of a hapten were addressed by immunizing animals against one hapten and then injecting a second, lower-affinity hapten. To select a suitable low-affinity hapten, several compounds with structural similarity to FL were tested in a competitive ELISA assay. Serial dilutions of potential competitors in PBS were pipetted from top to bottom on 96 well plates, while serial dilutions of standard anti-FL serum were pipetted lengthwise on the plates. Otherwise, the procedure was the same as for normal ELISA. Concentrations of each of these molecules required to inhibit 50% of binding of standard fluorescein anti-serum (IC_{50}) are presented in Table II. Based on these results and its excellent solubility, eosin Y (EY) was chosen as a low-affinity analog of FL to evaluate the effect of reduced affinity binding in animals having similar antibody titers. The structure of EY-EA conjugate is shown in Fig. 1 along with that of FL-EA. Antibody precipitation with $(NH_4)_2SO_4$ followed by Scatchard analysis with standard anti-FL serum resulted in affinity constants $2.4 \times 10^8\ M^{-1}$ for FL-EA and $1.2 \times 10^6\ M^{-1}$ for EY-EA. These values are in good agreement with the results obtained by competitive ELISA shown in Table II.

Pharmacokinetics of FL-EA

A total of 25 mice, representing five groups, were subject to the study. As controls, 4 non-immunized and 3 mock-immunized (immunized with KLH) mice were dosed. For three groups of immunized mice, 5, 8, and 5 mice represented low-titer, high-titer, and very high-titer groups, respectively. Three of the very high-titer mice were bled up to the 336-h point, while the other two were bled up to the 168-h point. Peritoneal ascites was also sampled from some of these mice at most of the time points.

Shown in Fig. 2 are time-dependent concentrations of the intact probe conjugate in the circulation for all five different groups of mice. Data obtained for the non-immunized and mock-immunized mice were fitted to a typical two-compartment pharmacokinetic model using WinNonlin. Data from immunized mice were fitted to the physiological model

Table I. Anti-FL Titer in Three Groups of Mice[a]

Immunization protocol	Number of mice	Titer range (fbu)	Mean titer (fbu)	Standard deviation	Relative SD
Low titer	5	0.06–0.15	0.11	0.04	40%
High titer	43	0.25–4.5	1.45	0.87	60%
Very high titer	5	5.0–16.3	9.30	4.8	52%

Note: 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 a reference monoclonal antibody against FL, FL-D6 (see Determination of Anti-Fluorescein Titers section). In the present study, approximately 1 fbu = 6.67 μM.

[a] Some of these mice were used in subsequent pharmacokinetic experiments.

Table II. Concentrations of Fluorescein Analogs and Derivatives Which Inhibit 50% Binding of FL-EA to Anti-FL Antibodies in a Competitive ELISA

Fluorescein analog	IC_{50} (μM)
FL-EA	1.2
Sodium Fluorescein	5.5
Fluorescein Maleimide	0.74
Erythrosin B	1500
Eosin B	3300
Eosin Y	350
Fluorescein Diacetate	250
Tetramethyl Rhodamine	580
Rhodamine B	1000

given in Fig. 3. Pharmacokinetic parameters thus obtained are listed in Table III. Statistics were carried out with two-tailed Student *t*-tests. Since mice in the very high-titer group were immunized over a long period of time, their body weight was greater than the rest. As expected, immunized mice showed significantly different level of anti-FL titers from non-immunized or mock-immunized mice ($P < 0.001$). Furthermore, the titers of the three immunized groups significantly differ from one another ($P < 0.05$). Both volume of distribution ($P < 0.01$) and AUC ($P < 0.01$) were different between the control groups and the immunized groups.

All of the very high-titer mice developed significant amounts of peritoneal ascites as a result of their hyper-immunization with FL-KLH. In all cases investigated, anti-FL titers in serum and in peritoneal ascites were found to be identical within measurement error. In low-titer mice, serum and peritoneal concentrations of FL-EA tended to equalize rapidly, while in the very high-titer mice, the equilibration was much slower, and peritoneal levels stayed well below serum levels throughout a seven-day period.

Because of the low blood volume per sample and the very low concentrations of unbound FL-EA for all time points. Nonetheless, a limited attempt was made to assess free FL-EA pharmacokinetics for a group of very high-titer mice. This was done by collecting 100-μL blood samples at half the normal number of time points. The total radioactivity of one 20-μL aliquot of a serum sample was directly counted, while a second 20 μL aliquot was precipitated with saturated $(NH_4)_2SO_4$, and the re-dissolved pellet was counted. Interestingly, for 7 days after dosing, the serum level of free FL-EA remained relatively constant at about 2×10^{-7}M, while total FL-EA level decreased (data not shown). Free drug represented about 5% of the total up to 12 h and then leveled off to about 10%.

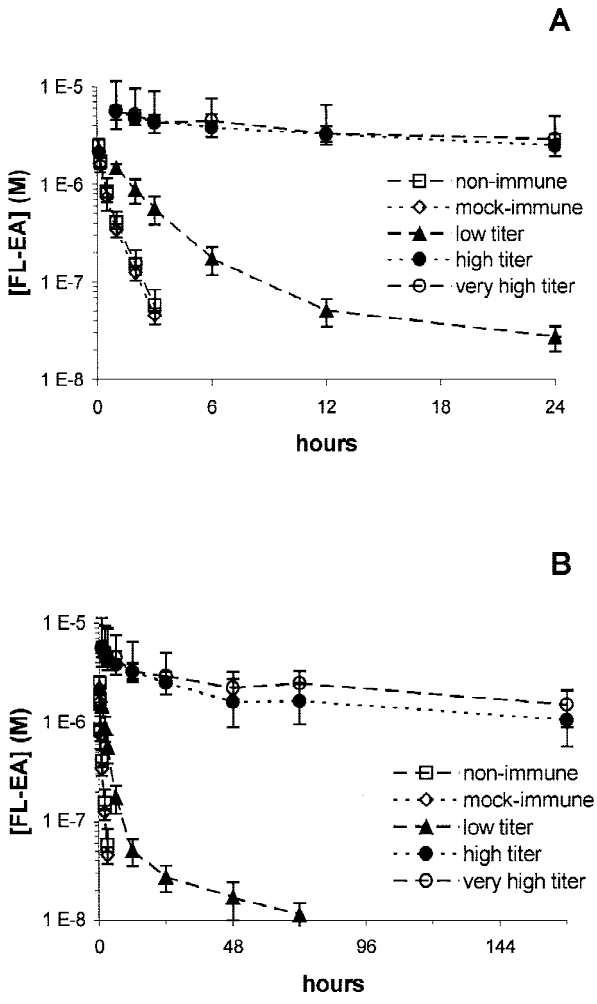


Fig. 2. Pharmacokinetics of FL-EA in non-immunized (n = 4), mock-immunized (n = 3), low-titer (n = 5), high-titer (n = 8), and very high-titer (n = 5) mice over the course of 24 h (A) and 168 h (B).

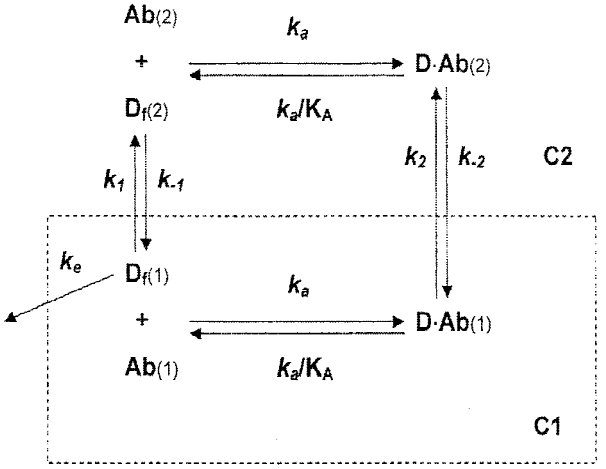


Fig. 3. Pharmacokinetic model used to fit the raw data from immunized mice shown in Fig. 2. Compartments C1 and C2 may represent the circulation and extravascular space. Not included in the analysis are; potential distribution to other compartment(s) from which antibodies are excluded, drug-receptor interaction, clearance of immune complex and antibody production. Abbreviations used are:

D_f = unbound hapten
Ab = unbound antibody
D-Ab = hapten-antibody complex
k_a = association rate constant for antibody-hapten binding
K_A = affinity constant (equilibrium constant) for antibody-hapten binding
k_e = elimination rate constant for unbound hapten
k_1 = extravasation rate constant for unbound hapten
k_{-1} = re-absorption rate constant for unbound hapten from tissue
k_2 = extravasation rate constant for immune complex
k_{-2} = tissue-to-blood transfer rate constant for immune complex

Pharmacokinetics of FL-EA Conjugate in Mice Immunized with EY and EY-EA in Mice Immunized with FL

Six mice were immunized as high-titer mice but with EY-KLH replacing FL-KLH. No attempt was made to determine anti-EY titers since no standard was available that would allow comparison between anti-EY and anti-FL titers. Instead, anti-FL titers were determined using the standard ELISA procedure based on the assumption that reduced affinity would be reflected in reduced titers. In terms of anti-FL titer, these mice showed a range of 0.05 to 0.2 *fbu,* compared to a range of 0.25–4.5 for mice immunized with FL-KLH. The pharmacokinetic profiles of these mice following a 100-μL dose of FL-EA are shown in Fig. 4A. As expected, there was a significant difference in blood level of FL-EA between the

Table III. Pharmacokinetic Parameters of Five Mouse Groups Administered FL-EA[a]

Mouse	wt. (g)	Titer (fbu)	Duration of expt.	Vd (mL/g)	AUC_{3h} nmol · hr/mL	AUC_{∞} nmol · hr/mL	
Non-Immune Mice							
EN-1	26.5	n.d.[b]	3 hr.	0.38	1.86	3.79	
EN-2	29.7	n.d.	3 hr.	0.39	1.19	4.17	
EN-3	27.6	n.d.	3 hr.	0.28	1.95	4.16	
EN-4	29.3	n.d.	3 hr.	0.26	1.20	2.44	
Average	28.3			0.33	1.55	3.64	
SD	±1.5			±0.07	±0.41	±0.82	
Mock-Immunized Mice							
EK-1	31.5	trace	3 hr.	0.30	1.48	2.95	
EK-2	30.8	trace	3 hr.	0.26	1.18	2.36	
EK-3	32.7	trace	3 hr.	0.35	1.44	2.88	
Average	31.7			0.30	1.37	2.73	
SD	±1.0			±0.05	±0.16	±0.32	
Low-Titer Mice							
EL-1	30.8	0.15	72 hr	0.28	4.78	7.09	8.57
EL-2	28.6	0.11	72 hr.	0.35	4.04	6.07	7.53
EL-3	29.6	0.07	72 hr.	0.14	5.81	6.89	7.60
EL-4	28.3	0.14	3 hr.	0.17	2.91	n/a	n/a
EL-5	28.3	0.06	72 hr.	0.12	7.17	8.90	10.6
Average	29.1	0.11		0.21	4.94	7.24	8.58
SD	±1.1	±0.04		±0.10	±1.63	±1.19	1.43
High-Titer Mice							
EI-1	32.7	0.72	3 hr.	0.10	13.7		
EI-2	29.5	2.3	3 hr.	0.12	13.6		
EI-5	30.1	1.5	3 hr.	0.13	13.3		
EI-9	29.5	1.1	48 hr.	0.16	16.2	51.8	
EI-10	30.2	0.75	48 hr.	0.21	13.1	40.7	
EI-12	36.4	1.2	168 hr.	0.12	20.1	56.8	192
EI-13	29.0	0.60	168 hr.	0.25	11.8	40.2	152
EI-14	33.8	1.8	168 hr.	0.10	17.7	57.6	232
Average	31.6	1.4		0.15	14.6	49.4	192
SD	±2.4	±0.6		±0.05	±2.6	±8.5	±40
Very High-Titer Mice							
7m-1	33.2	5.0	336 hr.	0.18	16.1	56.0	225
7m-3	36.2	7.4	336 hr.	0.12	15.3	47.4	204
7m-5	35.9	16.3	336 hr.	0.13	18.6	62.1	237
12m-1	35.6	5.7	168 hr.	0.12	13.2	40.1	145
12m-3	40.4	12.2	168 hr.	0.13	14.6	42.5	149
Average	36.3	9.3		0.14	15.6	49.6	192
SD	±2.6	±4.8		±0.03	±2.0	±9.3	±43

Note: Raw data from non-immunized and mock-immunized mice were processed based on a two-compartment model using WinNonlin, while those from immunized mice were fitted to the model given in Fig. 4.

[a] Dose = 100 μL of 0.3 mM FL-EA.

[b] Not detected.

groups immunized with FL and EY. For instance, $AUC_{0\text{-}12h}$ of FL-EA in the high-titer group was 49.4 ± 8.5 mmol · hr/mL (see Table III) whereas it was only 4.04 ± 0.46 mmol · hr/mL in EY-immunized mice.

In a converse manner, 3 mice with very high anti-FL titers were administered a 19-nmole dose of EY-EA and their blood and peritoneal fluid was sampled up to 168 h. Serum and peritoneal concentration-vs-time profiles are shown in Fig. 4B. Again, these mice showed much faster clearance of the mismatched probe. Equilibration of EY-EA between blood and peritoneal fluid was much more rapid than was equilibration of FL-EA in the same group of mice. The initial distribution volume of EY-EA in these mice, 0.33 ± 0.10 mL/g, was significantly larger than the 0.14 ± 0.05 mL/g observed for FL-EA (Table III). This is undoubtedly due to a high level of unbound EY-EA.

Effect of Dose

Three groups of high-titer mice were selected such that the average titer of the groups was as similar as possible. The groups were then randomly selected to receive doses of 15, 1.5 or 0.15 nmoles of FL-EA, and blood was sampled every hour for 6 h. Clearance for the middle and low dose mice was equal within experimental error (data not shown). Data from the high dose mice was unreliable, since all three failed to bleed

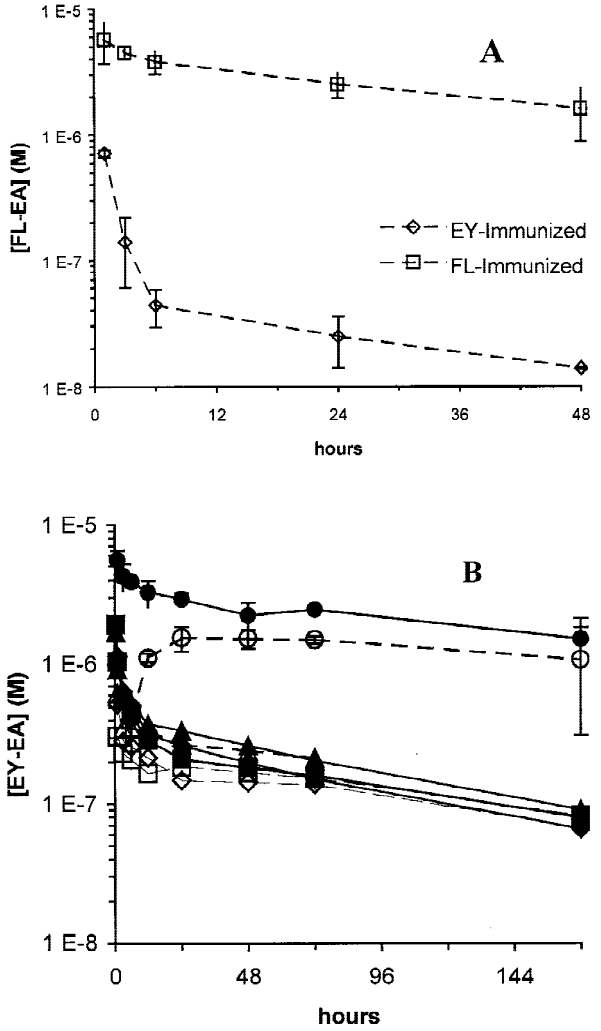


Fig. 4. (A) Pharmacokinetics of FL-EA in mice immunized against eosin Y (EY: lower curve with diamond) and in high anti-FL titer mice (upper curve with squares). (B) Pharmacokinetics of EY-EA in mice immunized with FL, where different closed symbols in the lower part represent results from each of three very high-titer mice. EY-EA levels in peritoneal fluid (open symbols) are also shown. For comparison, average data from very high-titer mice given FL-EA is shown in the upper part, where closed and open symbols represent FL-EA levels in serum and peritoneal fluid, respectively.

readily, and one died after an hour of pronounced lethargy. Similar, but more severe, toxicity was observed in immunized, but not in non-immunized mice, given a 30-nmole dose of FL-EA. Thus the toxicity appears to have been immune-mediated.

Pharmacokinetic Modeling

Data from immunized mice were fitted with a mathematical expression of the model given in Fig. 3. Based on the observation that FL-EA readily distributes outside the bloodstream where antibody titers are similar to those in the serum, the model incorporates two compartments and assumes similar binding interactions in the blood and the extravascular space. Since antibodies themselves can slowly move in and out of the bloodstream, immune complexes are assumed to do the same. Because WinNonlin was exceptionally slow in the analysis, a complex Excel spreadsheet was used to fit regressions to the data (details not given but available on request). Because of the large number of parameters, multiple good fits of the data were possible for all animals. For this reason, the elimination rate constant k_e was set equal to the average of the values obtained for the non-immunized mice. Additionally, we let $k_1 = k_{-1}$ and $k_2 = k_{-2}$. These changes allowed a reduction in the number of parameters such that unique estimates for the values of affinity constant (K_A) and antibody concentration could be obtained.

Only a limited number of immunized mice involved in pharmacokinetic experiments were subject to affinity determination. For the 4 high-titer mice, the K_A value estimated from the model analysis was approximately 5- to 10-fold lower than the experimentally determined values. In terms of titer, however, a meaningful comparison can be made in many mice between the estimated and determined anti-FL titer. As shown in Table I, the observed titers varied almost 100-fold, ranging from average 0.11 *fbu* in the low-titer group to 9.30 *fbu* for the very high-titer group. However, the values estimated from the model varied only a little: 0.36 ± 0.20 *fbu,* 0.79 ± 0.15 *fbu,* and 0.91 ± 0.36 *fbu,* for the low-, high-, and very high-titer groups, respectively. It was also interesting to notice that the model predicted a higher affinity in animals with high titers and that the range of variations was as much as 100-fold over the three groups treated in the model analysis.

Because of the bi-phasic disappearance of the conjugates, the time profiles of antibody-binding compounds have typically been fitted to an equation containing two exponential terms (15). Regressions from these two models, one based on Fig. 3 and the other on the empirical equation, are almost superimposable over the course of the experiment, and both seem to fit the data equally well. However, when the fits are extrapolated beyond the available data, the two models diverge considerably.

DISCUSSION

The goal of the present study is to better understand the pharmacokinetics of drug compounds that bind endogenous antibodies and to develop a pharmacokinetic model that could describe the disposition of these molecules in terms of measurable parameters. Our approach has been first to develop an animal system and then to observe the effects of altering parameters expected to affect the pharmacokinetic outcome. We have done this by altering the immunization protocol to yield different titers, by using a lower affinity hapten, and by altering the dose of hapten.

We chose to utilize active immunization because of our interest in endogenous antibodies as drug carriers. The active immunization model was BALB/c mice given a vaccine prepared from FL-KLH in Freund's adjuvants. By altering the volume and frequency of vaccine administration we were able to obtain three groups of mice with titers differing by about one log from one group to the next. Fairly consistent anti-FL titers were developed within the groups of mice immunized by the same protocol. Binding affinity was altered independently by administering a lower-affinity FL analog, eosin Y (EY), to mice immunized against FL.

The concentration-time profile of our radiolabeled conjugate probe, FL-EA, was remarkably consistent within the different titer groups. Relative standard deviations in serum

concentration at individual time points were typically as low in the immunized mouse groups as they were in the non-immunized group. A significant difference in pharmacokinetics was seen between the low-titer (~0.1 *fbu* or approximately 7×10^{-8} M^{-1}) and high-titer (~1 *fbu*) groups. By comparison, little change was seen between the high- titer and very high-titer (~10 *fbu*) groups. Mice with titers between these high- and low- titer groups had concentration-time curves that fell between those of the two groups (data not shown). These observations are consistent with the presence of a titer threshold below which the carrier effect drops off and above which total drug clearance is relatively unaffected by increased titer. The observed upper threshold may represent the antibody concentration and/or affinity sufficient to bind practically all of the conjugate introduced.

One might anticipate that a probe that binds to serum antibodies with low affinity would simply be cleared more rapidly. The pharmacokinetics of the low-affinity probe, EY-EA, in very high-titer mice turned out to be considerably more complex (Fig. 4B). Initially its concentration decreased quickly in the serum, but after 6 h the decline slowed substantially. The apparent half-life for the second phase was about 26 h, almost as long as that of FL-EA in the same group of mice (~34 h). Similar results were obtained with FL-EA administered to EY-immunized mice.

Measurement of EY-EA concentrations in peritoneal ascites, open symbols in the lower part of Fig. 4B, helps explain the biphasic decline of EY-EA. Anti-FL titers in serum and ascites from the same mouse were found to be almost equal, indicating that the very high-titer mice have a large reservoir of binding sites outside their bloodstream. Because antibodies could not readily cross the endothelium, antibody binding effectively hindered movement of the haptens across the barrier in both directions. Thus clearance of EY-EA was slowed not as much because antibody binding blocked excretion but because it slowed re-distribution. The extent to which this plays a role in the overall pharmacokinetics likely depends not only on how well the free hapten crosses the endothelium but also on binding factors such as antibody titer and affinity. The fact that the extent of re-distribution was greater with the low-affinity hapten suggests that the impact of reduced affinity in the bloodstream may, to a small degree, be balanced by more extensive re-distribution out of the bloodstream.

Because of experimental limitations, unbound drug pharmacokinetics were not assessed to any great extent in this study. In one experiment with very high-titer mice, unbound FL-EA measurements were surprisingly constant over the course of seven days, but these results may have been in part due to insensitivity of the assay at the very low concentrations measured. Nonetheless, some predictions can be made about free hapten levels based on computer modeling and on simple mathematics. As antibody concentration and affinity decrease, the unbound fraction of the hapten increases. Thus unbound hapten concentration at any particular time point and AUC over any given time interval should be less sensitive to changes in the binding parameters than total hapten.

For example, when the data from the various mouse groups are fitted to the model shown in Fig. 3, it can be seen that although the total hapten concentrations vary substantially, much less difference is seen in the estimated unbound hapten concentrations (analysis results not shown). Only in the very high-titer group is a dramatic difference seen, since the extensive antibody binding there more strongly impacts the unbound fraction than it does total hapten clearance (Fig. 2). When translated to antibody-binding drugs, the increase in unbound drug with increasing clearance may serve as a mechanism to blunt some of the effect of variability in binding.

In the high-titer mice, a 10-fold change of hapten dose had little or no impact on clearance, at least for the first 6 h after administration. Substantial toxicity was observed when high-titer mice were given doses of FL-EA higher than about 100 nanomoles. Mice were extremely lethargic and did not bleed readily when cut. Death, sometimes occurred, usually 1 to 2 h after dosing. Because non-immunized mice given the same doses showed no signs of toxicity, the observed morbidity is assumed to be related to an immune response.

The model chosen for fitting data was the minimal model that incorporated the factors known to be important in hapten pharmacokinetics, namely complexation in two compartments and hindered redistribution between those compartments. Nonetheless, the model is still sufficiently complex that multiple solutions are available for any given set of data. When constrained to a single solution, the fits were disappointing in the following two respects. First, for the model to apply universally, the estimates for the redistribution rate constants and for the volumes of distribution should have been similar from one group of mice to the next. In actuality, the estimates varied quite widely from group to group. Secondly, if the model were to have predictive value, then parameter estimates should be in line with measurements of those parameters. Estimates from the model were not consistently in good agreement with measurements of corresponding parameters. Estimates for both affinity and titer were lower than expected or determined.

Several factors likely contributed to the difference between measurements and estimates and to the deficiency of the model. First, measurements of the low-affinity binding constant and capacity were unreliable by Scatchard analysis of the data from the precipitation method. Given adequate antibody concentration, high-affinity binding is probably the most important determinant of hapten pharmacokinetics, but low-affinity/high-capacity binding may certainly play a significant role. Secondly, the actual concentration of antibodies of a given specificity is difficult to determine, so titer is usually used as a surrogate. Thirdly, highly substituted FL-BSA was used in the ELISA of titers and thus anti-FL antibodies are likely to bind di-valently with high avidity. Thus our assay must have measured the concentration of not only high-affinity antibodies but also of antibodies with more modest affinities. This could explain why the concentrations calculated from the measured titers were sometimes 5- to 10-fold higher than the estimates obtained from fitting the curves. Finally, being a small molecule, FL-EA should have access to many tissues from which antibodies are excluded. Such tissue might represent a third compartment contiguous to the central and peripheral compartments.

Despite these shortfalls, the current model represents, to our knowledge, the first experimentally tested attempt to incorporate measurable parameters into a mathematical description of hapten pharmacokinetics. As such, it can serve as a template for future development. For any given data set, a simple two-compartment model is likely to fit just as well as the model proposed. However, such an approach may have

no predictive value either in estimating what will happen in an animal or patient with different antibody titers or in predicting outcome beyond the end of the experiment. The basic constraint to continued development of the model has been keeping the model from becoming too complex for the data. The number of parameters had to be kept at a reasonable level so as to allow unique fits to data set. This difficulty could potentially be overcome by increasing the number of data set per experiment and/or by simultaneously fitting several data from several experiments.

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