Pharmacokinetics in the Rat of a Panel of Immunotoxins Made with Abrin A Chain, Ricin A Chain, Gelonin, and Momordin

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ABSTRACT

A panel of immunotoxins was constructed by chemically attaching the ribosome-inactivating proteins abrin A chain, ricin A chain, gelonin, and momordin to the monoclonal mouse IgG2a antibody Fib75 by means of a disulfide linkage. All the immunotoxins were toxic in tissue culture to the EJ human bladder carcinoma cell line expressing the antigen recognized by Fib75, inhibiting the incorporation of [35S]methionine by 50% at concentrations between 1 x 10^-10 M and 8 x 10^-10 M. The pharmacokinetics of the immunotoxins in the normal Wistar rat was determined following i.v. administration. The concentrations of intact immunotoxin in serum samples taken at various intervals after injection for up to 24 h were measured by solid-phase enzyme-linked immunosorbent assays specific for each of the four different ribosome-inactivating proteins. The Fib75 immunotoxins were cleared from the circulation with comparable, but not identical, biphasic kinetics best described by a two compartment open pharmacokinetic model. The a-phase half-lives of the panel, between 0.35 and 0.71 h, were similar. The b-phase half-life of Fib75-abrin A chain, 13.3 h, was significantly longer than the b-phase half-lives of Fib75-ricin A chain, Fib75-gelonin, and Fib75-momordin, between 7.5 and 8.6 h. Fib75-abrin A chain was found to be about 3- to 4-fold more resistant than the other immunotoxins to breakdown by reduction of the disulfide linkage between the A chain and the antibody with glutathione in vitro. This suggests that the longer serum half-life of Fib75-abrin A chain may have been due to greater stability against reduction in vivo. Analysis of serum samples obtained up to 24 h after injection of Fib75-abrin A chain revealed that the chemically intact immunotoxin present in the circulation retained full cytotoxic activity. An abrin A chain immunotoxin made with a different monoclonal mouse IgG2a antibody was also found to be more stable against reduction by glutathione in vitro than an analogous ricin A chain immunotoxin. Thus, abrin A chain may possess unique molecular properties that endow immunotoxins made with this A chain with greater stability in vivo than immunotoxins made with ricin A chain or other ribosome-inactivating proteins.

INTRODUCTION

ITs are hybrid protein molecules consisting of a toxic protein linked to an antibody. Many agents of this type can bind selectively to cells that express the target antigen recognized by the antibody component and kill the cells by the intracellular action of the toxic protein. Immunotoxins made using antibodies recognizing tumor-associated antigens have been shown to exert potent and selective cytotoxic effects in animal tumour models and are currently undergoing clinical trials in cancer patients (reviewed in Refs. 1-4).

Ricin A chain, a RIP isolated from the plant toxin ricin, has most frequently been used in the synthesis of ITs. The first experimental studies in which ricin A chain ITs were administered i.v. to rabbits and rats revealed that the IT was rapidly lost from the circulation (5-7). Subsequent studies clearly demonstrated that the cause of the initial rapid clearance after injection of the IT was specific receptor-mediated hepatic recognition of an oligomannose side chain present exclusively on the A3 form of native ricin A chain (8–10). Three strategies have been shown to minimize uptake of ricin A chain ITs by the liver in vivo: (a) saturation of the hepatic receptor system by coadministration of the IT and an excess of mannose-containing protein or carbohydrate (8, 9, 11); (b) chemical treatment of the native ricin to destroy the mannose residues (10); and (c) the use of the A1 form of native ricin A chain which lacks the oligomannose side chain (12).

An alternative approach to bypassing the problem of hepatic clearance is to construct ITs with RIPs other than ricin A chain. A large family of RIPs of diverse plant origin has now been identified (13). These RIPs resemble ricin A chain in size and mode of action but differ from ricin A chain and from one another in primary structure, pl, and the degree and type of glycosylation. Pharmacokinetic studies have been reported for ITs made with abrin A chain (14), pokeweed antiviral protein (15, 16), gelonin (16–21), saporin (17, 18, 22–25), and trichokirin (26). A common feature of the majority of these studies is the finding that all types of IT have a much shorter half-life in the bloodstream than the parent antibody. However, it is difficult to make direct comparisons between the results of the various studies because of major differences in experimental detail. These differences include: (a) the nature of the antibodies; (b) the methods of IT preparation and purification; (c) the animal species; (d) the times of the sampling points; (e) the procedures for IT quantification; and (f) the methods of pharmacokinetic analysis.

In this study, we have measured the blood clearance of a panel of ITs prepared using a single monoclonal antibody, Fib75, which recognizes an integral membrane glycoprotein (M, 19,000) present on most differentiated normal and neoplastic human cells and not expressed by rodent tissues. Cytotoxic ITs were prepared by similar chemical procedures using abrin A chain, native ricin A chain, gelonin, and momordin. Samples of IT were injected into normal rats i.v. and blood samples were taken at identical times after injection. The concentration of IT in serum was determined using RIP-specific ELISAs detecting only intact IT molecules. All the experimental data were analyzed by the same mathematical procedures. This study showed that the Fib75-abrin A chain IT had the longest blood half-life of the ITs in the panel. The disulfide bond linking abrin A chain to the Fib75 antibody was found to be less susceptible to reduction by glutathione in vitro than the disulfide linkages in the ITs made with the other RIPs suggest-
ing that the longer persistence of the abrin A chain IT may be attributed, in whole or in part, to a slower rate of breakdown in vivo.

MATERIALS AND METHODS

Materials

Castor bean cake derived from seeds of Ricinus communis of Kenyan origin was obtained from Croda Premier Oils, Hull, Humberside, England. Seeds of Abrus precatorius were obtained from the Department of Botany, University of Ghana, Accra, Ghana. Seeds of Gelonium multiformum and Momordica charantia were from United Chemicals and Allied Products, Calcutta, India.

The murine hybridoma LICR-LOND Fib75 secreting a mouse monoclonaal antibody of the IgG2a isotype was provided by Dr. R. A. J. Mellhiney. The antibody Fib75 was purified from the ascitic fluid of hybridoma-bearing mice by ammonium sulfate precipitation followed by chromatography on immobilized staphylococcal protein A using stepwise elution. The mouse monoclonal antibody 2AL-1, raised against vesicular stomatitis virus and also of the IgG2a isotype, was purified by a similar procedure.

Chromatography media, Sephade x G-25 (F), Sephacryl S-200, Sepharose 4B, protein A-Sepharose 4B, and CNBr-activated Sepharose 4B, were purchased from Pharmacia, Ltd., Milton Keynes, Buckinghamshire, England. TSK-G3000 HPLC columns were purchased from Anachem, Ltd., Luton, Bedfordshire, England.

Sheep anti-mouse immunoglobulin-gorseradish peroxidase (NA.931), sodium [35]iodide (IMS.30), and L-[4,5-3H]leucine (TRK.170) were purchased from Amersham International plc., Amersham, Buckinghamshire, England. EmulsiFyve-Safe liquid scintillation cocktail was obtained from Canberra Packard Ltd., Pangbourne, Berkshire, England. Iodo-Gen was purchased from Pierce (UK), Ltd., Chester, England.

Iodoacetamide was obtained from BDH, Ltd., Poole, Dorset, England. Glutathione and o-phenylenediamine were purchased from Sigma Chemical Co., Ltd., Poole, Dorset, England. SPDP was from Pharmacia, Ltd. All other reagents were of the highest purity available.

Tissue culture media, RPMI 1640 and Dulbecco's modified Eagle's medium, and fetal calf serum were purchased from Gibco, Ltd., Paisley, Scotland.

Flat-bottomed 96-well micro-ELISA plates (Immulon 2) were obtained from Dynatech Laboratories, Ltd., Billingham, Sussex, England. Sterile tissue culture plates (24 wells) were from Nunclon, Ltd., Uxbridge, Middlesex, England.

Normal male albino Wistar/CBI rats, 8–16 weeks old, were supplied by the M.R.C. Animal Breeding Unit, National Institute of Medical Research, London, England, and allowed free access to food and water. Male New Zealand White rabbits were obtained from J. & L. G. Phillips, Ltd., Petersfield, Hampshire, England.

Purification of Ribosome-inactivating Proteins

Ricin was purified from an aqueous extract of defatted castor bean cake by ammonium sulfate precipitation, affinity chromatography on acid-treated Sepharose 4B, and gel permeation chromatography on Sephacryl S-200 essentially as described by Nicolson and Blaustein (27) with the modifications previously described by Cumber et al. (28). Ricin A chain was isolated from the toxin by reductive cleavage and further purified by the method described by Forrester et al. (29). This preparation gave two bands with apparent molecular weights of about 30,000 and 32,000 when examined by SDS-PAGE corresponding to the two differently glycosylated forms of the A chain.

Abrin was purified from defatted meal of A. precatorius seeds and abrin A chain was isolated from the toxin and purified by procedures identical with those described for ricin A chain (28, 29). The preparation gave a single band on SDS-PAGE with an apparent molecular weight of about 29,000.

Gelonin was prepared as described by Stripe et al. (30). Momordin was prepared according to the method of Barbieri et al. (31). Purified gelonin and momordin both appeared as single bands with apparent molecular weights of about 30,500 and 31,000, respectively, by SDS-PAGE.

Preparation and Characterization of Immunotoxins

Abrin A chain and ricin A chain were attached to the Fib75 antibody using the methodology described in detail by Cumber et al. (28). Briefly, 2-pyrindyl disulfide groups were first introduced into Fib75 at an average derivatization level of about 1.8 groups/antibody by reaction with SPDP. The derivatized antibody was then reacted with an excess of freshly reduced toxin A chain. The reaction mixture was applied to a column of Sephacryl S-200 and the material which eluted at a position corresponding to IT (M, 180,000–210,000) was collected. Immunotoxins made with the 2AL-1 antibody were prepared by the same procedure.

Gelonin and momordin were modified by reaction with SPDP to introduce an average of 1.0 2-pyridyl disulfide groups/RIP molecule and were reduced with dithiothreitol before mixing with derivatized antibody as above. The gelonin and momordin ITs were purified free of unconjugated RIP by gel permeation chromatography on Sephacryl S-200 as for the toxin A chain ITs.

The IT preparations were analyzed by SDS-PAGE and gel permeation HPLC. Electrophoresis was performed on 4.0–12.5% gradient polyacrylamide gels in the presence of 0.2 g SDS/100 ml solution (32). Samples were prepared for electrophoresis in the absence of reducing agent to preserve the disulfide linkage between the antibody and RIP. Gel permeation HPLC was performed on a TSK-G3000SW column (7.5 mm inside diameter x 60 cm). Samples (0.1 ml) were applied to the column and eluted at a flow rate of 0.03 ml/min. The running buffer was 20 mm sodium phosphate-0.1 m sodium sulfate, pH 6.8, containing 0.05 g NaN3/100 ml solution. The column was calibrated using protein standards of known molecular weight.

The RIP content of the final IT preparations was determined as described by Cumber et al. (28). The content of unconjugated antibody and different IT species in the final preparations was estimated following SDS-PAGE by densitometry of Coomassie blue-stained protein bands using a Bio-Rad Model 620 video densitometer.

Preparation of Affinity-purified Anti-RIP Antibody

Rabbit antisera to abrin A chain, ricin A chain, gelonin, and momordin were produced by the procedure described by Worrell et al. (17). Abrin A chain- and ricin A chain-specific antibody was isolated from serum by affinity chromatography on columns of A. precatorius agglutinin and R. communis agglutinin, respectively, linked to CNBr-activated Sepharose 4B. Gelonin- and momordin-specific antibody was isolated from antiserum by affinity chromatography on a column of the appropriate RIP linked to CNBr-activated Sepharose 4B.

Measurement of Immunotoxin Concentration in Serum Samples by Enzyme-linked Immunosorbe nt Assay

The solid-phase ELISA procedure used was the method originally described for detecting intact ITs made with ricin A chain (33). Briefly, affinity-purified anti-RIP antibody was first immobilized on micro-ELISA plates. Serum samples containing IT were added to the plates and, following incubation and washing, the bound IT was detected using anti-mouse immunoglobulin-horseradish peroxidase in combination with an o-phenylenediamine substrate solution developing color at 492 nm. The mean absorbance at 492 nm of triplicate serum samples was used to calculate IT concentration at each time point by reference to a standard curve spanning a concentration range of 0.125–15 ng/ml. The maximum standard deviation from the determined mean values ranged between 5 and 9% in individual experiments.

Measurement of IT Breakdown in Vitro

ELISA. A stock solution of glutathione was prepared in PBS at a concentration of 10 mm and sterilized by passage through a 0.22-µm filtration unit (Millex GV). The concentration of reduced glutathione in the stock solution was confirmed by measuring the amount of reactive.
jugated A chains and the peak heights from linear standard curves
were prepared in PBS without the addition of carrier protein. Each IT solution (135 µl) was mixed with 15 µl of conjugated RIP per ml of glutathione solution at the three concentrations. After incubation for 1 h at 37°C under sterile conditions, iodoacetamide was added to a final concentration of 13 mM. Control samples were treated in identical fashion except that the glutathione was omitted. Samples (0.1 ml) of the IT solutions were analyzed by gel permeation HPLC on a TSK-G3000SWXL column (7.8 mm inside diameter x 30 cm) equilibrated with 20 mM sodium phosphate-0.1 M sodium sulfate, pH 6.8, containing 0.05 g of Na3VO4/100 ml of solution at a flow rate of 0.4 ml/min. The absolute amounts of ricin A chain or abrin A chain released from intact IT in each sample was determined by a solid-phase ELISA similar to the method described above. Rate constants for the slower phase of the reaction were calculated assuming pseudo-first order reaction kinetics.

Gel Permeation HPLC. Solutions of glutathione were prepared in PBS at concentrations of 0.1 M, 10 mM, and 1 mM and sterilized by filtration. Sterile IT solutions containing between 100 and 170 µg of conjugated RIP per ml were prepared in PBS without the addition of carrier protein. Each IT solution (135 µl) was mixed with 15 µl of glutathione solution at the three concentrations. After incubation for 1 h at 37°C under sterile conditions, iodoacetamide was added to a final concentration of 13 mM. Control samples were treated in identical fashion except that the glutathione was omitted. Samples (0.1 ml) of the IT solutions were analyzed by gel permeation HPLC on a TSK-G3000SWXL column (7.8 mm inside diameter x 30 cm) equilibrated with 20 mM sodium phosphate-0.1 M sodium sulfate, pH 6.8, containing 0.05 g of Na3VO4/100 ml of solution at a flow rate of 0.4 ml/min. The absolute amounts of ricin A chain or abrin A chain released from intact IT were determined by incubation with glutathione at the three concentrations. After incubation for 1 h at 37°C under sterile conditions, iodoacetamide was added to a final concentration of 13 mM. Control samples were treated in identical fashion except that the glutathione was omitted. Samples (0.1 ml) of the IT solutions were analyzed by gel permeation HPLC on a TSK-G3000SWXL column (7.8 mm inside diameter x 30 cm) equilibrated with 20 mM sodium phosphate-0.1 M sodium sulfate, pH 6.8, containing 0.05 g of Na3VO4/100 ml of solution at a flow rate of 0.4 ml/min. The absolute amounts of ricin A chain or abrin A chain released from intact IT were determined by incubation with glutathione at the three concentrations. After incubation for 1 h at 37°C under sterile conditions, iodoacetamide was added to a final concentration of 13 mM. Control samples were treated in identical fashion except that the glutathione was omitted. Samples (0.1 ml) of the IT solutions were analyzed by gel permeation HPLC on a TSK-G3000SWXL column (7.8 mm inside diameter x 30 cm) equilibrated with 20 mM sodium phosphate-0.1 M sodium sulfate, pH 6.8, containing 0.05 g of Na3VO4/100 ml of solution at a flow rate of 0.4 ml/min. The absolute amounts of ricin A chain or abrin A chain released from intact IT by coincubation with glutathione were calculated by comparing the peak heights of material eluting with the retention time of the unconjugated A chains and the peak heights from linear standard curves generated with samples of the appropriate A chain at concentrations of 1–15 µg A chain/ml. The total A chain content of the ITs was determined from the A chain released by incubation of the IT in the presence of 10 mM glutathione for 5 h at 37°C.

Cytotoxicity Experiments in Tissue Culture

Cytotoxicity experiments using the EJ human bladder carcinoma cell line were carried out essentially as described by Forrester et al. (29). Briefly, dilutions of IT solution were added to subconfluent monolayer cultures of EJ cells in triplicate and incubated for 24 h. [3H]Leucine (1 µCi) was added to each culture followed by incubation for a further 24 h. At the end of this period, the incorporation of [3H]leucine was determined by liquid scintillation counting. The results were expressed as a percentage of the [3H]leucine incorporated by control cultures not receiving IT. The incorporation of [3H]leucine by control cultures was greater than 80,000 cpm.

Blood Clearance Measurements

Immunotoxins. ITs were prepared in sterile solution at concentrations of conjugated RIP between 109 and 187 µg/ml. These preparations were checked by gel permeation HPLC shortly before administration to animals in order to ensure the absence of aggregated protein. Clearance studies were performed as described by Worrell et al. (7) following a single i.v. injection of 11–22 µg of conjugated RIP. The concentration of intact IT in serum samples was determined using the ELISA procedure described above. The experimental data were adjusted according to the amount of conjugated RIP injected and animal weight in each experiment and are expressed as the serum concentration based on the injection of 10 µg of conjugated RIP/300 g of rat weight.

Ribosome-inactivating Proteins. RIPs were radiolabeled to a specific activity of 200–400 µCi 125I/mg protein using the Iodo-Gen method (35). In the case of abrin A chain and ricin A chain, the intact toxins were labeled and the A chains were isolated subsequently using the usual procedure. Groups of rats, treated as above, received injections of 125I-RIP solution in PBS containing between 1.0 and 1.3 × 108 cpm. Samples of blood taken at intervals between 2 and 30 min after injection were weighed and the radioactivity present in the samples was determined in a Packard 5266 gamma counter.

Pharmacokinetic Analysis

Blood clearance curves were fitted to the determined serum levels of IT by a computerized nonlinear least-squares regression algorithm (36). The experimental data were most consistent with a two compartment open pharmacokinetic model described by the biexponential equation

$$C = Ae^{-at} + Be^{-bt}$$

where C is the concentration at time t and A, B and α, β are the concentration and rate constants, respectively. The weighting function $1/(Y + \hat{Y})^2$ was applied to all measurements (37). The C0 values were calculated from the fitted curves by back extrapolation, i.e., $C0 = A + B$. The α- and β-phase half-lives were calculated as $t1/2 = 0.6931/\alpha$ or $\beta$. The area under the serum concentration versus time curve at infinite time after injection (AUC) was calculated as the integral of the curve using the formula

$$AUC = A/\alpha + B/\beta$$

RESULTS

Characterization of the Fib75 Immunotoxins. Fib75 ITs made with abrin A chain, ricin A chain, gelonin, and momordin were synthesized using closely matched preparative conditions. In each case, the IT preparation was completely separated from unconjugated RIP and partially resolved from unmodified antibody by a single step of gel permeation chromatography on Sephacryl S-200. The four IT preparations all had a similar composition as judged by SDS-PAGE (Fig. 1). Densitometry was used to calculate the mean percentage content of the different molecular species and the standard deviations from the mean values. The predominant species present was the singly substituted antibody-RIP conjugate [51 ± 3% (SD)], containing one RIP molecule linked to one antibody molecule, with lesser amounts of multiply substituted antibody-RIP conjugates [23 ± 12%] and of unconjugated Fib75 antibody [26 ± 11%]. All the IT preparations were further analyzed by gel permeation HPLC. Fig. 2 shows the elution profile of Fib75-abrin A chain which is representative of the elution profiles seen for all the ITs in the figure. This HPLC analysis showed

![Fig. 1. SDS-PAGE analysis of Fib75 antibody and ITs. Samples were run on a 4–12.5% gradient polyacrylamide gel under nonreducing conditions. Protein bands were visualized by Coomassie Brilliant Blue staining. Lane 1, unconjugated Fib75; Lane 2, Fib75-abrin A chain; Lane 3, Fib75-ricin A chain; Lane 4, Fib75-gelonin; Lane 5, Fib75-momordin.](image-url)
the presence of multiply substituted IT species (Fig. 2, Peak A), singly substituted IT (Fig. 2, Peak B), and unconjugated antibody (Fig. 2, Peak C) in proportions consistent with the analysis by SDS-PAGE and of minimal amounts of aggregated protein or unconjugated RIP.

The Fib75 ITs were all toxic to the EJ human bladder carcinoma cell line in tissue culture. The concentrations at which [3H]leucine incorporation by cells was reduced by 50% for the panel of ITs ranged between approximately $1 \times 10^{-10}$ and $8 \times 10^{-18}$ M. In direct comparisons of cytotoxicity in vitro, such as in the representative experiment shown in Fig. 3, the ITs differed consistently in potency. The order of potency was: Fib75-abrin A chain > Fib75-ricin A chain > Fib75-momordin > Fib75-gelonin. The difference in cytotoxicity between Fib75-abrin A chain and Fib75-ricin A chain was statistically significant: $P < 0.02$ by Student's $t$ test. The unconjugated RIPs or unconjugated Fib75 antibody exerted no significant cytotoxic effects upon EJ cells at concentrations of $1 \times 10^{-8}$ M (not shown).

**Pharmacokinetics of the Fib75 Immunotoxins in the Rat.** The serum concentrations of the four Fib75 IT preparations in normal Wistar rats were determined at various intervals up to 24 h after i.v. injection. All four ITs were cleared from the circulation with biphasic kinetics (Fig. 4) that were best described by a two compartment open pharmacokinetic model. The pharmacokinetic data are shown in Table 1.

Fig. 2. Gel permeation HPLC of Fib75-abrin A chain. Fib75-abrin A chain was applied to a column of TSK-G3000SW and eluted as described under "Materials and Methods." Arrows, elution positions of molecular weight standards with values in thousands.

Fig. 3. Cytotoxic effects of Fib75 ITs upon EJ human bladder carcinoma cells in tissue culture. Monolayer cultures were incubated for 48 h in the presence of different concentrations of Fib75-abrin A chain (●), Fib75-ricin A chain (○), Fib75-gelonin (■), or Fib75-momordin (□). Points, mean values of triplicate measurements of [3H]leucine incorporated by the cells during the final 24 h of culture expressed as a percentage of the incorporation in untreated cultures. The standard deviations from the mean values were <8% and have been omitted for the sake of clarity.

In the case of Fib75-ricin A chain, a substantial amount of the IT had disappeared from the bloodstream by the time the first sample was taken at 2 min after the injection of the IT solution. This rapid loss was excluded from the analysis in order to allow fitting of the two compartment pharmacokinetic model to the data and afford a direct comparison with the other ITs in the panel (see "Discussion"). The calculated $\alpha$-phase half-lives of the abrin A chain, ricin A chain, and momordin ITs, 0.50, 0.70, and 0.71 h, respectively, were not significantly different. The gelonin IT had the shortest $\alpha$-phase half-life of 0.35 h. This value was significantly shorter than the half-lives of the ricin A chain and momordin ITs (0.1 > $P$ > 0.05). The $\beta$-phase half-lives of the ricin A chain, gelonin, and momordin ITs, 7.5, 8.0, and 8.6 h, respectively, were similar. In contrast, the $\beta$-phase half-life of Fib75-abrin A chain was 13.3 h. The longer half-life of the abrin A chain IT in the $\beta$-phase of clearance compared with those of the other ITs was highly significant (0.01 > $P$ > 0.001).

Fib75-gelonin did not suffer the early rapid clearance shown by Fib75-ricin A chain and thus was present at higher concentration in the bloodstream than the ricin A chain IT. However, when compared as a percentage of the calculated $C_0$ values after 4 and 24 h, the clearance of these two ITs was very similar. The serum concentration of Fib75-gelonin was lower than that of Fib75-momordin from 1 h after injection onwards because the gelonin IT had a shorter $\alpha$-phase half-life and because its $\alpha$-phase was prolonged compared with that of the momordin IT (see Table 1). This indicates that the principal difference in the clearance of the gelonin and momordin ITs occurred shortly after injection since the $\beta$-phase half-lives of the two ITs did not differ significantly. Fib75-abrin A chain persisted in the circulation longer than Fib75-momordin and was present in the highest amount after 24 h, at 18.3% of the calculated $C_0$. The concentration of the abrin A chain IT was comparable with that of the momordin IT at 4 h after injection but was about 1.5-fold higher at 24 h after injection indicating that the difference in the clearance of these two ITs occurred predominantly in the $\beta$-phase. As a result, the area under the serum concentration versus time curve for the abrin A chain IT was calculated to be 1.5-fold greater than that for the momordin IT.

The blood clearance of unconjugated abrin A chain, ricin A chain, gelonin, and momordin was also measured following i.v. administration of [125I]-labeled RIPs. In contrast with the rates...
of clearance of the ITs, all the RIPs cleared very rapidly from the bloodstream with an initial half-life of less than 5 min (not shown).

Breakdown of Fib75 and Control Immunotoxins in Vitro. The susceptibility of the panel of Fib75 ITs to breakdown in the presence of reducing agent was assessed by incubating a solution of each IT in the presence of 5 mM glutathione at 37°C in vitro. The amount of intact IT remaining after different times of exposure to the glutathione was measured using solid-phase ELISA. In each case, coincubation with glutathione led to a progressive decline in the amount of intact IT (Fig. 5) whereas there was no significant loss of IT as measured by the ELISA when glutathione was omitted from the incubation (not shown). The breakdown of the ITs in the presence of the large (approximately 500,000-fold) molar excess of glutathione used in the experiment would have been predicted to follow pseudo-first order reaction kinetics giving a linear relationship between the logarithm of the IT concentration and time. The biphasic kinetics actually observed indicated that the IT preparations might be heterogeneous with respect to the rate of cleavage of the disulfide bond. In the more rapid phase of breakdown, the decrease in the amount of intact IT was most pronounced in the case of Fib75-gelonin, greater than 50% loss occurring within 1 h of incubation, compared with the loss of only about 20% of Fib75-abrin A chain (Fig. 5). Fib75-ricin A chain and Fib75-momordin broke down in similar amounts intermediate between those of the abrin A chain and gelonin ITs. In the slower phase of breakdown, the Fib75 ITs made with ricin A chain, gelonin, and momordin all broke down with similar rate constants of between 0.14 and 0.17 h⁻¹ whereas Fib75-abrin A chain broke down with a 3–4-fold lower rate constant of 0.043 h⁻¹. The amount of Fib75-abrin A chain remaining was significantly greater than that of the other ITs at all time points and more than 50% of the starting amount of the abrin A chain IT was left intact after 8 h of incubation. A similar difference in the rates of breakdown of the abrin A chain and ricin A chain ITs could be detected in the presence of glutathione concentrations as low as 0.2 mM (not shown).

To confirm that the decrease in the amount of intact IT measured by the ELISA was, in fact, due to the cleavage of the disulfide bond, the release of the A chains from Fib75-abrin A chain and Fib75-ricin A chain upon treatment with glutathione was measured directly. Gel permeation HPLC was used to measure the absolute amount of A chain released upon incubation of the ITs in the presence of different concentrations of glutathione for 1 h at 37°C in vitro (Fig. 6A). The amount of A chain released by glutathione was about 2- to 3-fold lower in the case of Fib75-abrin A chain consistent with the greater stability of the abrin A chain IT measured by ELISA. The release of A chain by glutathione was also measured directly in vitro.

![Graph](image_url)

**Fig. 5. Instability of Fib75 ITs in the presence of glutathione. Solutions of Fib75-abrin A chain (○), Fib75-ricin A chain (•), Fib75-gelonin (□), and Fib75-momordin (△) were each incubated in the presence of 5 mM glutathione at 37°C in vitro. The concentration of intact IT was determined after various times of incubation using solid-phase ELISA as described under “Materials and Methods.” Points are expressed as the percentage of the amount of intact IT at the start of the incubation; bars, SD from the mean values unless smaller than the points as plotted.**

**Fig. 6. Release of A chain from Fib75 and 2AL-1 ITs made with abrin A chain and ricin A chain by glutathione. ITs made with abrin A chain () or ricin A chain (©) linked to the antibodies Fib75 (A) or 2AL-1 (B) were incubated in the presence of different concentrations of glutathione for 1 h at 37°C in vitro. The amount of A chain released was measured using gel permeation HPLC as described under “Materials and Methods.” Points are expressed as the percentage of A chain released relative to the total A chain content.**

### Table 1 Pharmacokinetic data for the blood clearance of the Fib75 immunotoxins

<table>
<thead>
<tr>
<th>IT</th>
<th>t_{ao} (h)</th>
<th>t_{ao} (h)</th>
<th>Zero-time intercepts (% of C₀)</th>
<th>Blood level (% of C₀) 4 h</th>
<th>Blood level (% of C₀) 24 h</th>
<th>AUC (μg/ml x h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fib75-abrin A</td>
<td>0.50 ± 0.08</td>
<td>13.3 ± 0.5</td>
<td>38.3 ± 3.2</td>
<td>63.6 ± 1.7</td>
<td>51.8</td>
<td>18.3</td>
</tr>
<tr>
<td>Fib75-ricin A</td>
<td>0.70 ± 0.16</td>
<td>7.5 ± 0.4</td>
<td>50.6 ± 6.0</td>
<td>49.0 ± 3.5</td>
<td>35.6</td>
<td>5.5</td>
</tr>
<tr>
<td>Fib75-gelonin</td>
<td>0.35 ± 0.08</td>
<td>8.0 ± 0.4</td>
<td>55.8 ± 8.1</td>
<td>46.4 ± 2.8</td>
<td>33.4</td>
<td>5.9</td>
</tr>
<tr>
<td>Fib75-momordin</td>
<td>0.71 ± 0.19</td>
<td>8.6 ± 0.2</td>
<td>80.3 ± 2.0</td>
<td>64.3</td>
<td>12.4</td>
<td>7.9</td>
</tr>
</tbody>
</table>

* *t_{ao} and t_{ao}, α- and β-phase half-lives in blood; A and B, concentration constants in the biexponential equation C = Ae^{-αt} + Be^{-βt} for the α- and β-phases of clearance, respectively; AUC, area under the serum concentration versus time curve.*
using abrin A chain and ricin A chain ITs made with a control monoclonal antibody of the same isotype, 2AL-1 (Fig. 6B). As with the Fib75 ITs, abrin A chain was released less rapidly from the 2AL-1 antibody than ricin A chain indicating that the relative resistance of Fib75-abrin A chain to reduction was not an idiosyncrasy of the Fib75 antibody. No release of A chains was detected in control experiments in which the ITs were incubated under the same conditions but in the absence of glutathione (not shown). Moreover, a Fib75-ricin A chain IT made with a nonreducible thioether linkage did not break down when coincubated with glutathione (not shown).

Cytoxic Activity of Fib75-Abrin A Chain in the Circulation. The cytotoxic activity of Fib75-abrin A chain remaining in the circulation after i.v. injection of IT into the normal Wistar rat was determined from serum samples isolated 2 min, 4 h, and 24 h after injection. The concentration of intact IT was measured by ELISA. The ability of the serum samples to inhibit the incorporation of [3H]leucine by EJ human bladder carcinoma cells in tissue culture was measured. In the case of serial serum samples isolated from two different animals, no significant difference could be detected between the cytotoxic activities of the serum samples isolated at the different times (Fig. 7). The IC50 values, between 1.5 × 10⁻¹⁰ and 2.5 × 10⁻¹⁰ M, were in accord with the determined potency of the abrin A chain IT before injection indicating that the activity of the IT was completely preserved throughout the entire period of the clearance experiment.

DISCUSSION

The major findings of the present study are as follows: (a) ITs made using four RIs with different physicochemical properties, abrin A chain, ricin A chain, gelonin, and momordin, were cleared from the bloodstream of the normal rat with comparable, although not identical, biphasic clearance kinetics; (b) Fib75-abrin A chain was eliminated from the bloodstream less rapidly in the β-phase of clearance than the Fib75 ITs made with ricin A chain, gelonin, and momordin; (c) the abrin A chain IT was less susceptible to cleavage via reduction of the disulfide bond by glutathione in vitro than the other ITs; (d) the Fib75-abrin A chain persisting in the circulation of the rat 24 h after injection retained the full cytotoxic activity of the injected IT.

The pharmacokinetic properties of a panel of ITs made with abrin A chain, ricin A chain, gelonin, and momordin were measured using procedures matched as closely as possible for each IT. The ITs were prepared using a single mouse monoclonal antibody, Fib75, derivatized with the same cross-linking agent, SPDP. The IT preparations used in the pharmacokinetic experiments were purified using similar procedures, had similar composition with respect to the content of singly and multiply substituted conjugate species, and exerted cytotoxic effects upon the EJ human bladder carcinoma cell line bearing the target antigen recognized by Fib75. The blood clearance rates of the ITs were determined by administering the ITs to the same strain of rat, sampling blood at identical intervals, and determining the concentration of IT present in isolated serum using analogous solid-phase ELISAs. The experimental data were analyzed using the same pharmacokinetic model and mathematical curve fitting procedures.

The early rapid clearance of a proportion of Fib75-ricin A chain within 2 min of injection was consistent with similar results reported previously by this laboratory (7, 12) and by other workers (5, 6). The preparation of native ricin A chain used in this study consisted of a mixture of the two differently glycosylated forms, A1 and A2, in a molar ratio of approximately 2:1. The A1 chain contains a single complex-type oligosaccharide side chain whereas the A2 chain carries an additional oligomannose side chain (38). We have shown previously that the rapid clearance of Fib75-ricin A chain was due to mannose-dependent receptor-mediated hepatic recognition (8). Fib75 IT molecules containing the A2 chain were eliminated virtually completely from the circulation of the rat within 1 h and those persisting in the circulation after this time were cleared with the same kinetics as a Fib75 IT made with purified ricin A1 chain (12). Fib75-gelonin did not show an early loss comparable with the ricin A chain IT preparation despite the fact that gelonin has been reported to contain a single oligomannose side chain (30, 39). This finding is consistent with previous studies of the blood clearance of ITs made with gelonin (16–21) and suggests that the oligosaccharide side chain of gelonin was not recognized avidly by the hepatic mannose receptor system in the rat. Nevertheless, the more rapid elimination of the gelonin IT in the α-phase compared with the other ITs in the panel could indicate that a proportion of the gelonin IT preparation was eliminated by a similar process. However, all the Fib75 ITs, including the IT made with abrin A chain, which is not glycosylated (40), had similar α-phase half-lives. This phase of clearance cannot, therefore, be adequately explained in terms of receptor-mediated uptake via carbohydrate recognition. Indeed, other studies have shown that Fib75-abrin A chain is taken up by hepatic parenchymal and nonparenchymal cells in only very low amounts both in vitro and in vivo (41).

The biphasic pharmacokinetics observed with the Fib75 ITs in the panel could have been the result of the structural heter-
ogeneity inherent in IT preparations synthesized by chemical procedures. First, the experiments measuring the rate of breakdown of the Fib75 ITs in vitro indicated the presence of IT molecules with different susceptibilities to reduction by glutathione. Thus, the $\alpha$-phase of clearance could reflect the preferential elimination of the fraction of IT molecules in which the disulfide bond is most readily cleaved. However, in a different study, Fib75-rinic A chain ITs made with a disulfide linkage or with a nonreducible thioether linkage had comparable $\alpha$-phase half-lives (33). Second, IT molecules within a single preparation differ with regard to the position of attachment of the RIP molecule to the antibody and may differ with respect to the relative configuration of the two components even when linked via the same position. The more rapid clearance of a proportion of IT molecules could have been due to an impairment to the ability of the antibody component to persist in the circulation. This might occur either by enhancement of interactions between the conjugated antibody and normal tissues mediated via natural mechanisms such as Fc receptor recognition or by novel interactions made possible by structural changes to the antibody as a result of conjugation.

The clearance rates of Fib75-rinic A chain, Fib75-gelonin, and Fib75-momordin were very similar in the $\beta$-phase of clearance suggesting that the different modes of construction necessarily used in the synthesis of ITs with toxin A chains on the one hand, compared with single-chain RIPs on the other, did not have a major influence upon the $\beta$-phase half-life. In contrast, the $\beta$-phase half-life of Fib75-abrin A chain was significantly longer than that of the other ITs in the panel. A possible explanation for the shorter $\beta$-phase half-life of ITs made with ricin A chain, gelonin, and momordin is that these ITs may all contain a complex-type oligosaccharide side chain (38, 39) and that the presence of this structural feature somehow enhances their clearance. One way to test this hypothesis would be to determine the effect of removing the oligosaccharide side chain from the RIPs upon the rate of clearance of IT. However, it has been reported that the complex-type oligosaccharide side chain of ricin A chain cannot be cleaved enzymically without loss of ribosome-inactivating activity (38). Similarly, we were unable to remove the oligosaccharide side-chains from gelonin and momordin without first denaturing the RIPs. We have recently found that a Fib75 IT made with an aglycosyl recombinant ricin A chain had a $\beta$-phase half-life similar to that of Fib75-rinic A chain suggesting that the presence of the complex-type oligosaccharide side chain had no influence in this phase of clearance.

The rate at which the disulfide bond linking the antibody and RIP can be cleaved by reduction has been shown previously to influence IT half-life in the $\beta$-phase. Thus, ITs made with cross-linking agents such as SPDB (33) and 4-succinimidyloxycarbonyl-\(\alpha\)-methyl-\(\alpha\)-(2-pyridylthio)toluene (42), which contain hindered disulfide bonds that are less susceptible to chemical reduction, have significantly longer $\beta$-phase half-lives than the corresponding ITs made with nonhindered linkages. In this study, we found that Fib75-abrin A chain was cleaved less rapidly (by 3-4-fold) than glutathione in vitro than the Fib75 ITs made with ricin A chain, gelonin, or momordin. Direct evidence for the slower cleavage of the abrin A chain IT was obtained by measuring the actual release of A chain by glutathione using a quantitative HPLC method. Since glutathione is the most abundant reducing molecule in the circulation (43), this finding strongly suggests that the longer half-life of Fib75-abrin A chain in the $\beta$-phase of clearance compared with the other Fib75 ITs may be due, either in part or in whole, to a slower rate of cleavage of the disulfide bond in vivo. In our experiments, a high concentration of glutathione was used to give readily measurable rates of breakdown for comparison. The concentration of glutathione in peripheral blood is relatively low but it is present at higher levels in the liver, the site of glutathione synthesis (43). It is therefore possible that the major site of IT breakdown by reduction is within the hepatic circulation.

The slower rate of breakdown of the IT made with abrin A chain in vitro could be associated with the physicochemical properties of this RIP. Ablin A chain has a considerably more acidic isoelectric point, pl 4.6 (40), than ricin A chain, gelonin, and momordin, pl 7.5, 8.15, and 8.6, respectively (39, 40). It is possible that a localized electrostatic interaction between abrin A chain and the Fib75 antibody could induce the hybrid molecule to adopt a more compact conformation in which the disulfide bond is partially shielded from attack by reducing agents. A similar explanation was suggested previously for the apparently slower breakdown of an IT made with saporin (44) although subsequent pharmacokinetic analyses claimed that there was no difference in the rate of splitting compared with an analogous ricin A chain IT (24). An alternative explanation is that the proximity of negatively charged side chains of amino acid residues of abrin A chain in the vicinity of the disulfide bond causes electrostatic repulsion of glutathione which is negatively charged at physiological pH. Such a mechanism of protection would be a unique feature of abrin A chain since all the other RIPs that have been used for IT construction carry a net positive charge at physiological pH. The relative resistance to reduction of the abrin A chain IT made with Fib75 was not a peculiarity of the Fib75 antibody because an abrin A chain IT made with another mouse monoclonal antibody of the same isotype (IgG2a) was also cleaved less readily in vitro than the corresponding ricin A chain IT. It remains to be determined whether this phenomenon is restricted to antibodies of the IgG2a isotype.

The ability of an IT made with abrin A chain to persist longer in the circulation than ITs made with ricin A chain, gelonin, and momordin would be of no advantage were IT molecules that are reduced less readily in vitro to possess a diminished cytotoxic potency. The analysis of samples obtained from rats up to 24 h after injection of Fib75-abrin A chain clearly showed that the IT present in the circulation remained fully active. The cytotoxic activity of ITs during the course of blood clearance experiments has rarely been reported. In one study, it was found that the rate of disappearance of a ricin A chain IT was mirrored exactly by the rate of loss of cytotoxic activity (9). In contrast, clearance studies of a gelonin IT revealed that the cytotoxic activity of the IT remaining in the bloodstream was substantially diminished (20).

In conclusion, we have demonstrated that an IT made with abrin A chain had the highest cytotoxic potency and the longest serum half-life of a panel of ITs made with different RIPs and that the IT retained full cytotoxic activity in the circulation. Our results suggest that the slower clearance of the abrin A chain IT may have been due to its greater resistance to reduction by glutathione. These findings indicate that abrin A chain can form ITs with greater stability than ITs made with other RIPs.

Unpublished results.

and merits further investigation as a component in the assembly of ITs which are designed to have pharmacokinetic properties optimal for therapy.

REFERENCES


Pharmacokinetics in the Rat of a Panel of Immunotoxins Made with Abrin A Chain, Ricin A Chain, Gelonin, and Momordin


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