Biochemical Pharmacology, Vol. 35, No. 3, pp. 417–423, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00
Pergamon Press Ltd.

FATE OF AN ANTIBODY–RICIN A CHAIN CONJUGATE ADMINISTERED TO NORMAL RATS*

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(*Received* 28 *May* 1985; *accepted* 13 *August* 1985)

Abstract—The pharmacokinetics and catabolism of ricin A chain, a mouse monoclonal antibody (LICR-LOND-Fib 75) and a disulphide linked conjugate of the two have been studied following their intravenous administration to normal rats. Results indicate that the conjugate was removed from the circulation much more rapidly than the antibody but less quickly than the free ricin A chain. Disappearance of the conjugate from the circulation appeared to be biphasic with an early rapid initial phase followed by a much slower phase. The fate of a conjugate with a ^{125}I iodide label in the antibody component was compared with that of a conjugate similarly labelled but in the ricin A chain component. The results indicate that breakdown of the conjugate involves both cleavage of the disulphide linkage and complete catabolism of the whole conjugate molecule with the release of ^{125}I iodide. Rapid cleavage of the disulphide bond in the vasculature does not appear to be responsible for the initial rapid disappearance of the conjugate from the circulation.

The concept of using antibodies as carriers of pharmacological agents has become more practical since the advent of monoclonal antibody technology. *In vitro* studies have shown that immunospecific conjugates can indeed be prepared and while encouraging results have been reported for a variety of antibody–drug conjugates [1] particularly impressive results have been achieved using antibodies conjugated to plant and bacterial toxins [2–5]. Many studies have centred on the plant toxin ricin which consists of two polypeptide chains, one denoted B chain which binds to cell surface galactose residues and an A chain which upon entry to the cytosol causes cell death by the inactivation of protein synthesis [6]. Galactose residues are present on the surface of most cells and as a consequence conjugates with ricin holotoxin have been found to have considerable non-specific cytotoxicity [7]. It has been argued that preparation of conjugates with the A chain of the toxin is one way in which this problem can be overcome and several sets of experiments have borne out this contention [8]. While linkage of ricin A chain to antibodies generally results in a potent cytotoxic conjugate it appears that linkage via a sulphide bond results in inactive conjugates [9, 10]. This may be due to a requirement for release of the A chain from the antibody before its ribosome damaging function can be expressed [11]. A number of studies *in vivo* have demonstrated that antibody–ricin A chain conjugates are effective in significantly prolonging the survival of animals injected with tumour cells [12–14]. In general, however, results have been less successful than expected on the basis of *in vitro* studies. These results may reflect the possibility that only low levels of the conjugate reach the tumour cells in an active form. A number of reasons for this have been suggested including limited ability of the conjugate to extravasate, instability of the antibody–ricin A chain linkage or rapid catabolism of the conjugate [15–17]. We have investigated the pharmacokinetics and *in vivo* fate of ricin A chain, a mouse monoclonal antibody and a disulphide bonded conjugate of these following administration to rats. The results indicate that both breakdown of the linkage and rapid catabolism of the conjugate occur.

MATERIALS AND METHODS

Materials. The hybridoma producing LICR-LOND-Fib 75, a mouse monoclonal antibody of the IgG 2a subclass, was provided by R.A.J. McIlhinney (Ludwig Institute for Cancer Research, London Branch). This antibody recognizes a widely distributed human antigen and has no known specificity for any rat tissues. Castor bean cake derived from the seed of *Ricinus communis* of Kenyan origin, was a kind gift of Croda Premier Oils, Hull. *N*-Succinimidyl 3-(2-pyridyldithio)propionate (SPDP) was prepared as described by Carlsson *et al.* [18]. Protein A Sepharose 4B, Sephacryl S-200, CNBr Sepharose 4B and Sephadex G-25 were purchased from Pharmacia Ltd. (Milton Keynes, Bucks., U.K.). Sodium (^{125}I) iodide was supplied by Amersham International (Amersham, Bucks., U.K.) and Iodogen by Pierce (UK) Ltd. (Chester, U.K.). Monoiodotyrosine was purchased from Sigma (Poole, Dorset). Rabbit antiserum to ricin A chain was produced by immunizing animals at two intramuscular sites with a total of 100 μg A chain emulsified with Freund's complete adjuvant, followed 5 weeks later by 200 μg of A chain in PBS divided between four subcutaneous sites. Animals were bled out 7 days after the subcutaneous boost. Specific antibody was isolated from the antiserum by affinity chromatography on immobilized *Ricinus communis* agglutinin. This antibody was subsequently coupled

* This work was supported by grants from the Medical Research Council and the Cancer Research Campaign, United Kingdom.

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to CNBr activated Sepharose to provide the anti-ricin A chain adsorbent used in these experiments. All other reagents were of analytical grade.

Preparation of conjugates. The Fib 75 hybridoma was grown intraperitoneally in Balb/c mice and the immunoglobulin fraction from the ascitic fluid purified by precipitation with ammonium sulphate (50% saturation at 4°) followed by affinity chromatography on staphylococcal protein A immobilized on Sepharose 4B [19].

Ricin was prepared from castor bean cake by the method of Nicholson and Blaustein [20] with modifications as previously described [21].

Proteins were radiolabelled using the Iodo-gen method [22]. Labelled ricin A-chain was prepared by reductive cleavage of iodinated holotoxin [21]. The specific activities of the labelled proteins ranged from 200 to 400 μCi/mg.

The preparation of conjugate between antibody and the A chain of ricin has previously been described in detail [21]. Briefly, the antibody was reacted with SPDP under the conditions for an average 1.5–2 equivalents of the 3-(2-pyridyldithio)-propionyl group to be incorporated for each molecule of antibody. Unreacted reagent was removed by gel chromatography and the derivatized antibody mixed with a >2-fold molar excess of freshly reduced ricin A chain and left overnight at room temperature. Two conjugates were prepared, the first with ^{125}I-labelled ricin A chain (Ig–ricin A*) and the second with ^{125}I-labelled antibody (Ig*–ricin A). Both conjugates were purified by gel filtration chromatography on Sephacryl S-200 to remove free ricin A chain. Because of the small difference in molecular weight it was not possible to fully free the Ig*–ricin A of uncoupled antibody by this means.

The molecular composition of the conjugates was examined by polyacrylamide gradient gel electrophoresis in the presence of sodium dodecyl sulphate under reducing and non-reducing conditions.

Affinity chromatography. Further purification of the conjugate prepared with ^{125}I-labelled antibody was performed by affinity chromatography on a column of rabbit anti-ricin A chain conjugated to Sepharose 4B. As described by Vitetta *et al.* [23], this procedure does not affect the integrity of either component of the conjugate. The column (1 ml), equilibrated with 100 mM disodium hydrogen phosphate, 100 mM sodium chloride, 1 mM EDTA adjusted to pH 7.5 with 1 M hydrochloric acid, was saturated with cold Fib 75 and extensively washed with the buffer. Conjugate was applied to the column, unbound material eluted and the column washed at a flow rate of 6 ml/hr with buffer (four column volumes) followed by 0.1 M sodium chloride (four column volumes). The bound material was displaced at 37° with three column volumes of 3.5 M magnesium chloride at an elevated flow rate (about 10 ml/hr). The eluted conjugate was immediately dialysed against distilled water for 1 hr then against PBS for 18 hr.

A smaller column of immobilized rabbit anti-ricin A chain (0.5 ml) was used to estimate the proportion of intact ^{125}I-labelled antibody conjugate in samples of serum taken from animals treated with the conjugate and samples of serum and plasma incubated with the conjugate *in vitro* (see below). Samples (100 μl) were applied to the column at room temperature and left for 5 min. The column was then washed with buffer until no radioactivity was present in the eluate. The column was of sufficient capacity to allow for analysis of all the samples without desorption of the bound material.

Animals. Male Wistar albino rats (200–300 g) were supplied by the Chester Beatty Rodent Breeding Unit and allowed free access to food and water.

Clearance studies. Rats were lightly anaesthetized with ether followed by an intramuscular injection of fentanyl citrate (315 μg/kg) and fluanisone (10 mg/kg). The proteins under study were injected into the right jugular vein. Each animal received 0.8–1.1 $\times$ 10^6 cpm of radiolabelled material in the case of the antibody, ricin A chain, the ^{125}I-labelled antibody conjugate and the sodium ^{125}I iodide and 8 $\times$ 10^6 cpm of radiolabelled material in the case of the ^{125}I-labelled ricin A chain conjugate. Each dose was contained in approximately 0.1 ml Dulbecco's phosphate buffered saline. Samples of blood (approximately 0.3 ml) were taken at time intervals from the left jugular vein, weighed and the radioactivity determined in a Packard 5266 gamma counter. Blood samples at selected time points were allowed to clot overnight at 4° and serum samples from two animals were pooled for chromatographic analysis. Repeated induction of anaesthesia was required for sampling the blood at the later time points.

Samples of each of the radiolabelled proteins were added to freshly drawn rat blood and the samples subsequently treated in an identical manner to the samples from the dosed animals. These samples provided a control for any breakdown of the proteins subsequent to withdrawal from the animal.

Excretion studies. Rats were anaesthetized using ether. The ^{125}I-labelled materials (0.8–1.1 $\times$ 10^6 cpm in approximately 0.1 ml PBS) were injected into the sublingual vein and the animals housed individually in metabolic cages (Lab Care Precision, Ashford, Kent). Urine and faeces were collected separately and the total radioactivity excreted by each route measured.

Gel permeation high pressure liquid chromatography. Samples of serum taken and urine collected from animals at various times after injection with the radiolabelled materials were applied to a 7.5 $\times$ 600 mm G3000 S.W. T.S.K. gel permeation column (L.K.B. Ltd., Croydon, Surrey, U.K.). The separation was performed at a flow rate of 1 ml/min in 20 mM sodium dihydrogen phosphate, 100 mM sodium sulphate, 0.05% sodium azide adjusted to pH 6.8 with 1 M sodium hydroxide. Fractions (0.5 ml) were collected and the radioactivity measured. The retention times for the peaks of radioactivity were compared with those of standard proteins of known molecular weight (Biorad Ltd., Watford, Herts., U.K.).

Sephadex G-25 chromatography. Samples of serum and urine from animals injected with ^{125}I-labelled materials were analysed on a Sephadex G-25 column (18 mm $\times$ 830 mm), equilibrated in 0.2 M sodium acetate. Fractions (1.7 ml) were collected at a flow rate of 40 ml/hr and the radioactivity measured. Elution profiles of monoiodotyrosine and dextran

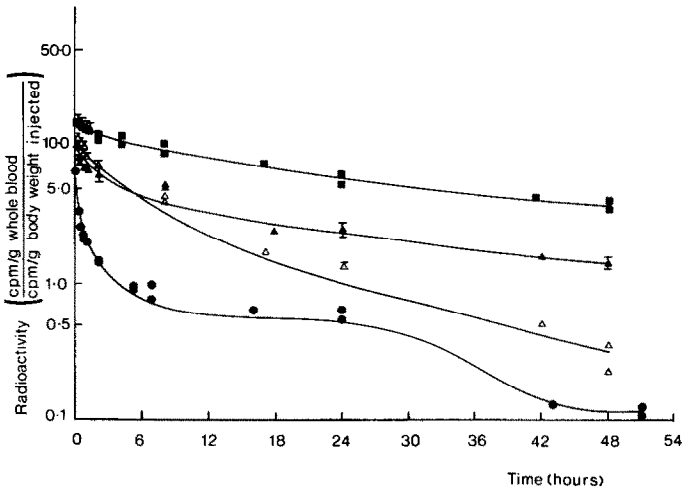

Fig. 1. Blood levels of radioactivity after i.v. injection of: antibody, (■) approximately 40 μg/rat, ricin A chain, (●) approximately 8 μg/rat, Ig*–ricin A, (▲) approximately 40 μg/rat with respect to antibody and Ig–ricin A*, (△) approximately 7 μg/rat with respect to ricin A chain. Results are for three animals. Where samples were taken at the same time point results are expressed as the mean ±S.D.; where samples were taken at different time points individual data points are plotted.

blue (by u.v. absorbance at 280 nm) and sodium ^{125}I iodide (by radioactivity measurement) were obtained for comparison.

In vitro *studies*. ^{125}I-labelled antibody conjugate was added to control rat EDTA-anticoagulated blood to give a final concentration approximately equal to the initial blood concentration seen on *in vivo* administration of the conjugate. The blood was incubated at 37° for 48 hr and samples were taken at time intervals, centrifuged and the plasma analysed by affinity chromatography (see above). A similar incubation was set up using serum containing the same concentration of EDTA.

RESULTS

Conjugate composition

The final Ig–ricin A* preparation was contaminated by <1% free ^{125}I labelled ricin A chain (see Fig. 2b) while the final Ig*–ricin A contained <1.5% unconjugated antibody (see Table 2, column 3). The elution profiles of the Ig*–ricin A and Ig–ricin A* on gel permeation HPLC analysis were very similar (data not shown). Sodium dodecyl sulphate polyacrylamide gel electrophoresis demonstrated that 60–70% of the labelled material in each conjugate preparation ran as a single band with a molecular weight consistent with one molecule of ricin A chain coupled to one of antibody. The remaining ^{125}I labelled material comprised conjugate with more than one ricin A chain coupled to each immunoglobulin molecule. When the samples were treated with reducing agent the radioactivity ran either as the heavy and light chains of the immunoglobulin (Ig*–ricin A) or as ricin A chain (Ig–ricin A*) (data not shown).

Blood levels

Blood radioactivity levels were monitored after a single i.v. injection of antibody, ricin A chain, Ig–ricin A* and Ig*–ricin A. Figure 1 plots the level of radioactivity in whole blood on a logarithmic scale against time after administration of each of the proteins. In each instance an initial rapid drop in blood radioactivity was followed by a slower phase. Blood levels of radioactivity were also monitored after a single i.v. injection of 3-(2-pyridyldithio)propionylated antibody. No differences were observed between the blood levels of radioactivity after administration of the modified and unmodified antibody (results not shown).

Gel permeation high pressure liquid chromatography

The gel permeation column allowed separation in the molecular weight range 1000–800,000. A semi-log plot of molecular weight against retention time was linear over the range 17,000–670,000. Vitamin B-12 (molecular weight 1350) had a retention time of 27 min. Material with a retention time longer than this was assumed to be of a molecular weight <1350.

Figure 2a shows the elution profile of ricin A chain applied to the column in serum. The radioactivity eluted as two peaks, one at the void volume of the column (molecular weight >800,000) the second between 21 and 26 min. The second peak (22.5 min) corresponds to a molecular weight of approximately 27,000 and was the only peak seen when ricin A chain was applied to the column directly in buffer.

Figure 2b shows the elution profile of Ig–ricin A* applied to the HPLC column in the buffer described above. Ninety-nine per cent of the radioactivity eluted between 12 and 20.5 min with a peak at 16.5 min. This retention time was as expected for a 1:1 conjugate on the basis of molecular weight markers.

Figure 2c was the elution profile of a serum sample taken 24 hr after dosing with Ig–ricin A*. Radioactivity eluted as three peaks with 68% eluting between 12 and 20.5 min with a peak at 16 min, 1.5% between 20.5 and 26 min with a peak at 22 min and 31% between 26 and 30 min with a peak at 28 min.

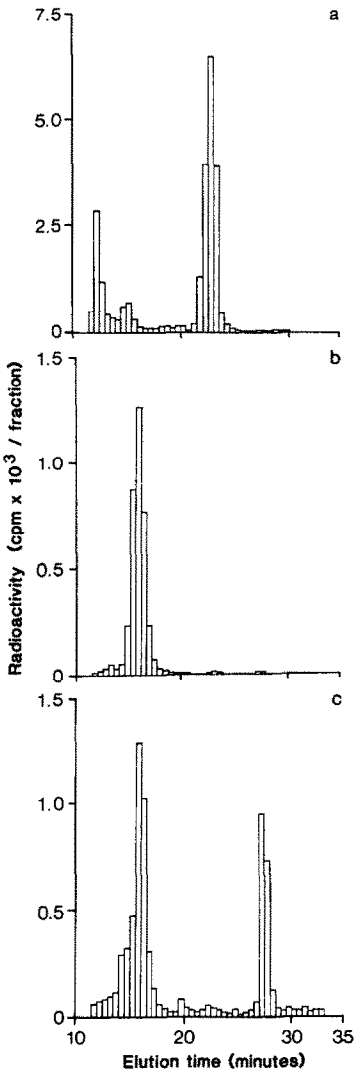

Fig. 2. Elution profiles of (a) ricin A chain added to serum, (b) Ig–ricin A* in elution buffer, (c) serum sample taken at 24 hr after injection of Ig–ricin A* analysed on a G3000 S.W. T.S.K. gel permeation column. The bars represent the recovered radioactivity present in each fraction.

Analysis of serum samples from animals dosed with ricin A chain revealed that by 1 hr the major radioactive species present (approximately 60%) was of a molecular weight of <1350. The remainder of the radioactivity eluted as two peaks as indicated in Fig. 2a. The rapid removal of radioactivity from the blood compartment of animals dosed with ricin A chain made accurate HPLC analysis of the samples at later time points difficult. The results indicate that by 2 hr and at later time points >80% of the radioactivity was of a molecular weight <1350 while the remainder of the radioactivity eluted at the void volume of the column in a position corresponding to the high molecular weight material found after incubating ricin A chain with serum *in vitro*. No counts were seen at the position corresponding to ricin A chain.

In contrast, HPLC analysis of serum samples taken over a 48-hr period from animals dosed with radiolabelled antibody showed that all of the radioactivity in the samples appeared as a single band with an elution profile and retention time identical to that of the injected material.

Table 1. Gel permeation HPLC analysis of serum after i.v. administration of Ig–ricin A*

Time after dosing (hr)	Percentage radioactivity eluting between the indicated retention times 12–21 (m)	22–26 (m)	27–30 (m)
0†	96	3.4	0.6
0.0333	96	3.7	0.3
1.0	92	2.9	4.7
4.0	92	2.9	4.7
4.0	90	1.2	8.7
8.0	84.9	1.5	13.6
24.0	68.2	1.5	31.2
48.0	65.0	3.5	31.7

† Ig–ricin A* added to blood as a control for breakdown before analysis.

Table 1 shows the results of HPLC analysis of serum samples taken at various times after dosing with Ig–ricin A*. The results are presented as the percentage radioactivity eluting between the two indicated retention times. Over the 48-hr time course after injection of the conjugate there was a progressive increase in the percentage of the total radioactivity eluting as material of molecular weight less than 1350. There was no evidence for an increase in the amount of ricin A chain present in the serum with time, the value being at a consistently low level, nor for any radioactive material eluting at the void volume of the column. Serum samples from animals dosed with Ig*-ricin A were also analysed by HPLC. Radioactivity appeared in two peaks, one in the molecular weight range 100,000–600,000 and the other as material of a molecular weight <1350. Over the 48-hr period of investigation the percentage of radioactivity in the circulation associated with material of a molecular weight <1350 never exceeded 10% of the total. These results are shown in column 4 of Table 2. Since mixtures of conjugate and immunoglobulin could not be resolved by this technique, further aliquots of these samples were analysed by affinity chromatography (see below). Urine samples from animals dosed with each of the radiolabelled proteins were analysed by HPLC. Greater than 99% of the radioactivity in each case eluted as material of molecular weight <1350 with the exception of the 0–3 hr urine collection from the animals treated with ricin A chain where 2% of the radioactivity eluted between 22 and 26 min.

Affinity chromatography

Analysis of serum samples from animals injected with Ig*–ricin A on an anti-ricin A chain column facilitated the distinction between materials containing and not containing ricin A chain. The percentage of radioactivity which bound to the column is shown in column 2 of Table 2. On the basis of the HPLC data the unbound fraction is known to comprise 2 components, one of molecular weight approximately 150,000 and one of molecular weight

Table 2. Gel permeation HPLC analysis and affinity chromatography of serum after i.v. administration of Ig*–ricin A

Sample	% Radioactivity binding to –ricin A column	% Radioactivity not binding to –ricin A column	
		Mol. wt >100,000	Mol. wt <1350
Ig*	<4.0	>96.0	—
Ig*–ricin A	98.7	1.3	—
Time after dosing (hr)			
0.0333	94.7	4.6	0.7
1.0	87.7	9.8	2.5
4.0	75.2	19.5	5.3
8.0	60.0	32.7	7.3
24.0	27.7	65.2	7.1
48.0	10.9	82.0	7.1

<1350. Column 3 in Table 2 shows the percentage of the radioactivity in the unbound fraction after subtraction of the radioactivity associated with the low molecular weight material (shown in column 4 of Table 2). This presumably represents free immunoglobulin. It is apparent from Table 2 that there is a decrease in the proportion of radioactivity associated with the ricin A chain containing material and a concomitant increase in the amount of free immunoglubin.

Sephadex G-25 chromatography

Gel filtration analysis on Sephadex G-25 was used to examine further the nature of the low molecular weight radioactive species present in the urine and serum of animals injected with Ig–ricin A*. Figure 3a shows the elution profiles of blue dextran, monoiodotyrosine and sodium ^{125}I iodide and Fig. 3b that of a serum sample taken at 24 hr after dosing with Ig–ricin A*. One hour after dosing with Ig–ricin A* 96% of the radioactivity eluted at the void volume of the column. By 48 hr 63% of the radioactivity still eluted at the void volume with 37% co-eluting with sodium iodide. At all time points examined radioactivity either eluted at the void volume or co-eluted with sodium iodide. Some samples were examined using both HPLC and G-25 chromatography. The percentage of radioactivity which co-eluted with sodium iodide on the G-25 column agreed closely with the percentage radioactivity eluting as material of a molecular weight <1350 on the HPLC column (Table 1, column 3). Most of the radioactivity in the urine co-eluted with sodium iodide with less than 0.1% of the radioactivity appearing in the void volume. Serum and urine samples from animals dosed with ^{125}I–ricin A chain and with ^{125}I-antibody were also analysed by Sephadex G-25 chromatography. The radioactivity in the samples either appeared at the void volume of the column or co-eluted with sodium iodide. After administration of ricin A chain ^{125}I iodide rapidly appeared in both serum and urine while administration of the antibody resulted in a slow appearance of ^{125}I iodide in the urine only. In no case was any radioactivity seen in fractions associated with monoidotyrosine.

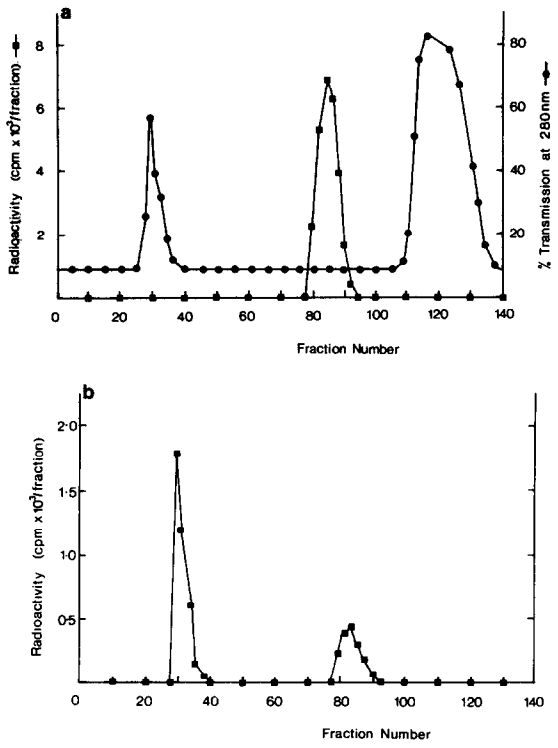

Fig. 3. Elution profiles of (a) sodium ^{125}I iodide, monoiodotyrosine and dextran blue, and (b) serum sample taken at 24 hr after injection of Ig–ricin A*, analysed on a Sephadex G-25 column.

Pharmacokinetic analysis

From the chromatography data blood levels of intact Ig–ricin A* were calculated. These results when plotted on a semi-log scale obeyed biphasic kinetics. A computerized non-linear least squares analysis was used to estimate alpha and beta phase half-lives [24]. These were estimated for each animal and then the mean and standard deviation calculated. Half-lives for the alpha and beta phase were estimated as 0.70 hr ± 0.06 and 9.61 hr ± 0.54 respectively.

Excretion studies

The total radioactivity excreted in urine and faeces after a single i.v. dose of each protein was measured over a time course of 96 hr. For each of the proteins

more than 95% of the excreted radioactivity was found in the urine. Figure 4 shows the cumulative excretion of radioactivity for each of the administered substances. At 96 hr post-injection the cumulative radioactivity excreted in the urine as a percentage of that administered was 67% in the case of ricin A chain, 54% in the case of Ig–ricin A*, 49% for Ig*–ricin A and 19% for the antibody. The excretion of intravenously administered sodium ^{125}I iodide was investigated for comparison with the labelled proteins. Again greater then 95% of the excreted radioactivity was found in the urine. The cumulative radioactivity excreted in the first 96 hours as a percentage of the injected dose was 80%.

In vitro *studies*

Table 3 shows the results of affinity chromatography on samples of Ig*–ricin A chain incubated *in vitro* at 37° with anticoagulated blood and serum. There was an increasing appearance of radiolabelled material not containing ricin A chain over the course of the experiment. At the conclusion of the incubation, chromatography on Sephadex G-25 demonstrated that all the radioactivity was still associated with the high molecular weight material.

DISCUSSION

The biological half-life of LICR-LOND Fib 75, calculated from the linear part of a semi-log plot, was estimated to be approximately 125 hr. This result agrees reasonably well with that of Houston *et al.* [25], who measured a biological half-life of 6 days for mouse monoclonal IgG 2a when administered to mice and that of Peppard and Orlans [26], who found that the biological half-life of rat IgG 2a administered to rats was 106 hr.

Administration of ricin A chain resulted in a rapid

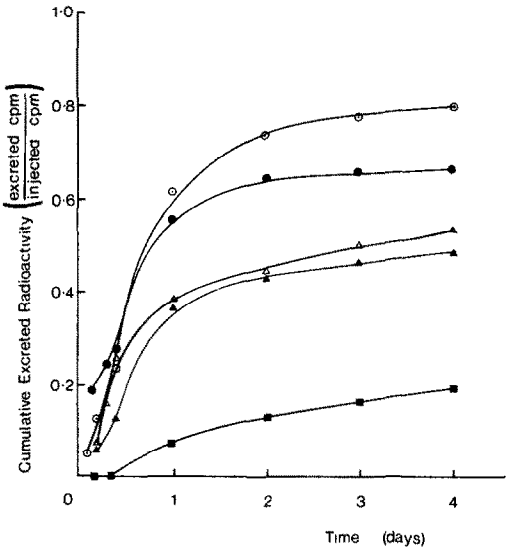

Fig. 4. Cumulative urinary excretion of radioactivity after i.v. injection of antibody (■), ricin A chain (●), Ig*–ricin A (▲) and Ig–ricin A* (△) as described in the legend to Fig. 1 and sodium ^{125}I iodide (⊙). Results are the mean for two animals and the pairs of values differed from each other by not more than 10%.

Table 3. *In vitro* decomposition of Ig*–ricin A in serum and anticoagulated blood at 37°

Time (hr)	% Radioactivity not binding to –ricin A chain column	
	Blood	Serum
1	3.7	1.5
4	8.9	4.0
8	12.6	7.7
24	18.5	15.3
48	34.4	26.7

disappearance of radioactivity from the circulation and the formation of an as yet unidentified high molecular weight species. While, the number of radioactive species present in the blood after injection of ricin A chain made pharmacokinetic analysis of the results difficult, our results are in agreement with those of Cushley *et al.* [27] who saw a rapid decrease in total blood radioactivity after administration of iodinated ricin A chain.

Two hours after administration of both Ig*–ricin A and Ig–ricin A* approximately 50% of the administered radioactivity had disappeared from the circulation. The disappearance of radioactivity from the circulation in the second phase was, however, considerably slower for Ig*–ricin A than for Ig–ricin A*, reflecting the appearance of labelled non-conjugated antibody in the circulation which then persisted. The disappearance of conjugate from the circulation was much more rapid than that of the unconjugated antibody.

Urinary excretion studies following administration of Ig*–ricin A and Ig–ricin A* showed that the rate of urinary excretion of ^{125}I iodide over the first 96 hr after administration of the two conjugates was very similar with approximately 50% of the injected radioactivity excreted in the urine by 96 hr.

These results indicate that at least three processes are involved in the removal of intact conjugate from the circulation; firstly, a distribution of the conjugate into other compartments of the body, secondly, a process resulting in circulating free antibody, and thirdly a process resulting in the release of ^{125}I iodide into the circulation and its subsequent appearance in urine.

The release of free antibody in the absence of any free ricin A chain may be due either to intracellular cleavage of the linkage with release of the free immunoglobulin only, or to cleavage of linkage in the circulation with persistence of the free immunoglobulin and rapid catabolism of the ricin A chain. Cleavage of the linkage in both blood and serum has been observed *in vitro*, and may be of significance with respect to the *in vivo* results. It has been suggested by a number of authors that cleavage of the linkage in the circulation is an important factor in the inactivation *in vivo* of conjugates [9, 16, 17]. While it is clear that breakdown of the linkage is indeed occurring we feel that our results indicate that intravascular cleavage of the linkage is not responsible for the rapid early disappearance of the conjugate from the circulation.

The release of ^{125}I iodide is generally considered to result from proteolytic degradation of an iodinated

protein followed by dehalogenation of the released monoiodotyrosine [28]. Since no other labelled products of proteolysis are seen in either blood or urine, complete catabolism of some conjugate molecules must be occurring. Complete catabolism of the conjugate could follow uptake by the reticuloendothelial system as a result of mannose receptor recognition of the ricin A chain part of the conjugate as suggested by Vitetta *et al.* [29] or be the result of perturbation of the normal route of clearance of the antibody as a result of the considerable modification of the molecule by the introduction of the ricin A chain.

When blood levels of intact conjugate were calculated the disappearance of the conjugate from the circulation obeyed biphasic kinetics with alpha and beta phase half-lives estimated as 0.70 ± 0.06 and 9.61 ± 0.54 hr respectively. Since disappearance of the conjugate from the circulation is much faster than that of the antibody the behaviour of the conjugate in the alpha phase cannot merely be a consequence of distribution into two compartments. This suggests that there may be a faster removal of some conjugate molecules from the circulation than of others. Heterogeneity of the conjugate could be due to variation in the point of attachment of the ricin A chain to the antibody, variation in the number of ricin A chain molecules attached per molecule of antibody or to heterogeneity of the ricin A chain, particularly with respect to its glycosylation [30].

The length of time taken for optimal *in vivo* tumour localization of appropriate antibody has been reported to be of the order of 24 hr [31]. The total amounts of antibody and intact conjugate remaining in the circulation at 24 hr calculated as a percentage of the injected dose, assuming a blood volume of 6% of the total body weight, are 29% and 1% respectively. These results may provide an explanation for the poor therapeutic results seen when ricin A chain conjugates have been used *in vivo* [16, 17].

Prolonging the *in vivo* persistence of a conjugate may lead to an improvement in its efficacy. There are a number of ways in which this may be achieved. By modifying the bridging structure between the A chain and antibody it may be possible to increase its stability. Alternatively, if saccharide recognition is an important factor in the rapid removal of ricin A chain conjugates from the circulation, conjugates with the non-glycosylated A chain of abrin or the non-glycosylated ribosome-inactivating protein saporin may well prove more effective anti-tumour agents *in vivo* [32, 33].

Acknowledgements—The authors wish to thank Mr A. Mirza for the synthesis of SPDP and Mr B. Gwynne for sublingual vein injections.

REFERENCES

1. T. Ghose and A. H. Blair, *J. natn. Cancer Inst.* **61**, 657 (1978).
2. F. L. Moolten and S. R. Cooperband, *Science* **169**, 68 (1970).
3. P. E. Thorpe, W. C. J. Ross, A. J. Cumber, C. A. Hinson, D. C. Edwards and A. J. S. Davies, *Nature, Lond.* **271**, 752 (1978).
4. P. E. Thorpe, A. J. Cumber, N. Williams, D. C. Edwards, W. C. J. Ross and A. J. S. Davies, *Clin. exp. Immunol.* **43**, 195 (1981).
5. R. J. Youle and D. M. Neville Jr., *Proc. natn. Acad. Sci. U.S.A.* **77**, 5483 (1980).
6. S. Olsnes and A. Pihl, in *Molecular Action of Toxins and Viruses* (Eds. Cohen and Van Heyningen), p. 51. Elsevier Biochemical Press, Amsterdam (1982).
7. S. Olsnes, E. Saltvedt and A. Pihl, *J. biol. Chem.* **249**, 803 (1974).
8. H. E. Blythman, P. Casellas, O. Gros, P. Gros, F. K. Jansen, F. Paolucci, B. Pau and H. Vidal, *Nature, Lond.* **290**, 145 (1981).
9. F. K. Jansen, H. E. Blythman, D. Carriere, P. Casellas, O. Gros, P. Gros, J. C. Laurent, F. Paolucci, B. Pau, P. Poncelet, G. Richer, H. Vidal and G. A. Voisin, *Immunological Rev.* **62**, 185 (1982).
10. S. Ramakrishnan and L. L. Houston, *Cancer Res.* **44**, 201 (1984).
11. S. Olsnes and A. Pihl, *Pharmac. Ther.* **15**, 355 (1982).
12. K. A. Krolick, J. W. Uhr, S. Slavin and E. S. Vitetta, *J. exp. Med.* **155**, 1797 (1982).
13. M. Seto, N. Umemoto, M. Saito, Y. Masuho, T. Hara and T. Takahashi, *Cancer Res.* **42**, 5209 (1982).
14. K. Kishida, Y. Masuho, M. Saito, T. Hara and H. Fuji, *Cancer Immunol. Immunother.* **16**, 93 (1983).
15. P. E. Thorpe, D. C. Edwards, A. J. S. Davies and W. C. J. Ross, in *Monoclonal Antibodies In Clinical Medicine* (Eds. A. J. McMichael and J. W. Fabre), p. 168. Academic Press, London (1982).
16. I. S. Trowbridge and D. L. Domingo, *Cancer Surveys* **1**, 542 (1982).
17. M. Strand, D. A. Scheinberg and O. Gansow, in *Cell Fusion: Gene Transfer and Transformation Miles International Symposium No. 14* (Eds. R. F. Beers and E. G. Bassett), p. 385. Raven Press, New York (1983).
18. J. Carlsson, D. Drevin and R. Axen, *Biochem. J.* **173**, 727 (1978).
19. J. A. Forrester, D. P. McIntosh, A. J. Cumber, G. D. Parnell and W. C. J. Ross, *Cancer Drug Deliv.* **1**, 283 (1984).
20. G. Nicolson and J. Blaustein, *Biochim. biophys. Acta* **266**, 543 (1972).
21. A. J. Cumber, J. A. Forrester, B. M. J. Foxwell, W. C. J. Ross and P. E. Thorpe, *Meth. Enzymol.* **112**, 207 (1985).
22. P. J. Fraker and J. C. Speak, *Biochem. biophys. Res. Commun.* **80**, 849 (1978).
23. E. S. Vitetta, W. Cushley and J. W. Uhr, *Proc. natn. Acad. Sci. U.S.A.* **80**, 6332 (1983).
24. J. Sampson, in *University of California Publications in Automatic Computations*, No. 3, *BMD Biomedical Computer Programs X-Series Supplement* (Ed. W. J. Dixon), p. 177. University of California Press, Berkley, CA (1970).
25. L. L. Houston, R. C. Nowinski and I. D. Bernstein, *J. Immunol.* **125**, 837 (1980).
26. J. V. Peppard and E. Orlans, *Immunology* **40**, 683 (1980).
27. W. Cushley, M. J. Muirhead, F. Silva, J. Greathouse, T. Tucker, J. W. Uhr and E. S. Vitetta, *Toxicon* **22**, 265 (1984).
28. R. M. Campbell, D. P. Cuthbertson, C. M. Matthews and A. S. McFarlane, *Int. J. appl. Radiation Isotopes* **1**, 66 (1956).
29. E. S. Vitetta and J. W. Uhr, *Transplantation* **37**, 535 (1984).
30. B. M. J. Foxwell, T. A. Donovan, P. E. Thorpe and G. Wilson, *Biochim. biophys. Acta*, **840**, 193 (1985).
31. L. L. Houston, R. C. Nowinski and I. D. Bernstein, *J. Immunol.* **125**, 837 (1980).
32. K. M. Hwang, K. A. Foon, P. H. Cheung, J. W. Pearson and R. K. Oldham, *Cancer Res.* **44**, 457 (1984).
33. P. E. Thorpe, A. N. F. Brown, J. A. G. Bremner, B. M. J. Foxwell and F. Stirpe, *J. natn. Cancer Inst.* **75**, 151 (1985).