Uptake of injected ^{125}I -ricin by rat liver *in vivo*

Subcellular distribution and characterization of the internalized ligand

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Subcellular-fractionation techniques were used to characterize the endocytic pathway followed by ricin in rat liver *in vivo* and tentatively identify the site(s) at which the ricin interchain disulphide bridge is split. After injection of ^{125}I -ricin, hepatic uptake of radioactivity was maximum at 30 min (40 % of injected dose). At 5 min, about 80 % of the radioactivity in the homogenate was recovered in the microsomal (P) fraction, but later on the recovery of the radioactivity in the mitochondrial-lysosomal (ML) fractions progressively increased (50 % at 30 min) at the expense of that in the P fraction. Subfractionation of the P and ML fractions on analytical sucrose-density gradients revealed a time-dependent translocation of the radioactivity from low- to high-density endocytic structures, with median relative densities at 5 and 60 min of about 1.15 and 1.16 (P fraction) and 1.19 and 1.22 (ML fraction) respectively. The late distribution of the radioactivity in the ML fraction was similar to that of the lysosomal marker acid phosphatase. Studies with co-injected lactose and mannan showed that ricin was internalized mainly via the mannose receptor. In the presence of mannan, the late recovery of radioactivity in the ML fraction was decreased, and the distribution of the radioactivity associated with the P fraction was shifted toward lower densities (median relative density 1.13), indicating a different pathway of endocytosis. Analysis of the radioactivity associated with the ML and S fractions by SDS/PAGE revealed a time-dependent increase in the amount of intact A- and B-chains and low-molecular-mass products. When ML fractions containing partially processed ricin were incubated at 37 °C at pH 5 or at pH 7.2 in the presence of ATP, only low-molecular-mass products were generated. We conclude that internalized ricin associates with endocytic structures whose size and density of equilibration increase with time, and that, although detectable in these structures, reduction of the ricin interchain disulphide bridge occurs to a large extent in the cytosol.

INTRODUCTION

Ricin is a plant toxin which consists of two polypeptide chains, A and B ($M_r \approx 30000$ each), linked by a single disulphide bond. The B-chain is a lectin which binds to cell-surface galactose-containing glycoproteins and glycolipids (Baenziger & Fiete, 1979; Turpin *et al.*, 1984). The A-chain is a specific *N*-glycosidase which inhibits protein synthesis by removing a single adenine residue from the 28 S ribosomal RNA (Endo & Tsurugi, 1987; Endo *et al.*, 1987). Ricin has been widely used in the preparation of immunotoxins, in which either the entire toxin or the A-chain are conjugated to monoclonal antibodies directed against tumour antigens (Hertler & Frankel, 1989; Olsnes *et al.*, 1989).

The fate and action of ricin have been studied in a variety of cells using native or derivatized ricin. Biochemical and morphological studies have shown that ricin undergoes adsorptive endocytosis after binding via its B-chain to the cell surface. Ultrastructurally, internalized ricin has been shown to associate first with coated (van Deurs *et al.*, 1985) or non-coated (Moya *et al.*, 1985; Sandvig *et al.*, 1987) vesicles, and then with endosomes, lysosomes and elements of the Golgi complex (Nicolson *et al.*, 1975; Gonatas *et al.*, 1977; van Deurs *et al.*, 1985; Sandvig *et al.*, 1987). More recently, the association of ricin with the *trans*-Golgi network has been emphasized (van Deurs *et al.*, 1988; Hansen *et al.*, 1989). Endocytosed ricin is in part recycled to the cell surface and released in the extracellular space in an intact form (McIntosh *et al.*, 1990; Magnusson *et al.*, 1991).

One first requirement for ricin action is the translocation of ricin or ricin A-chain into the cytosol. On the basis of the

observations that ricin cytotoxicity is optimal at neutral pH (Sandvig & Olsnes, 1982) and is enhanced by acidotropic drugs (Sandvig *et al.*, 1979; Olsnes & Pihl, 1982) and in acidification-defective mutant cell lines (Moehring & Moehring, 1983; Marnell *et al.*, 1984; Robbins *et al.*, 1984), it was previously suggested that ricin enters the cytosol from a neutral vacuolar compartment. In some studies ricin cytotoxicity has been shown to be functionally linked to ricin delivery to the Golgi complex (Sandvig *et al.*, 1986; van Deurs *et al.*, 1987; Youle & Colombatti, 1987; Yoshida *et al.*, 1990, 1991), implicating this organelle as the site of entry. However, with certain cell systems, such as with mannose-receptor-mediated uptake in macrophages (Simmons *et al.*, 1986), ricin toxicity is inhibited by acidotropic drugs, suggesting entry from acidic endosomes. Ultrastructural evidence in favour of a translocation of ricin across the endosomal membrane has also been presented (Calafat *et al.*, 1988).

Another requirement for ricin A-chain action is its physical separation from the B-chain (Montesano *et al.*, 1982) or, at least, a reduction of the ricin interchain disulphide bridge (Oda & Funatsu, 1979; Barbieri *et al.*, 1982). However, it is unclear whether this process occurs before, during or after translocation of ricin into the cytosol.

Previous studies have shown that, when injected in rodents, ricin is to a large extent taken up by the liver (Fodstad *et al.*, 1976; Skilleter *et al.*, 1981; Thorpe *et al.*, 1985; Ramsden *et al.*, 1989). Evidence has also been presented that liver non-parenchymal cells (Kupffer and endothelial cells) accumulate ricin to a greater extent and are much more sensitive to ricin toxicity than are parenchymal cells (hepatocytes); uptake of ricin

Abbreviations used: ML, mitochondrial-lysosomal fraction; N, nuclear fraction; P, microsomal fraction; S, soluble fraction (cytosol).

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by the former was shown to involve mainly terminal mannose residues of the toxin (Skilleter *et al.*, 1985).

In the present study, cell-fractionation techniques have been used to characterize the endocytic pathway followed by ricin in rat liver *in vivo* and to tentatively identify the site at which the ricin interchain disulphide bond is split.

EXPERIMENTAL

Materials

Ricin was prepared with *Ricinus communis* (castor bean) seeds (var. *sanguineus*; Bertrand Frères, Angers, France) as described by Nicolson & Blaustein (1972). The preparation was homogeneous when tested by PAGE at pH 7.5, ultracentrifugation and immunoelectrophoresis. Its haemagglutinating activity against human O-erythrocytes was 15–17 μg (minimum haemagglutinating dose). The ricin concentration was determined by absorbance measurements at 280 nm at pH 7, using $A_{1\text{cm}}^{1\%} = 14$ (Zentz *et al.*, 1978).

Carrier-free Na^{125}I was from CIS-Bioindustries (Gif sur Yvette, France) and carrier-free Na^{131}I was from Amersham International. Rat serum albumin, lactose (4-*O*- β -D-galactopyranosyl-D-glucopyranose), lactulose (4-*O*- β -D-galactopyranosyl-D-fructofuranose), cellobiose (4-*O*- β -D-glucopyranosyl-D-glucopyranose), palatinose (6-*O*- α -D-glucopyranosyl-D-fructofuranose), *N*-ethylmaleimide, 1,10-phenanthroline, Mops, ATP, creatine kinase and phosphocreatine were from Sigma. Iodo-Gen

was from Pierce. Trisacryl GF 05 and Amberlite IRA 400 resin were from I.B.F. (Villeneuve la Garenne, France) and Sigma respectively.

Preparation, purification and characterization of ^{125}I -ricin and ^{131}I -albumin

Iodination of ricin with ^{125}I was performed by using the Iodo-Gen method. Ricin (160 μg) was allowed to react in an Iodo-Gen-coated tube with Na^{125}I (59 MBq; 1.6 mCi) in 130 μl of 0.1 M-sodium phosphate buffer containing 0.15 M-NaCl and 0.15 M-methyl β -D-galactopyranoside in order to protect the ricin saccharide-binding sites. After 5 min at 23 $^{\circ}\text{C}$, ^{125}I -ricin was purified by gel filtration on a Trisacryl GF 05 column (1 cm $\times$ 14 cm) and subsequent chromatography on Amberlite IRA-400 (1 cm $\times$ 5 cm).

The specific radioactivity of ^{125}I -ricin was 6×10^6 c.p.m./ μg and its haemagglutinating activity was 18–20 $\mu\text{g}/\text{ml}$. ^{125}I -Ricin was kept at -20°C in the presence of 0.25% BSA and used within 1 month.

On SDS/PAGE ^{125}I -ricin migrated as a major component with a M_r 60000 and only 4–6% of the radioactivity was recovered as components of M_r 30000–34000 (Fig. 1a). The electrophoretic migration of ^{125}I -ricin was unchanged by incubation at 37 $^{\circ}\text{C}$ for 120 min or boiling with 2.5% SDS and 4 M-urea for 4 min or less. Addition of 1% (v/v) β -mercaptoethanol caused a complete shift of the radioactivity towards the 30000–34000- M_r components, indicating that the latter consist of free A- and B-chains (Fig. 1b; Emmanuel *et al.*, 1988). Incubation of ^{125}I -ricin with either GSH or cysteine (5 mM each) for 30 min at 37 $^{\circ}\text{C}$ also caused ricin reduction, albeit partial (59 and 35% respectively).

Rat serum albumin was iodinated with ^{131}I at a specific radioactivity of 10–20 $\mu\text{Ci}/\mu\text{g}$ using the lactoperoxidase method. The labelled protein was separated from ^{131}I that had not reacted by gel filtration on a Sephadex G-25 column (0.6 cm $\times$ 10 cm) equilibrated with 0.1 M-sodium phosphate buffer, pH 7.4, containing 0.1% (w/v) BSA.

Animals and injections

Male Sprague-Dawley rats, weighing 180–200 g, were obtained from Charles River France and fasted for 18 h before injections. Routinely, 6×10^7 c.p.m. ^{125}I -ricin (approx. 8 μg) diluted in 0.5 ml of a 0.15 M-NaCl solution containing 0.1% (w/v) BSA, were injected over 10 s in the penis vein under diethyl ether anaesthesia. For cytosol studies, 1.8×10^8 c.p.m. (30 μg) were injected. Animals were killed from 5 min to 4 h after injection. Although the injected ricin doses were in the range 10–40 LD₅₀, no symptoms of toxicity were observed. This is related to the long incubation time necessary for the development of ricin toxicity in animals (Balint, 1974).

In experiments designed to estimate the contribution of the blood radioactivity to the liver radioactivity, ^{131}I -serum albumin [$(1-2) \times 10^6$ c.p.m.] was co-injected with ^{125}I -ricin.

Subcellular fractionation

Liver subcellular fractions were prepared from homogenates using established procedures with minor modifications (Janicot & Desbuquois, 1987). In some experiments, *N*-ethylmaleimide (5 mM) and 1,10-phenanthroline (5 mM) were included in the homogenization medium to prevent a further potential reduction and degradation of ricin and/or ricin chains during cell fractionation. Mitochondrial-lysosomal (ML), nuclear (N), microsomal (P) and supernatant (S) fractions were prepared from homogenates in 0.25 M-sucrose by differential centrifugation as described by Aronson & Touster (1974). ML and P fractions were further subfractionated on analytical sucrose-density gradients as described by Janicot & Desbuquois (1987). Plasma-

Fig. 1. SDS/PAGE characterization of ^{125}I -ricin in the absence (a) or the presence (b) of 1% β -mercaptoethanol

The results are expressed as percentage of the total radioactivity recovered in the gel.

membrane and lysosomal fractions were prepared as described by Neville (1968) and Wattiaux *et al.* (1978) respectively. Low-density P subfractions (relative densities 1.03–1.08, 1.08–1.11 and 1.11–1.14) and a low-density ML subfraction (density 1.05–1.18) were isolated from the respective parental fractions using discontinuous sucrose-density gradients. Low-density P subfractions were further fractionated by using iso-osmotic Percoll density gradients (final Percoll concentration, 17.2%, v/v) as described previously (Janicot & Desbuquois, 1987). After isolation, subcellular fractions were analysed for protein, marker enzymes and/or radioactivity, with results expressed as relative specific activity and/or recovery. In analytical density-gradient studies, results were expressed as recoveries as a function of density and used to calculate median densities as described previously (Janicot & Desbuquois, 1987).

In some experiments, the amount of ^{125}I -ricin in liver-cell fractions was corrected for the contribution of ^{125}I -ricin associated with the blood. This contribution was calculated from measurements of ^{125}I -ricin and ^{131}I -albumin in liver-cell fractions and peripheral blood, assuming that the distribution of the radioactivity between peripheral and liver blood was the same for both tracers and that ^{131}I -albumin associated with cell fractions arose solely from the blood.

Characterization of the radioactivity associated with subcellular fractions

This was achieved either on freshly isolated fractions (*'in vivo'* studies) or after incubation of subcellular fractions for 30–120 min at 37 °C in 125 mM-KCl, 5 mM-MgCl₂ and either 25 mM-Mops/KOH, pH 5, or 25 mM-Mops/KOH, pH 7.2, in the absence or presence of 1 mM-ATP, creatine kinase (0.5 mg/ml) and 5 mM-phosphocreatine (cell-free studies). In most experiments the radioactivity was characterized by using discontinuous PAGE in the presence of SDS and urea as described by Laemmli (1970); separating gels contained 12.5% (w/v) acrylamide. Once obtained, subcellular fractions were boiled for 2 min in the presence of 2.5% (w/v) SDS and 4 M-urea, and stored at –20 °C. On the day scheduled for electrophoresis, they were thawed and reboiled for 1 min. After electrophoresis, the distribution of the radioactivity along the gel was analysed by counting sequential slices (0.25 cm; 45 slices per gel). In cell-free studies, the radioactivity was also characterized by precipitation with trichloroacetic acid (5%, w/v).

Protein and enzyme assays

Protein was measured according to the method of Lowry *et al.* (1951), with BSA as a standard. 5'-Nucleotidase, acid phosphatase and galactosyltransferase activities were measured by the method of Heppel & Hilmoie (1951), Trouet (1974) and Beaufay *et al.* (1974) respectively. The specific activities and recoveries of these enzymes in the N, ML, P, S and low-density microsomal fractions have been reported previously (Janicot & Desbuquois, 1987).

RESULTS

^{125}I -ricin uptake by rat liver

In rats injected with ^{125}I -ricin, the uptake of radioactivity into the liver occurred rapidly and reached a maximum by 30 min. At this time, 0.03% of the injected dose was recovered per mg of homogenate protein, corresponding to 6% of the dose per g of liver ($\approx 40\%$ in the whole organ). Later on the radioactivity in the homogenate declined progressively, with an approximate half-life of 60 min (Fig. 2a).

To assess the nature of the binding sites involved in the interaction of ricin with liver cells, various carbohydrates were

Fig. 2. ^{125}I -ricin uptake into liver homogenates

(a) Time course of radioactivity uptake into homogenates. Rats were killed at the indicated times after injection of ^{125}I -ricin. Livers were then removed and homogenized as described in the Experimental section. (b) Effect of saccharide co-injection on ^{125}I -ricin uptake. Rats were killed 5 min after injection of ^{125}I -ricin in the absence (control) or the presence of the indicated mono- and di-saccharides (50 mmol) or mannan (0.5 mg). The dark portion of the bars represents the uptake after correction for contribution of blood radioactivity. Results are means $\pm$ S.E.M. of three determinations. Abbreviation: Gal, galactopyranoside.

co-injected with ^{125}I -ricin and examined for their ability to affect the initial uptake of the radioactivity into liver homogenates (Fig. 2b). At 5 min after injection, uptake was reduced by 2-fold by the co-injection of mannan, a ligand of the mannose receptor. Conversely, it was increased about 2.5-fold by lactose, an inhibitor of ricin binding to galactose-containing cell-surface glycoconjugates. When co-injected simultaneously, mannan and lactose inhibited ricin uptake to approximately the same extent as mannan alone.

Control experiments were performed with saccharides containing or not containing galactose. Whereas methyl β -D-galactopyranoside and lactulose enhanced the uptake of ^{125}I -ricin to an extent similar to that obtained with lactose, the injection of non-galactose-containing disaccharides (cellobiose and palatinose) did not affect the uptake of ^{125}I -ricin by the liver (Fig. 2b).

Subcellular distribution of ^{125}I -ricin taken up by the liver

The distribution of ricin taken up by the liver was first assessed using differential centrifugation (Fig. 3). At 5 min after injection, about 80% of the radioactivity in the homogenate was associated with the P fraction, the remainder being found mainly in the ML and S fractions (7 and 8% respectively). Later on there was a progressive increase in the amount of radioactivity associated with the ML fraction (up to half of the total at 1 h), with a concurrent decrease in that associated with the P fraction. Throughout the time of the study, there were little changes in the radioactivity associated with the N and S fractions.

The distribution of the radioactivity associated with the P and ML fractions was further studied on analytical sucrose-density

Fig. 3. Subcellular distribution of ^{125}I -ricin in liver homogenates after differential centrifugation

Rats were killed at the indicated times after injection of ^{125}I -ricin in the absence or the presence of lactose (50 mM) or mannan (0.5 mg). Livers were removed, homogenized and fractionated as described in the Experimental section. (a) Time course of distribution of radioactivity among the N (●), ML (○), P (△) and S (■) fractions in control rats. Results are expressed as the percentage of total recovered radioactivity. (b–c) Effect of saccharide co-injection on ^{125}I -ricin distribution 5 min (b) and 30 min (c) after injection. Results are presented as relative specific activity plotted against the protein content as described by de Duve *et al.* (1955). Results are means $\pm$ S.E.M. of three to five determinations. For the S fraction, results have been corrected for the contribution of blood radioactivity (about 60 and 40% of the total at 5 and 30 min respectively). Results for the N, ML and P fractions have not been corrected, since within these fractions less than 1% of the radioactivity originated from the blood.

gradients and compared with that of enzymic markers for major cell organelles (Fig. 4 and Table 1). For the P fraction, the radioactivity migrated as one single broad component, with

Fig. 4. Distribution of ^{125}I -ricin associated with the P and ML fractions on analytical sucrose density gradients

P (a, c) and ML (b, d) fractions were isolated in rats killed 5 min (a, b) and 30 min (c, d) after injection of ^{125}I -ricin in the absence (thin line) or the presence (thick line) of mannose. After centrifugation in linear sucrose density gradients, about 20 subfractions were collected and analysed for density and radioactivity, with the results expressed as percentage of the total recovered by normalized relative-density interval of 0.01. Histograms are representative of two or three separate fractionations. Arrows on the abscissa indicate the median densities of the radioactivity in the absence (clear arrow) or the presence (black arrow) of mannose. Arrows above the histograms indicate the median densities of galactosyltransferase (GT), 5'-nucleotidase (N) and acid phosphatase (AP), as derived from density distribution studies on the same fractions (histograms not shown). Values of median densities and radioactivity and enzymes are given in Table 1.

equilibration relative densities of about 1.15 at 5 min and 1.16 at 60 min. Regardless of time, the radioactivity equilibrated at higher densities than 5'-nucleotidase (a plasma-membrane marker) and galactosyltransferase (a Golgi marker), but at lower densities than acid phosphatase (a lysosomal marker). For the ML fraction the radioactivity migrated at 5 min as two overlapping components and at 30–60 min as one single broad component with equilibration relative densities of 1.19 and 1.22 respectively. Only at late times did this distribution coincide with that of acid phosphatase.

Although not easily seen on analytical sucrose-density gradients, a time-dependent increase in the association of radioactivity with low-density P subfractions (relative density 1.08–1.14) enriched in galactosyltransferase was clearly detectable in preparative procedures (Table 2). However, these subfractions were only 6-fold enriched in radioactivity and contained less than 3% of the total present in the homogenate. Further fractionation of these Golgi-enriched fractions by centrifugation on Percoll gradients showed that, at 5 min, the radioactivity was mostly recovered in the upper half of the gradient, partially overlapping (of relative density fractions 1.08–1.11) with or similar (of relative density 1.11–1.14) to galactosyltransferase distribution. At 30 min, the radioactivity was broadly distributed throughout the gradient, overlapping to a large extent with acid phosphatase (Fig. 5). These results suggest that, with time, the radioactivity associated with the low-density P subfraction is transferred from early, low-density endosomes to late, high-density endosomes.

The enrichment of radioactivity in a plasma-membrane fraction (Neville, 1968), known to originate mainly from hepatocytes, was low (≤ 2) and showed little if any time-dependence.

To assess whether the endocytic pathway described above was affected by the type of binding site involved in ricin recognition, the effects of co-injected mannose and lactose on the subcellular distribution of the radioactivity were examined (Fig. 3 and 4; Table 1). Neither on differential centrifugation nor on analytical sucrose-density gradients did lactose affect the subcellular distribution of the radioactivity (results not shown). However, this distribution was affected by mannose in two ways. First, on differential centrifugation, the recovery of radioactivity in the ML fraction at 30 min was decreased and, concurrently, the

Table 1. Median relative densities of protein, ¹²⁵I-ricin and enzymes associated with the P and ML fractions in sucrose gradients

Rats were killed at the indicated times after injection of ¹²⁵I-ricin alone (control) or in the presence of mannan or lactose. Liver ML and P fractions were prepared and subfractionated by sucrose-gradient centrifugation, with each subfraction analysed for relative density and one or more of the constituents indicated. Median relative densities for ¹²⁵I-ricin at 5 and 30 min are derived from the density-distribution histograms of Fig. 3; median densities for other constituents have been similarly determined. Most results shown are the mean $\pm$ S.E.M. of triplicate determinations in separate fractionations or the mean $\pm$ half-the-range (*) of duplicate determinations in separate fractionations.

Constituent	Median relative density			
	P fraction			ML fraction
	Control	Lactose	Mannan	Control
Protein	1.160 $\pm$ 0.003	1.157 $\pm$ 0.003*	1.158 $\pm$ 0.002	1.214 $\pm$ 0.002
¹²⁵ I-ricin				
5 min	1.149 $\pm$ 0.005		1.129	1.192
30 min	1.155 $\pm$ 0.004	1.153 $\pm$ 0.002*	1.126 $\pm$ 0.008*	1.217 $\pm$ 0.003
60 min	1.162			1.210
5'-Nucleotidase	1.139 $\pm$ 0.006	1.134 $\pm$ 0.002*	1.138 $\pm$ 0.003	
Galactosyltransferase	1.121 $\pm$ 0.002	1.110	1.125 $\pm$ 0.002	
Acid phosphatase	1.171 $\pm$ 0.001	1.167 $\pm$ 0.003*	1.168 $\pm$ 0.001	1.205 $\pm$ 0.001

Table 2. Uptake of ¹²⁵I-ricin into low-density microsomal subfractions

Rats were killed at the indicated times after ¹²⁵I-ricin injection. Two sets of low-density microsomal subfractions were prepared from the total microsomal fraction and analysed for radioactivity. Relative specific activities (RSA) and recoveries of radioactivity have been expressed relative to the radioactivity present in the homogenate. Results shown are the mean $\pm$ S.E.M. of at least three determinations.

Relative density	Parameter	Time...	5 min	30 min	60 min
1.08–1.11	RSA		1.12 $\pm$ 0.14	6.76 $\pm$ 0.71	6.25 $\pm$ 0.54
	Recovery (%)		0.32 $\pm$ 0.04	1.36 $\pm$ 0.24	1.03 $\pm$ 0.08
1.11–1.14	RSA		2.37 $\pm$ 0.42	6.85 $\pm$ 0.75	4.64 $\pm$ 0.69
	Recovery (%)		0.54 $\pm$ 0.08	1.05 $\pm$ 0.14	0.86 $\pm$ 0.12

recovery of radioactivity in the P and, to a lesser extent, the S, fraction was increased (Fig. 3). Secondly, on analytical sucrose gradients, the radioactivity associated with the P fraction at 5 and 30 min was shifted toward lower densities (Fig. 4). Thus blockade of the mannose receptor markedly affected the pathway(s) involved in ricin endocytosis.

Characterization of ¹²⁵I-ricin associated with liver-cell fractions

The radioactivity associated with liver subcellular fractions isolated various times after ricin injection was first characterized on freshly isolated fractions (Fig. 6). Since subcellular fractions, especially the S fraction, can reduce added ¹²⁵I-ricin *in vitro*, cell fractionation was performed in the presence of *N*-ethylmaleimide, which blocks free SH groups, and 1,10-phenanthroline, which inhibits the activity of metalloproteinases. [When added to freshly isolated subcellular fractions, ¹²⁵I-ricin underwent partial reduction (about 30% in the S fraction and 15% in crude particulate fractions). However, such reduction was observed only when cell fractions were immediately processed for electrophoresis and was no longer detectable or markedly attenuated after a 30 min incubation at 37 °C.] With the ML fraction (Fig. 6a–6c), most of the radioactivity was associated with components

Fig. 5. Distribution of the radioactivity and enzymes associated with low-density microsomal subfractions on Percoll density gradients

P subfractions of relative density 1.08–1.11 (a and c) and 1.11–1.14 (b and d) isolated from control rats (a and b) or from rats killed 5 or 30 min (c and d) after injection of ¹²⁵I-ricin were subjected to centrifugation in Percoll density gradients; 15 fractions (0.5 ml each) were collected and analysed for enzyme activity and radioactivity. (a) and (b) Distribution of galactosyltransferase (thin line) and acid phosphatase (thick line, hatched histograms) activities. (c) and (d) Distribution of radioactivity at 5 min (thin line, dark histograms) and 30 min (thick line) after ricin injection. The profiles shown are mean results for two experiments in separate fractionations.

of *M_r* 60000 or above, and only at 60 min was the amount of radioactivity associated with A- and B-chains and in the gel front increased (about 13 and 9–10% of the total radioactivity recovered respectively). With the S fraction, a time-dependent generation of labelled products migrating as intact A- and B-chains was more readily detectable, with about 15% of the total generated at 15 min and 25–30% at 30 and 60 min (Figs. 6d–6f). With low-density P subfractions, the migration pattern of the

Fig. 6. SDS/PAGE characterization of ^{125}I -ricin associated with liver fractions

Homogenization and fractionation were performed in the presence of *N*-ethylmaleimide and 1,10-phenanthroline as described in the Experimental section. (a-c), ML fraction isolated 15 min (a), 30 min (b) and 60 min (c) after injection. (d-f), S fraction isolated 15 min (d), 30 min (e) and 60 min (f) after injection. The results are expressed as percentages of the total radioactivity recovered in the gel, and arrows indicate the migration positions of 60000- (left) and 30000- (right) M_r proteins.

radioactivity was virtually undistinguishable from that observed with intact ricin (cf. Fig. 1a). These results identify endocytic structures sedimenting with the ML fraction and the cytosol as two potential sites of ricin reduction *in vivo*.

Processing of internalized ricin in cell-free endocytic structures

Because ricin chains generated in endocytic structures *in situ* may rapidly be translocated into the cytosol, subcellular fractions containing ricin *in vivo* internalized were examined for their ability to process ricin in a subsequent incubation at 37 °C. In these experiments, crude ML fractions, ML-derived endosomal and lysosomal fractions and P-derived endosomal fractions were used. In each case, incubations were carried out at pH 5 and at pH 7.2 in the presence or absence of ATP, the substrate of the vacuolar proton ATPase.

Incubation of crude ML fractions isolated 30 min after ricin injection resulted in a time-dependent increase in the recovery of low-molecular-mass products, along with a decrease in the recovery of intact ricin; recovery of intact ricin chains also slightly decreased after 15 min (Figs. 7a-7c and Figs. 8a-8c). The generation of low-molecular-mass products was highest at pH 5 and, when assayed at pH 7.2, was higher in the presence than in the absence of ATP, suggesting a functional linkage with ATP-dependent acidification. Analysis of incubation mixtures by trichloroacetic acid precipitation revealed a time-dependent generation of trichloroacetic acid-soluble products which paralleled the generation of labelled products migrating at the gel front (results not shown).

Incubation of ML-derived endosomal fractions (Figs. 7d-7f and 8d-8f) and lysosomal fractions (results not shown) also resulted in a time- and pH-dependent generation of low-molecular-mass products, as well as in a slight decrease in the

Fig. 7. SDS/PAGE characterization of ^{125}I -ricin associated with the ML fraction and a ML-derived endosomal fraction: effect of incubation *in vitro*

A total ML fraction and a low-density ML subfraction were prepared from rats killed 30 min after injection of ^{125}I -ricin. In these experiments, neither *N*-ethylmaleimide nor 1,10-phenanthroline were included in the homogenization medium. The total ML fraction (a,b,c) and the low-density ML subfraction (d,e,f) were resuspended in 0.15 M-KCl/5 mM-MgCl₂/25 mM-Mops/KOH and subjected to SDS/PAGE under three conditions: (i) immediately after resuspension (a,d); (ii) after incubation for 30 min at 37 °C at pH 5 (b,e); (iii) after a similar incubation at pH 7.2 in the presence of 1 mM-ATP, creatine kinase (0.5 mg/ml) and 5 mM-phosphocreatine (c,f). The results of one experiment (representative of two experiments) are expressed as percentage of the total radioactivity recovered in the gel, and arrows indicate the migration positions of 60000- (left) and 30000- (right) M_r proteins. The percentages of radioactivity migrating as intact ricin, ricin chains and low- M_r products are shown in Fig. 8.

recovery of ricin chains. However, under comparable conditions, ML-derived endosomal fractions degraded ricin to a lesser extent than total ML and ML-derived lysosomal fractions. With P-derived endosomal fractions, virtually no changes in the migration profile of the radioactivity relative to injected ^{125}I -ricin were observed regardless of time and pH, even after a 120 min incubation (results not shown).

DISCUSSION

In the present work we used cell-fractionation techniques in association with SDS/PAGE to identify the cell compartments

Fig. 8. Time-dependence of the generation of radiolabelled products from ^{125}I -ricin associated with the ML fraction and a ML-derived endosomal fraction on incubation *in vitro*

Subcellular fractions prepared as described in legend to Fig. 7, were resuspended in 0.15 M-KCl/5 mM-MgCl₂ and one of the following additives: 25 mM-Mops/KOH, pH 5 (▲); 25 mM-Mops/KOH, pH 7.2 (●); and 25 mM-Mops/KOH, pH 7.2, plus 1 mM-ATP, creatine kinase (0.5 mg/ml) and 5 mM-phosphocreatine (○). After incubation at 37 °C for the indicated times, aliquots were subjected to SDS/PAGE and the percentage of radioactivity migrating as intact ricin (a,d), ricin chains (b,e) and low-*M_r* products (c,f) was determined. Gel profiles obtained at 0 min and after incubation at pH 5 and 7.2 in the presence of ATP are shown in Fig. 7.

involved in the endocytosis and processing of ricin taken up by rat liver *in vivo*. Our results show that internalized ricin associates with endocytic structures whose size and density of equilibration increase with time. They also show that reduction of the ricin interchain disulphide bridge occurs both in high-density endocytic structures and in the cytosol.

On the basis of measurements of ricin content in separate liver-cell populations and on the effects of various glycoproteins or carbohydrates on ricin uptake, it has been shown that most of ricin taken up by rat liver *in vivo* is recovered in non-parenchymal cells and recognized by mannose receptors (Skilleter *et al.*, 1981; Thorpe *et al.*, 1985). Although the distribution of injected ricin between individual cell types has not been measured in the present study, the effects of co-injected mannan and lactose on ricin uptake clearly indicate that this process is mediated to a large extent by the mannose receptor and thus involves non-parenchymal cells. However, when co-injected simultaneously, mannan and lactose did not inhibit ricin uptake by more than 70%. Assuming that blockade of the mannose and galactose was complete, the residual uptake might reflect in part the interaction of ricin fucose residues (Kimura *et al.*, 1988) with fucose-recognition systems present at the surface of liver cells (Furbish *et al.*, 1980; Haltiwanger *et al.*, 1986).

The time-dependent changes in the subcellular distribution of ricin taken up by the liver indicate that this ligand undergoes

endocytosis. At 5 min, ricin was associated with structures of relative density 1.15 sedimenting mainly in the P fraction. Although distributing on sucrose gradients like the plasma-membrane marker 5'-nucleotidase, these structures were probably of endosomal origin, since, unlike 5'-nucleotidase, they were not concentrated in the N fraction. Later on, ricin was translocated to structures of high density, sedimenting mainly with the ML fraction, with a distribution becoming similar to that of the lysosomal marker acid phosphatase. Only limited amounts of ricin were recovered in low-density endocytic structures sedimenting with the P fraction.

The ricin endocytic pathway as described above resembles that described for other ligands taken up by endothelial cells, either via the mannose receptor, such as mannosylated albumin (Quintart *et al.*, 1983), ovalbumin (Kindberg *et al.*, 1990) and lysosomal enzymes (Jadot *et al.*, 1986) or via scavenger receptors, such as formaldehyde-treated albumin (Eskild *et al.*, 1989). On the other hand, it differs from the pathway for ligands taken up by parenchymal cells, such as asialoglycoproteins (Limet *et al.*, 1985). Differences between these two pathways result from the fact that internalized ligands associate more rapidly with lysosomes in non-parenchymal cells than in parenchymal cells (Kindberg *et al.*, 1990), and/or that endosomes originating from the former equilibrate at higher densities than endosomes originating from the latter.

When mannan was co-injected with ricin, less radioactivity was recovered in high-density endocytic structures sedimenting with the ML fraction and, concurrently, more was recovered in low-density structures sedimenting with the P fraction. Along with the inability of lactose to affect the subcellular distribution of ricin, these findings further support the view that endocytosis of ricin is mediated to a large extent via the mannose receptor. They also indicate that, when this receptor is blocked, ricin endocytosis involves a pathway which resembles that described for ligands internalized into parenchymal cells (Limet *et al.*, 1985). Despite the fact that lactose did not further inhibit ricin uptake in the presence of mannan, endocytosis via galactose residues remains possible. An alternative possibility remains of a single endocytic pathway which is both partially blocked and slowed down by mannan.

Analysis of the labelled ricin associated with cell fractions by SDS/PAGE showed that the material associated with the ML and supernatant fractions was reduced in a time-dependent manner, whereas that associated with the low-density P sub-fractions retained essentially complete integrity. Although reduction of internalized ricin appeared to proceed more rapidly in the S fraction than in the ML fraction, owing to a higher recovery of ligand, the total amount of ricin chains associated with the ML fraction was about twice as high as that associated with the S fraction. However, when the ML fraction and a ML-derived endosomal fraction containing ricin internalized *in vivo* were examined for their ability to process ricin in a subsequent incubation at 37 °C, no further reduction of ricin was observed; instead, only low-molecular-mass products were formed. There are at least two potential explanations for these findings. One first possibility is that, as suggested for diphtheria toxin (Moskaug *et al.*, 1987), ricin reduction occurs at the cytoplasmic side of the endosomes and involves cytosolic components. One second possibility is that, under cell-free conditions, reducing systems associated with endosomes progressively lose their ability to reduce internalized ricin (see the note in square brackets in the first paragraph of the subsection 'Characterization of ^{125}I -ricin associated with liver-cell fractions' above).

The presence of intact ricin in the S fraction suggests that, unlike what is generally assumed, the reduction of ricin within endosomes is not an obligatory requirement for its entry into the

cytosol. Although it has been suggested that only the A-chain enters the cytosol, at least two lines of evidence suggest that this may also be the case for the whole ricin molecule. First, hydrophobic photolabelling experiments have shown that both subunits of ricin can penetrate artificial lipid membranes (Ishida *et al.*, 1983). Secondly, post-embedding electron-microscopic studies have shown that, in breast-cancer cells, some internalized ricin is translocated into the cytosol under an immunoreactive, presumably intact, form (Calafat *et al.*, 1988). Finally, ricin micro-injected in the cytosol of *Xenopus* oocytes has been shown to inhibit protein synthesis (Saxena *et al.*, 1989).

Although unable to document ricin reduction in endocytic structures, our 'cell-free' studies clearly show that, under conditions ensuring acidification of these structures, internalized ricin is progressively degraded into low-molecular-mass fragments. These findings indicate that the endocytic structures which concentrate internalized ricin in liver are acidic and are compatible with the view that acidity may be required for translocation of ricin or ricin A-chain into the cytosol. On the basis of the effects of NH_4Cl on ricin toxicity in macrophages, it has previously been suggested that, whereas ricin internalized via its galactose-binding sites enters the cytosol from a neutral compartment, ricin bound to the mannose receptor passes through an acidic compartment before translocation (Simmons *et al.*, 1986).

Like ricin, several bacterial toxins, such as diphtheria toxin and cholera toxin, are activated upon endocytosis by cleavage of their interchain disulphide bonds. Although reduction of diphtheria toxin has been suggested to occur in the cytosol (Moskaug *et al.*, 1987), cell-free studies comparable with those described here have shown that the A-subunit of cholera toxin is reduced in endosomes maximally at low pH (Janicot *et al.*, 1991). Evidence for the involvement of intracellular organelles, presumably Golgi elements, in the endocytic reduction of a model compound, poly(D-lysine) disulphide linked to radioiodinated tyramine, has also been presented (Feener *et al.*, 1990).

Reduction of disulphide bonds in internalized ricin or other proteins could either occur non-enzymically or be catalysed by enzymes such as thiol:protein disulphide oxidoreductase or thioredoxin (reviewed by Feener *et al.*, 1990). At least two observations suggest that reduction of ricin in the cytosol is non-enzymic. First, both cysteine and GSH, at concentrations comparable with those achieved in the cytosol, can reduce ricin (see the Experimental section). Secondly, the ability of ricin to inhibit protein synthesis in intact cells is blocked by diethyl maleate, which decreases intracellular glutathione levels (Lewis & Youle, 1986).

In summary, the present results show that ricin taken up by liver *in vivo* is recognized to a large extent by the mannose receptor. Upon internalization, ricin associates with endocytic structures whose size and density of equilibration increase with time; these structures are presumably derived from non-parenchymal cells. Although some ricin associated with endocytic structures is recovered as intact A- and B-chains, reduction of the ricin interchain disulphide bridge appears to occur to a large extent in the cytosol.

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